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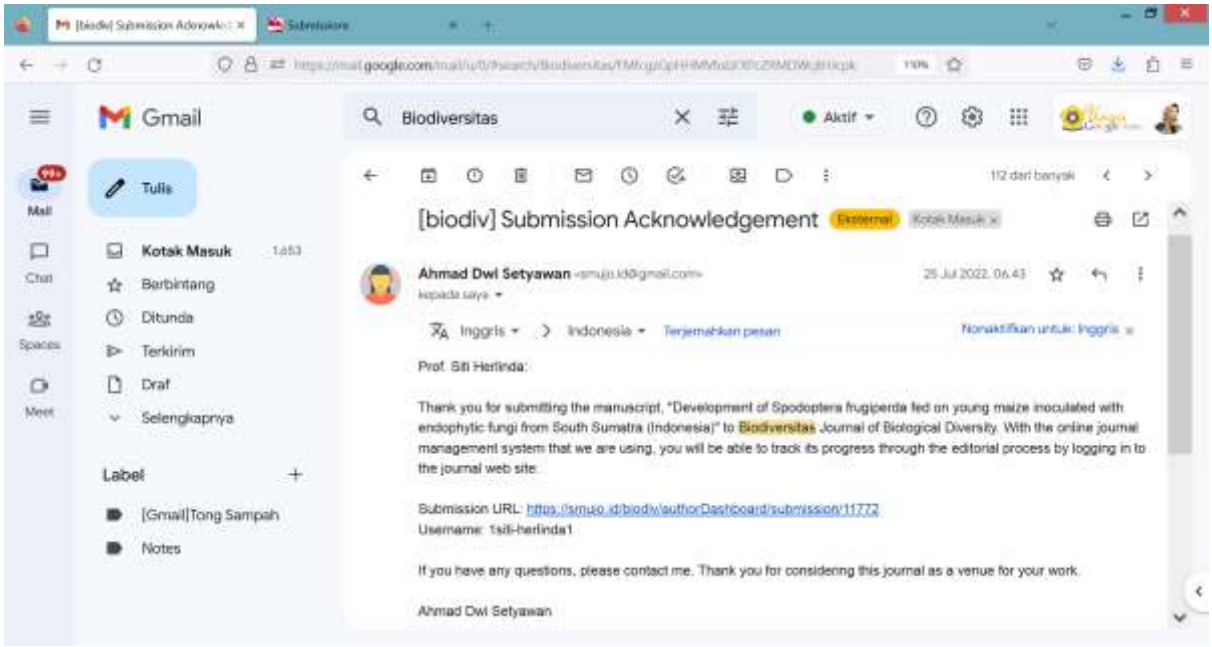

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Development of *Spodoptera frugiperda* fed on young maize inoculated with endophytic fungi from South Sumatra (Indonesia)

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This study highlights a finding that first report of *Beauveria bassiana* and *Metarhizium anisopliae* isolated from maize and red pepper from South Sumatra Indonesia in seed treated young maize have negative effects on development of *Spodoptera frugiperda*. The other finding highlights the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda.*

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Development of *Spodoptera frugiperda* fed on young maize inoculated with endophytic fungi from South Sumatra (Indonesia)

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Abstract. To control the hiding larvae of *Spodoptera frugiperda* is needed the endophytic entomopathogenic fungi. The objective of this research was to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhance the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

Key words: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

Abbreviations (if any): -

Running title: Development of *Spodoptera frugiperda* treated with endophytic fungi

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano *et al.*, 2018). This pest comes from South America (Otim *et al.*, 2018). The FAW came into Africa in 2016 (Goergen *et al.*, 2016) and crossed over to Europe in 2017 (Early *et al.*, 2018). In Asia, the FAW discovered for the first time in India in 2018 (Ganiger *et al.*, 2018; Mahat *et al.*, 2021) and on March 26, 2019 the pest came into Indonesia for the first time in West Sumatra (Sartiami *et al.*, 2020). More recently, it has spread throughout Indonesia (Maharani *et al.*, 2019; Ginting *et al.*, 2020; Supartha *et al.*, 2021) and becomes a new invasive pest in Indonesia (Herlinda *et al.*, 2021b). As the polyphagous insect, the FAW can attack 353 host plant species from 76 plant families (Montezano *et al.*, 2018). In Indonesia, the most severely attacked crop is maize (*Zea mays* L.) and it causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun *et al.*, 2021) and 26.50% to 70% in Lampung (Lestari *et al.*, 2020), and reaching 100% in South Sumatra (Herlinda *et al.*, 2021b). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano *et al.*, 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaves' midribs at daylight (Gustianingtyas *et al.*, 2021) and this behavior makes the FAW larvae difficult to be controlled.

The FAW commonly controls using synthetic insecticides due to the fast action and easy application (Kumela *et al.*, 2018) but the insecticide application causes the negative affect for the human health and environment (Harrison *et al.*, 2019) and the resistances againts the pest (Zhang *et al.*, 2021). An alternative eco-friendly control for FAW is by utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos, 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda *et al.* 2020).

Beauveria bassiana (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam *et al.*, 2020). The fungal topical application is less effective in the field (Gustianingtyas *et al.*, 2021) because at daylight up to night the FAW larvae hide in the corn midribs (Herlinda *et al.*, 2021a). To control the hiding larvae is needed the entomopathogenic fungi that are able to colonize in plant tissues (endophytic fungi) (Gustianingtyas *et al.*, 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their host hosts (Lira *et al.*, 2020), can stimulate the plant growth and depress the insect growth (Russo *et al.*, 2020).

The results of previous studies showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda *et al.*, 2021a). However, there is no information on development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. In this study, the effect of young maize inoculated with endophytic fungi on *S. frugiperda* development was investigated. So, the objective of this research is to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of *Spodoptera frugiperda*

Eggs of *S. frugiperda* obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya which have been mass-rearing since January 2020 (Herlinda *et al.*, 2020) and was identified molecularly by (Herlinda *et al.*, 2021b). The FAW were mass-reared in the laboratory at 28.81°C temperature, and 82.94% relative humidity (RH) and the lighting set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda *et al.*, 2021a). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage placed also fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the collection of the Laboratory of Entomology and they were identified molecularly by Herlinda *et al.* (2021a). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073) (Table 1). The fungi were originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, assesing the ability of the fungi colonizing in maize tissue was carried out by maize seeds treated. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 day. Before treated with the fungi, the 45 corn seeds were surface sterilized by using the method of Russo *et al.* (2020). Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas the seed control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti *et al.* (2020). For confirming the fungi as endophytes, detecting the fungi colonizing the young maize tissues was carried out by cutting the tip leaves of 7 and 14-day old young maize and then the tip leaves were grown onto the SDA medium for detecting the mycelia of the endophytic fungi whitin the leaves. The rest young maize leaves were used for bioassays. Before the leaves grown onto the SDA medium, they were first surface-sterilized by immersion in 70% ethanol, sodium hypochlorite for 2 minutes, and rinsed twice in sterile distilled water (Russo *et al.*, 2020). Finally, the last rinse water was also grown onto SDA medium. If on the last rinse water, no fungal growth was found, it corfirmed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungus growing on treated medium were endophytes.

The bioassay for assessing the effect of young maize inoculated with endophytic fungi on development of *Spodoptera frugiperda*

Assessing the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda* was carried out at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged 28−29 °C and 82−83%, respectively.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	(Herlinda *et al.*, 2021a)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	(Herlinda *et al.*, 2021a)
Tanjung Payang. Pagar	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	(Herlinda *et al.*, 2021a)

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on development of *S. frugiperda* followed the method of Russo *et al.* (2019). The leaves of young maize colonized by the endophytic fungi were provided to be consumed by the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda*, whereas for control, the non-treated leaves of young maize were consumed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves eaten up. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm) and fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae and replaced with new ones everyday. This experiment consisted of three fungal isolates and control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae were recorded everyday. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs and the dead larvae and pupae were grown in SDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily and their sex were recorded. The adults were placed in the wire mesh cage for copulation with fresh maize leaves inside for providing egg laying. Eggs laid by the adults were counted everyday. The adult longevity was determined by counting the time (days) from emergence until death.

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's test or Tukey's Honestly Significant Difference (HSD) test or was applied to determine the significant differences among the isolates at $p = 0.05$. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The young maize leaves colonized by the fungi when they were grown on to the SDA medium, the fungus grew and their mycelia covered the leaves (Figure 1). However, no mycelia were found on the leaves of untreated maize, and also on the final flushing water. This corfirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophyte originating from the maize tissues. The results showed that a percentage of fungal colonization in leaves after being inoculated by seed immersion treatment increased from 7 to 14 days after seed inoculation, and at the 14 days after seed inoculation, all maize leaves colonized by the fungi (100%) (Table 2).

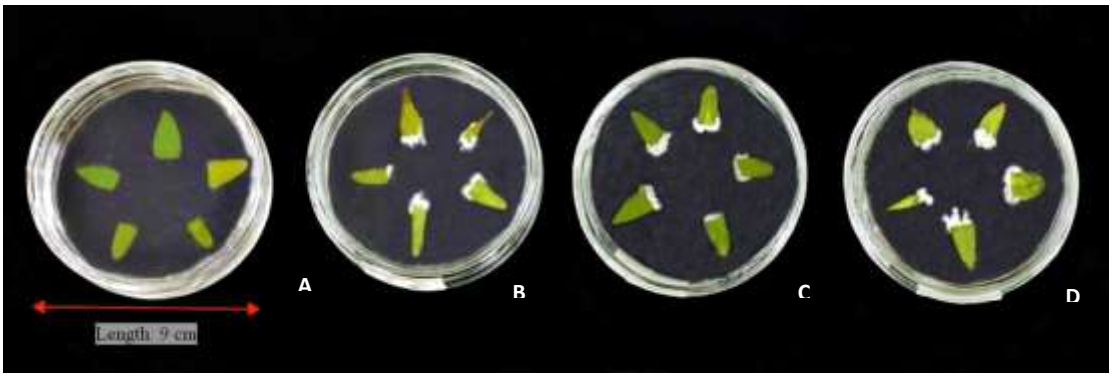

Figure 1. Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (A), *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

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 fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00^{c}	0.00^{b}
JgSPK	*Beauveria bassiana*	73.33^{b}	100.00^{a}

JgCrJr	*Beauveria bassiana*	53.33[b]	100.00[a]
CaTpPga	*Metarhizium anisopliae*	100.00[a]	100.00[a]
F-value		145.02[**]	143.40[**]
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

Effect of young maize colonized and non-colonized by fungi on the development of *Spodoptera frugiperda*

The first instar neonate larvae fed on leaves of fungal colonized maize caused the next instar significantly shifting their developmental time (P<0.0001) (Table 3). The developmental time of all instar fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time, however the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the lifespan of *S. frugiperda* increased significantly (P<0.0001). The longest lifespan of *S. frugiperda* found in the treatment of *M. anisopliae* (46.87 days) among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize was significantly longer (P<0.0001) compared to those fed on leaves of non-colonized maize.

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

		The developmental time (days)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d
JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		4.66×10^{-08}	3.31×10^{-08}	1.43×10^{-09}	3.99×10^{-07}	9.13×10^{-09}	3.61×10^{-09}
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Tabel 4. Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

		The developmental time (days)					
Isolate	Species	Prepupae	Pupae	Female adult	Male adult	Egg	Total of lifespan
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		2.71×10^{-08}	6.29×10^{-06}	2.51×10^{-05}	1.02×10^{-07}	1.91×10^{-04}	1.33×10^{-09}
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize (control) (Table 5). The mortality of the 6th larvae or the last instar fed on young maize colonized by *M. anisopliae* (57.67%) was the highest among other treatments, but it was not significantly different from those fed on maize colonized by *B. bassiana* of JgSPK isolate (51.33%). Mean of percentage of pupae and adults emergence of fungal treatments were significantly lower (P<0.0001) than those of control (Table 6). The percentage of pupae and adults emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize colonized with the fungi was not significantly different from those of control. The maize colonized with fungi significantly decreased eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 5. Mean of mortality of different instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

		Mean of mortality of different instar larvae (%)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae

Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*
P-value		0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value		-	5.98	4.70	3.83	5.06	6.42

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 young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08**	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and colour change, such as no appetite and muddy body colour, and the dead larvae showed unique symptoms, specifically shrunken and hardens like a mummy, the larvae body was covered with fungal mycelia and became white or green colour depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colour, respectively (Figure 2). The result indicated that the fungal isolate from re-isolation from the cadavers was the same as the fungal isolate used for seed treatment of maize seeds. The colony morphology of the fungi resulted from re-isolation from the cadavers had white and greenish white colour for *B. bassiana* and *M. anisopliae*, respectively (Figure 3). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae and mycelia, and globose conidia, nevertheless the morphology of *M. anisopliae* had the green hyphae and mycelia, and cylindrical conidia. The dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves not only could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* to be malformation (Figure 4, 5, and 6). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown in colour, and their integuments were harder than the healthy ones. The sick larvae could produce the abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and inability to fly.

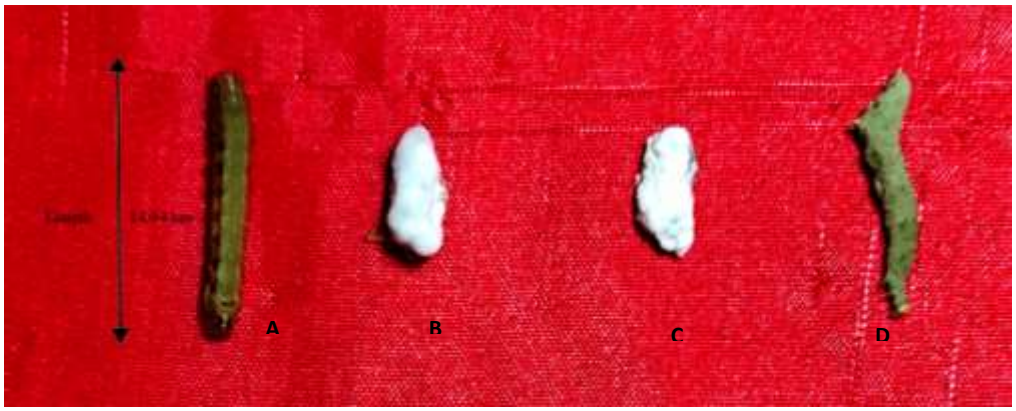

Figure 2. The cadavers from larvae fed on maize leaves untreated with fungi or control (A), and the cadavers from larvae fed on maize leaves colonized by *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

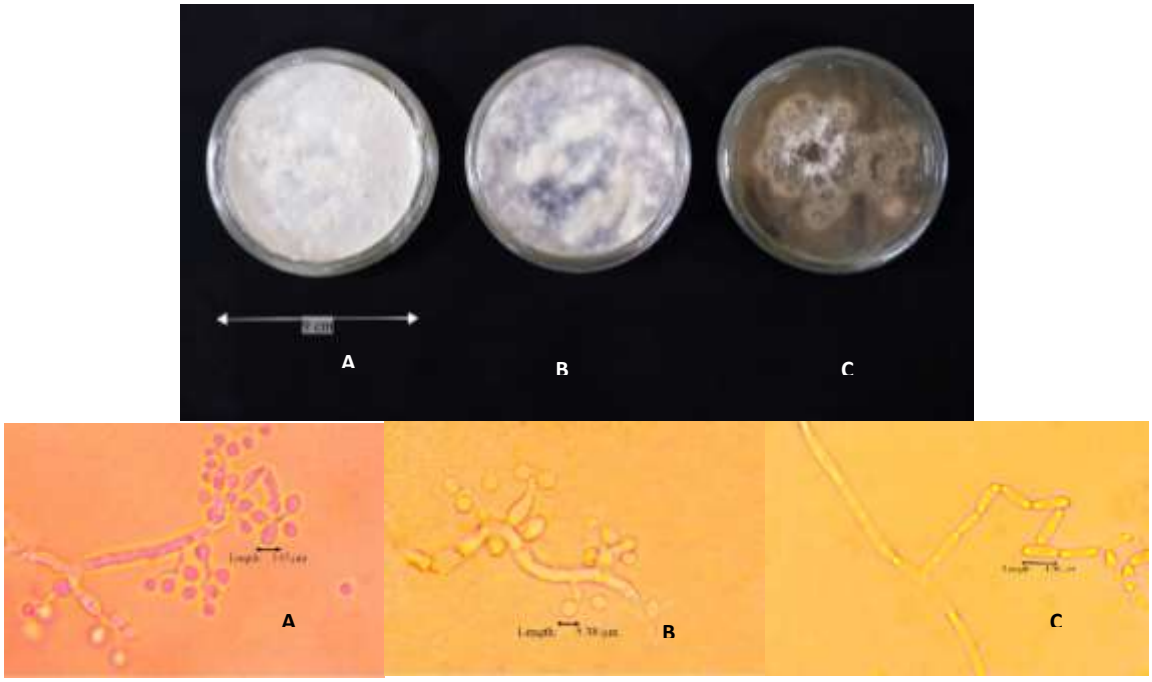

Figure 3. Colony morphology of endophytic fungi isolated from the cadavers, and cultured on SDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D) and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

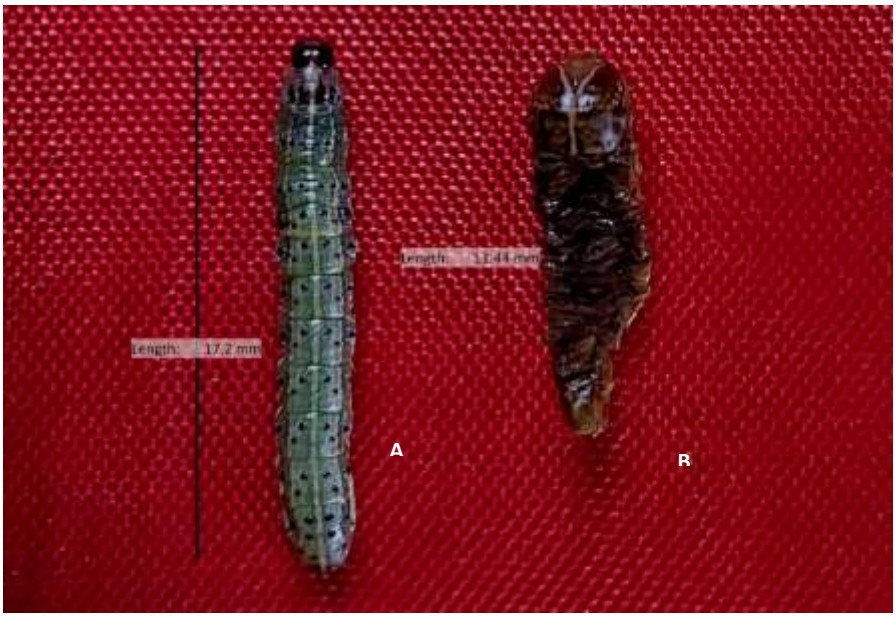

Figure 4. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

Figure 5. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 6. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia were able to colonize within the maize tissue including the leaves that were consumed by the neonate larvae. The fungal mycelia were not found whitin the leaves uninoculated by seed immersion treatment (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached 100% of leaves at 14 days after seed inoculation. The fungi, *B. bassiana* and *M. anisopliae* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo *et al.*, 2020). The fungal endophytes remained to colonize more than 14 days after inoculation, the fungi were found within roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina *et al.*, 2020). *B. bassiana* could be detected within entire plant growth cycle (120–140 days after sowing) and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga *et al.*, 2014). *B. bassiana* and *M. anisopliae* isolates in the present study could be detected within entire maize tissue and this obtained finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is very susceptible to be attacked by *S. frugiperda* larvae (Supartha *et al.*, 2021), so the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effective to be controlled by using the endophytic fungi compared to the fungal topical application (Gustianingtyas *et al.*, 2021; Herlinda *et al.*, 2021a).

B. bassiana and *M. anisopliae* in the present study inoculated as seed treatments of maize seeds prolonged developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased, however the adult longevity decreased. The obtained finding was in accordance with some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword, 2015; Hussain *et al.*, 2009) due to the fungi reduced the conversion of digested and ingested food that could stimulate the larvae to develop more slowly (Hussain *et al.*, 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in current study caused negative effects on the *S. frugiperda* development. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs, and enhance the larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo *et al.*, 2020) due to the fungi produced secondary metabolites

and caused mycosis on insect body (Vidal and Jaber, 2015). The fungal mycelia within the plant tissue fed on the larvae could produce blastospores in the larvae hemolymph, and the blastospores could produce secondary metabolites with their toxins that disrupted the normal cell metabolism and finally killed the insects (Mancillas-Paredes *et al.*, 2019). The endophytic fungi also reduced the larvae appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas *et al.*, 2021) because the fungi could produce secondary metabolites *in planta* resulting antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley, 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo *et al.*, 2020). When, the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber, 2015). The current study showed that the mycosis occured on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The other previous study also showed that the mycosis could occure on the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo *et al.*, 2020).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhance the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

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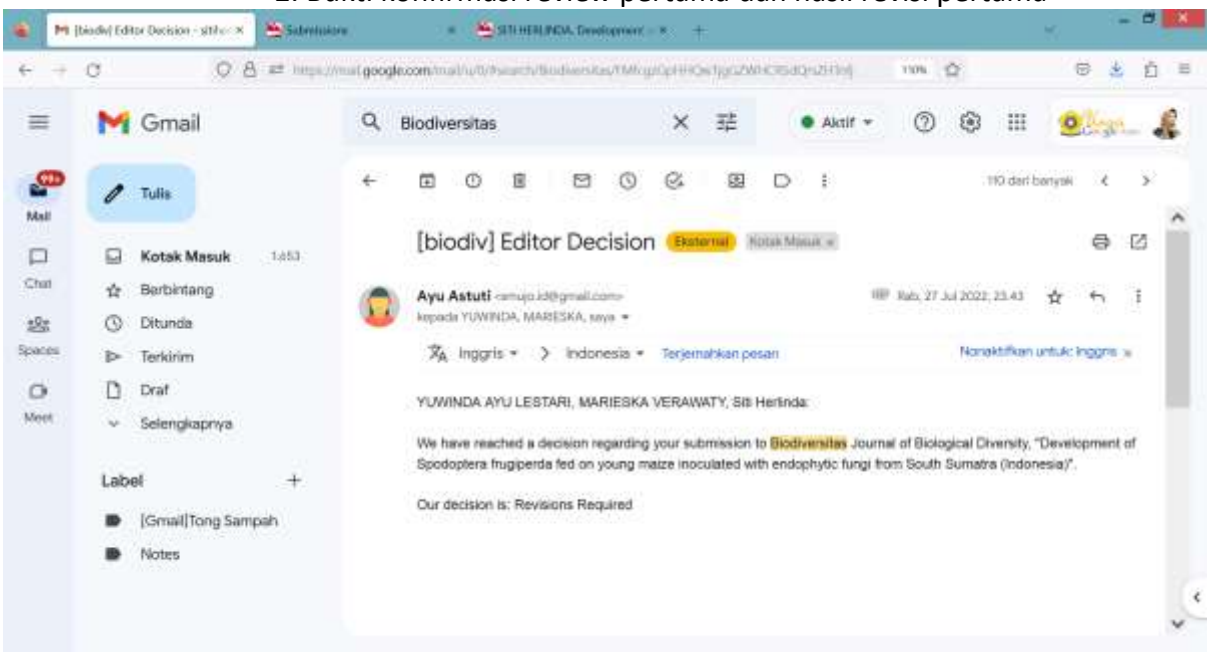
2. Bukti konfirmasi review pertama dan hasil revisi pertama



Development of *Spodoptera frugiperda* fed on young maize inoculated with endophytic fungi from South Sumatra (Indonesia)

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Abstract. To control the hiding larvae of *Spodoptera frugiperda* is needed the endophytic entomopathogenic fungi. The objective of this research was to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhance the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

Key words: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

Abbreviations (if any): -

Running title: Development of *Spodoptera frugiperda* treated with endophytic fungi

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano et al., 2018). This pest comes from South America (Otim et al., 2018). The FAW came into Africa in 2016 (Goergen et al., 2016) and crossed over to Europe in 2017 (Early et al., 2018). In Asia, the FAW discovered for the first time in India in 2018 (Ganiger et al., 2018; Mahat et al., 2021) and on March 26, 2019 the pest came into Indonesia for the first time in West Sumatra (Sartiami et al., 2020). More recently, it has spread throughout Indonesia (Maharani et al., 2019; Ginting et al., 2020; Supartha et al., 2021) and becomes a new invasive pest in Indonesia (Herlinda et al., 2021b). As the polyphagous insect, the FAW 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). In Indonesia, the most severely attacked crop is maize (*Zea mays* L.) and it causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun et al., 2021) and 26.50% to 70% in Lampung (Lestari et al., 2020), and reaching 100% in South Sumatra (Herlinda et al., 2021b). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano et al., 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaves' midribs at daylight (Gustianingtyas et al., 2021) and this behavior makes the FAW larvae difficult to be controlled.

The FAW commonly controls using synthetic insecticides due to the fast action and easy application (Kumela et al., 2018) but the insecticide application causes the negative affect for the human health and environment (Harrison et al., 2019) and the resistances againts the pest (Zhang et al., 2021). An alternative eco-friendly control for FAW is by utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos, 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda et al. 2020). *Beauveria bassiana* (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam et al., 2020). The fungal topical application is less effective in the field (Gustianingtyas et al., 2021) because at daylight up to night the FAW larvae hide in the corn midribs (Herlinda et al., 2021a). To control the hiding larvae is needed the entomopathogenic fungi that are able to colonize in plant tissues (endophytic fungi) (Gustianingtyas et al., 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their host hosts (Lira et al., 2020), can stimulate the plant growth and depress the insect growth (Russo et al., 2020).

The results of previous studies showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda et al., 2021a). However, there is no information on development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. In this study, the effect of young maize inoculated with endophytic fungi on *S. frugiperda* development was investigated. So, the objective of this research is to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of *Spodoptera frugiperda*

Eggs of *S. frugiperda* obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya which have been mass-rearing since January 2020 (Herlinda et al., 2020) and was identified molecularly by (Herlinda et al., 2021b). The FAW were mass-reared in the laboratory at 28.81°C temperature, and 82.94% relative humidity (RH) and the lighting set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda et al., 2021a). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage placed also fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the collection of the Laboratory of Entomology and they were identified molecularly by Herlinda et al. (2021a). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073) (Table 1). The fungi were originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, assesing the ability of the fungi colonizing in maize tissue was carried out by maize seeds treated. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 day. Before treated with the fungi, the 45 corn seeds were surface sterilized by using the method of

Russo et al. (2020). Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas the seed control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti et al. (2020). For confirming the fungi as endophytes, detecting the fungi colonizing the young maize tissues was carried out by cutting the tip leaves of 7 and 14-day old young maize and then the tip leaves were grown onto the SDA medium for detecting the mycelia of the endophytic fungi whitin the leaves. The rest young maize leaves were used for bioassays. Before the leaves grown onto the SDA medium, they were first surface-sterilized by immersion in 70% ethanol, sodium hypochlorite for 2 minutes, and rinsed twice in sterile distilled water (Russo et al., 2020). Finally, the last rinse water was also grown onto SDA medium. If on the last rinse water, no fungal growth was found, it corfirmed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungus growing on treated medium were endophytes.

The bioassay for assessing the effect of young maize inoculated with endophytic fungi on development of *Spodoptera frugiperda*

Assessing the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda* was carried out at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged 28−29 °C and 82−83%, respectively.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	Herlinda et al. (2021a)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	Herlinda et al. (2021a)
Tanjung Payang. Pagar Alam	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	Herlinda et al. (2021a)

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on development of *S. frugiperda* followed the method of Russo et al. (2019). The leaves of young maize colonized by the endophytic fungi were provided to be consumed by the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda*, whereas for control, the non-treated leaves of young maize were consumed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves eaten up. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm) and fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae and replaced with new ones everyday. This experiment consisted of three fungal isolates and control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae were recorded everyday. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs and the dead larvae and pupae were grown in SDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily and their sex were recorded. The adults were placed in the wire mesh cage for copulation with fresh maize leaves inside for providing egg laying. Eggs laid by the adults were counted everyday. The adult longevity was determined by counting the time (days) from emergence until death.

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's test or Tukey's Honestly Significant Difference (HSD) test or was applied to determine the significant differences among the isolates at $p = 0.05$. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The young maize leaves colonized by the fungi when they were grown on to the SDA medium, the fungus grew and their mycelia covered the leaves (Figure 1). However, no mycelia were found on the leaves of untreated maize, and also on the final flushing water. This corfirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophyte originating from the maize tissues. The results showed that a percentage of fungal colonization in leaves after being inoculated by

seed immersion treatment increased from 7 to 14 days after seed inoculation, and at the 14 days after seed inoculation, all maize leaves colonized by the fungi (100%) (Table 2).

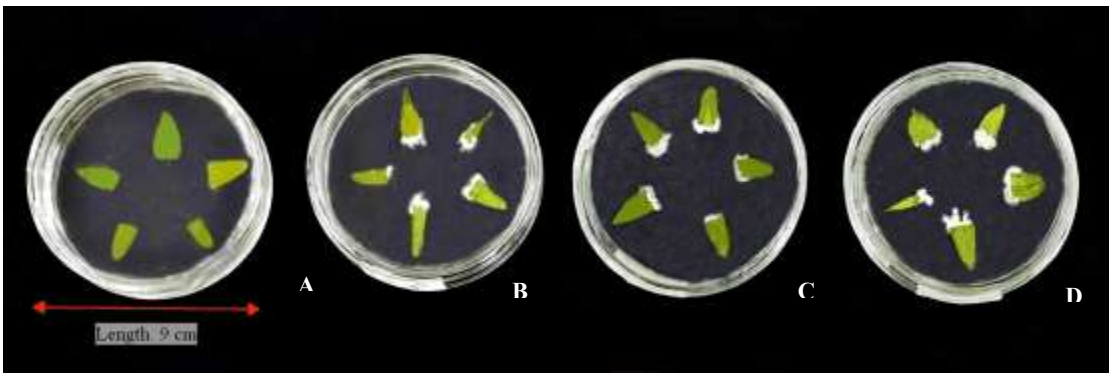

Figure 1. Colony morphology of endophytic fungi from the leaves of maize where seed treatment was given and control (untreated seeds): Control (A), *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

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 fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00^{c}	0.00^{b}
JgSPK	*Beauveria bassiana*	73.33^{b}	100.00^{a}
JgCrJr	*Beauveria bassiana*	53.33^{b}	100.00^{a}
CaTpPga	*Metarhizium anisopliae*	100.00^{a}	100.00^{a}
F-value		145.02^{**}	143.40^{**}
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

Effect of young maize colonized and non-colonized by fungi on the development of *Spodoptera frugiperda*

The first instar neonate larvae fed on leaves of fungal colonized maize caused the next instar significantly shifting their developmental time (P<0.0001) (Table 3). The developmental time of all instar fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time, however the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the lifespan of *S. frugiperda* increased significantly (P<0.0001). The longest lifespan of *S. frugiperda* found in the treatment of *M. anisopliae* (46.87 days) among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize was significantly longer (P<0.0001) compared to those fed on leaves of non-colonized maize.

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d
JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		$4.66\text{x}10^{-08}$	$3.31\text{x}10^{-08}$	$1.43\text{x}10^{-09}$	$3.99\text{x}10^{-07}$	$9.13\text{x}10^{-09}$	$3.61\text{x}10^{-09}$
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Tabel 4. Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)

		Prepupae	Pupae	Female adult	Male adult	Egg	Total of lifespan
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		$2.71x10^{-08}$	$6.29x10^{-06}$	$2.51x10^{-05}$	$1.02x10^{-07}$	$1.91x10^{-04}$	$1.33x10^{-09}$
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize (control) (Table 5). The mortality of the 6th larvae or the last instar fed on young maize colonized by *M. anisopliae* (57.67%) was the highest among other treatments, but it was not significantly different from those fed on maize colonized by *B. bassiana* of JgSPK isolate (51.33%). Mean of percentage of pupae and adults emergence of fungal treatments were significantly lower (P<0.0001) than those of control (Table 6). The percentage of pupae and adults emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize colonized with the fungi was not significantly different from those of control. The maize colonized with fungi significantly decreased eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 5. Mean of mortality of different instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

		Mean of mortality of different instar larvae (%)					
Isolate	Species	1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*
P-value		0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value		-	5.98	4.70	3.83	5.06	6.42

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 young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08**	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and colour change, such as no appetite and muddy body colour, and the dead larvae showed unique symptoms, specifically shrunken and hardens like a mummy, the larvae body was covered with fungal mycelia and became white or green colour depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colour, respectively (Figure 2). The result indicated that the fungal isolate from re-isolation from the cadavers was the same as the fungal isolate used for seed treatment of maize seeds. The colony morphology of the fungi resulted from re-isolation from the cadavers had white and greenish white colour for *B. bassiana* and *M. anisopliae*, respectively (Figure 3). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae and mycelia, and globose conidia, nevertheless the morphology of *M. anisopliae* had the green hyphae and

mycelia, and cylindrical conidia. The dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves not only could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* to be malformation (Figure 4, 5, and 6). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown in colour, and their integuments were harder than the healthy ones. The sick larvae could produce the abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and inability to fly.

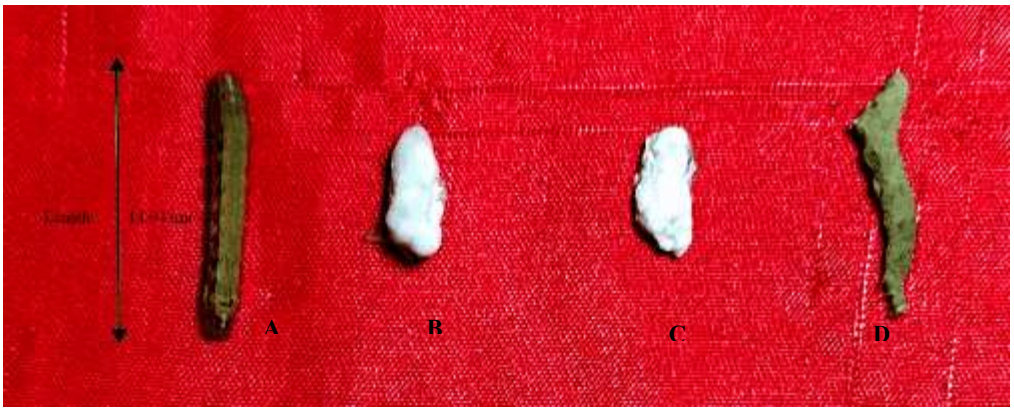

Figure 2. The cadavers from larvae fed on maize leaves untreated with fungi or control (A), and the cadavers from larvae fed on maize leaves colonized by *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

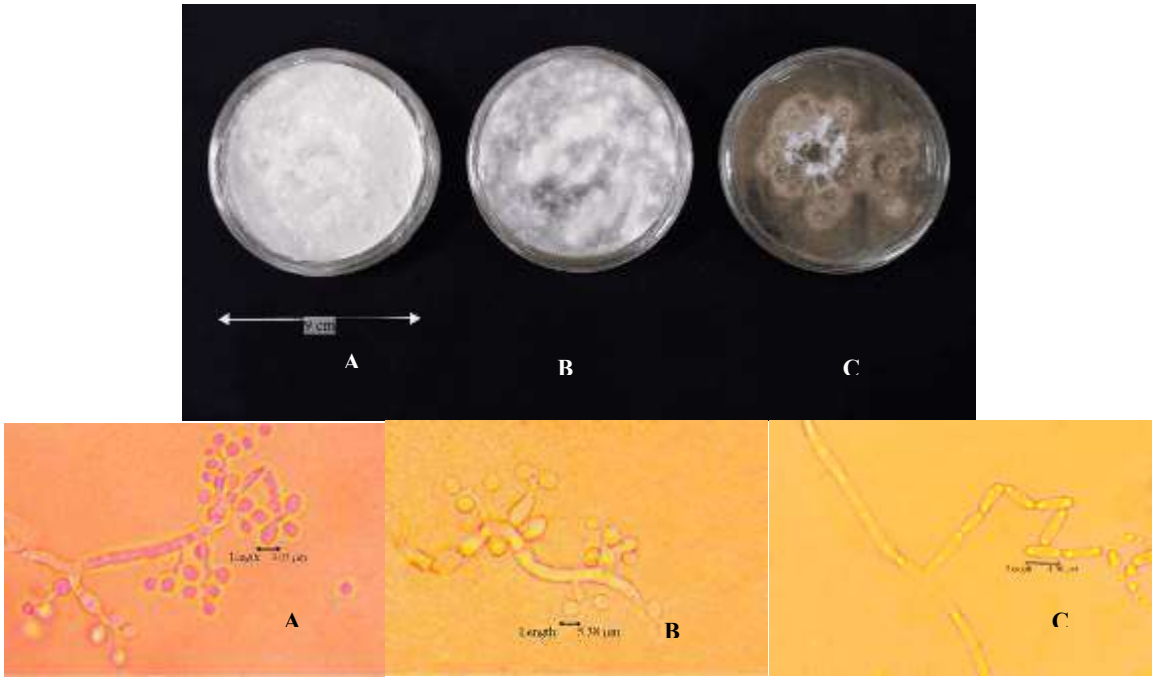

Figure 3. Colony morphology of endophytic fungi isolated from the cadavers, and cultured on SDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D) and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

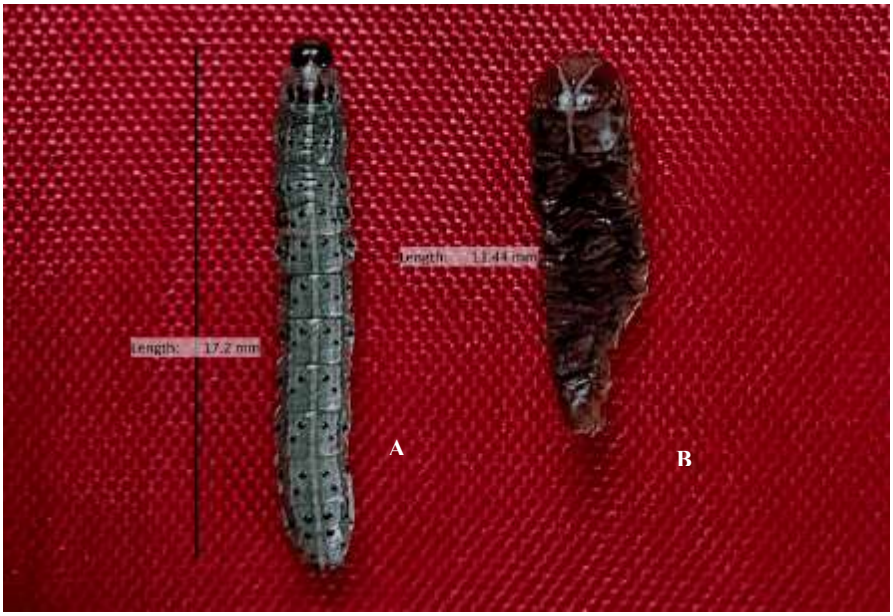

Figure 4. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

Figure 5. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 6. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia were able to colonize within the maize tissue including the leaves that were consumed by the neonate larvae. The fungal mycelia were not found whitin the leaves uninoculated by seed immersion treatment (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached

100% of leaves at 14 days after seed inoculation. The fungi, *B. bassiana* and *M. anisopliae* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo et al., 2020). The fungal endophytes remained to colonize more than 14 days after inoculation, the fungi were found within roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina et al., 2020). B. bassiana could be detected within entire plant growth cycle (120–140 days after sowing) and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga et al., 2014). *B. bassiana* and *M. anisopliae* isolates in the present study could be detected within entire maize tissue and this obtained finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is very susceptible to be attacked by *S. frugiperda* larvae (Supartha et al., 2021), so the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effective to be controlled by using the endophytic fungi compared to the fungal topical application (Gustianingtyas et al., 2021; Herlinda et al., 2021a).

B. bassiana and *M. anisopliae* in the present study inoculated as seed treatments of maize seeds prolonged developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased, however the adult longevity decreased. The obtained finding was in accordance with some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword, 2015; Hussain et al., 2009) due to the fungi reduced the conversion of digested and ingested food that could stimulate the larvae to develop more slowly (Hussain et al., 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in current study caused negative effects on the *S. frugiperda* development. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs, and enhance the larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo et al., 2020) due to the fungi produced secondary metabolites and caused mycosis on insect body (Vidal and Jaber, 2015). The fungal mycelia within the plant tissue fed on the larvae could produce blastospores in the larvae hemolymph, and the blastospores could produce secondary metabolites with their toxins that disrupted the normal cell metabolism and finally killed the insects (Mancillas-Paredes et al., 2019). The endophytic fungi also reduced the larvae appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas et al., 2021) because the fungi could produce secondary metabolites in planta resulting antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley, 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo et al., 2020). When, the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber, 2015). The current study showed that the mycosis occured on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The other previous study also showed that the mycosis could occure on the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo et al., 2020).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhance the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda.*

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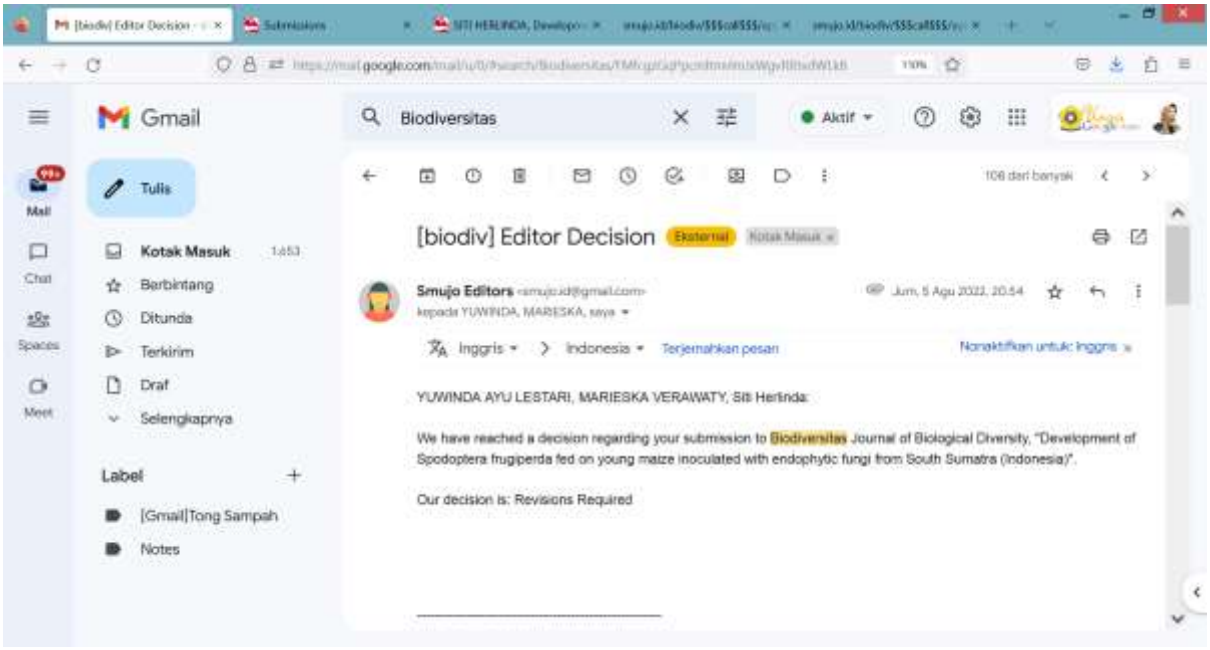
3. Bukti konfirmasi review kedua dan hasil revisi kedua



Development of *Spodoptera frugiperda* fed on young maize inoculated with endophytic fungi from South Sumatra (Indonesia)

Abstract. To control the hiding larvae of *Spodoptera frugiperda* is needed the endophytic entomopathogenic fungi. The objective of this research was to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda.*

Keywords: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

Abbreviations (if any): -

Running title: Development of *Spodoptera frugiperda* treated with endophytic fungi

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano et al., 2018). This pest comes from South America (Otim et al., 2018). The FAW came into Africa in 2016 (Goergen et al., 2016) and crossed over to Europe in 2017 (Early et al., 2018). In Asia, the FAW discovered for the first time in India in 2018 (Ganiger et al., 2018; Mahat et al., 2021) and on March 26, 2019 the pest came into Indonesia for the first time in West Sumatra (Sartiami et al., 2020). More recently, it has spread throughout Indonesia (Maharani et al., 2019; Ginting et al., 2020; Supartha et al., 2021) and becomes a new invasive pest in Indonesia (Herlinda et al., 2021b). As the polyphagous insect, the FAW 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). In Indonesia, the most severely attacked crop is maize (*Zea mays* L.) and it causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun et al., 2021) and 26.50% to 70% in Lampung (Lestari et al., 2020), and reaching 100% in South Sumatra (Herlinda et al., 2021b). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano et al., 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaves' midribs at daylight (Gustianingtyas et al., 2021) and this behavior makes the FAW larvae difficult to be controlled.

The FAW commonly controls using synthetic insecticides due to the fast action and easy application (Kumela et al., 2018) but the insecticide application causes the negative affect for the human health and environment (Harrison et al., 2019) and the resistances againts the pest (Zhang et al., 2021). An alternative eco-friendly control for FAW is by utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos, 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda et al. 2020). *Beauveria bassiana* (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam et al., 2020). The fungal topical application is less effective in the field (Gustianingtyas et al., 2021) because at daylight up to night the FAW larvae hide in the corn midribs (Herlinda et al., 2021a). To control the hiding larvae is needed the entomopathogenic fungi that are able to colonize in plant tissues (endophytic fungi) (Gustianingtyas et al., 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their hosts (Lira et al., 2020), can stimulate the plant growth and depress the insect growth (Russo et al., 2020).

The results of previous studies showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda et al., 2021a). However, there is no information on development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. In this study, the effect of young maize inoculated with endophytic fungi on *S. frugiperda* development was investigated. So, the objective of this research is to assess the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of *Spodoptera frugiperda*

Eggs of *S. frugiperda* obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya which have been mass-rearing since January 2020 (Herlinda et al., 2020) and was identified molecularly by (Herlinda et al., 2021b). The FAW were mass-reared in the laboratory at 29±1°C temperature, and 83% relative humidity (RH) and the lighting set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda et al., 2021a). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage placed also fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the collection of the Laboratory of Entomology and they were identified molecularly by Herlinda et al. (2021a). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073) (Table 1). The fungi were originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, assesing the ability of the fungi colonizing in maize tissue was carried out by maize seeds treated. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 days. Before treated with the fungi, the 45 corn seeds were surface sterilized by using the method of Russo et al. (2020). Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas the seed control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti et al. (2020). For confirming the fungi as endophytes, detecting the fungi colonizing the young maize tissues was carried out by cutting the tip leaves of 7 and 14-day old young maize and then the tip leaves were grown onto the SDA medium for detecting the mycelia of the endophytic fungi whitin the leaves. The rest young maize leaves were used for bioassays. Before the leaves grown onto the SDA medium, they were first surface-sterilized by immersion in 70% ethanol, sodium hypochlorite for 2 minutes, and rinsed twice in sterile distilled water (Russo et al., 2020). Finally, the last rinse water was also grown onto SDA medium. If on the last rinse water, no fungal growth was found, it corfirmed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungus growing on treated medium were endophytes.

The bioassay for assessing the effect of young maize inoculated with endophytic fungi on development of *Spodoptera frugiperda*

Assessing the effect of young maize inoculated with endophytic fungi on development of *S. frugiperda* was carried out at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged 28−29 °C and 82−83%, respectively.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	Herlinda et al. (2021a)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	Herlinda et al. (2021a)
Tanjung Payang. Pagar Alam	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	Herlinda et al. (2021a)

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on development of *S. frugiperda* followed the method of Russo et al. (2019). The leaves of young maize colonized by the endophytic fungi were provided to be consumed by the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda*, whereas for control, the non-treated leaves of young maize were consumed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves eaten up. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm) and fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae and replaced with new ones everyday. This experiment consisted of three fungal isolates and control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae were recorded everyday. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs and the dead larvae and pupae were grown in SDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily and their sex were recorded. The adults were placed in the wire mesh cage for copulation with fresh maize leaves inside for providing egg laying. Eggs laid by the adults were counted everyday. The adult longevity was determined by counting the time (days) from emergence until death.

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's test or Tukey's Honestly Significant Difference (HSD) test or was applied to determine the significant differences among the isolates at p = 0.05. All data were calculated using software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The young maize leaves colonized by the fungi when they were grown on to the SDA medium, the fungus grew and their mycelia covered the leaves. However, no mycelia were found on the leaves of untreated maize, and also on the final flushing water. This confirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophyte originating from the maize tissues. The results showed that a percentage of fungal colonization in leaves after being inoculated by seed immersion treatment increased from 7 to 14 days after seed inoculation, and at the 14 days after seed inoculation, all maize leaves colonized by the fungi (100%) (Table 2).

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 fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00c	0.00b
JgSPK	*Beauveria bassiana*	73.33b	100.00a
JgCrJr	*Beauveria bassiana*	53.33b	100.00a
CaTpPga	*Metarhizium anisopliae*	100.00a	100.00a
F-value		145.02*	143.40*
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

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

Effect of young maize colonized and non-colonized by fungi on the development of *Spodoptera frugiperda*

The first instar neonate larvae fed on leaves of fungal colonized maize caused the next instar significantly shifting their developmental time (P<0.0001) (Table 3). The developmental time of all instars fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time, however the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the total of lifespan of *S. frugiperda* increased significantly (P<0.0001). The longest total lifespan or generation time of *S. frugiperda* found in the treatment of *M. anisopliae* (46.87 days) among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize was significantly longer (P<0.0001) compared to those fed on leaves of non-colonized maize.

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d
JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		$4.66x10^{-08}$	$3.31x10^{-08}$	$1.43x10^{-09}$	$3.99x10^{-07}$	$9.13x10^{-09}$	$3.61x10^{-09}$
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Tabel 4. Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		Prepupae	Pupae	Female adult	Male adult	Egg	Total of lifespan
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		$2.71x10^{-08}$	$6.29x10^{-06}$	$2.51x10^{-05}$	$1.02x10^{-07}$	$1.91x10^{-04}$	$1.33x10^{-09}$
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize (control) (Table 5). The mortality of the last larval instar fed on young maize colonized by *M. anisopliae* (57.67%) was the highest among other treatments, but it was not significantly different from those fed on maize colonized by *B. bassiana* of JgSPK isolate (51.33%). Mean of percentage of pupae and adults emergence of fungal treatments were significantly lower (P<0.0001) than those of control (Table 6). The percentage of pupae and adults emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize colonized with the fungi was not significantly different from those of control. The maize colonized with fungi significantly decreased eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 5. The cummulative mortality of different instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The cummulative mortality of mortality of different instar larvae (%)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*
P-value		0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value		-	5.98	4.70	3.83	5.06	6.42

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 young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08**	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and colour change, such as no appetite and muddy body colour, and the dead larvae showed unique symptoms, specifically shrunken and hardens like a mummy, the larvae body was covered with fungal mycelia and became white or green colour depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colour, respectively (Figure 1). The result indicated that the fungal isolate from re-isolation from the cadavers was the same as the fungal isolate used for seed treatment of maize seeds. The colony morphology of the fungi resulted from re-isolation from the cadavers had white and greenish white colour for *B. bassiana* and *M. anisopliae*, respectively (Figure 2). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae and mycelia, and globose conidia, nevertheless the morphology of *M. anisopliae* had the green hyphae and mycelia, and cylindrical conidia. The dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves not only could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* could cause malformation (Figure 3, 4, and 5). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown in colour, and their integuments were harder than the healthy ones. The sick larvae could produce the abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and inability to fly.

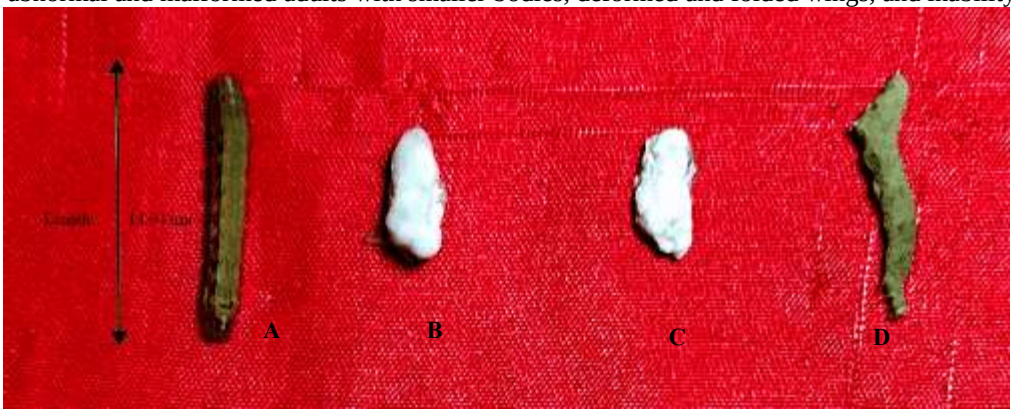


Figure 1. The cadavers from larvae fed on maize leaves untreated with fungi or control (A), and the cadavers from larvae fed on maize leaves colonized by *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

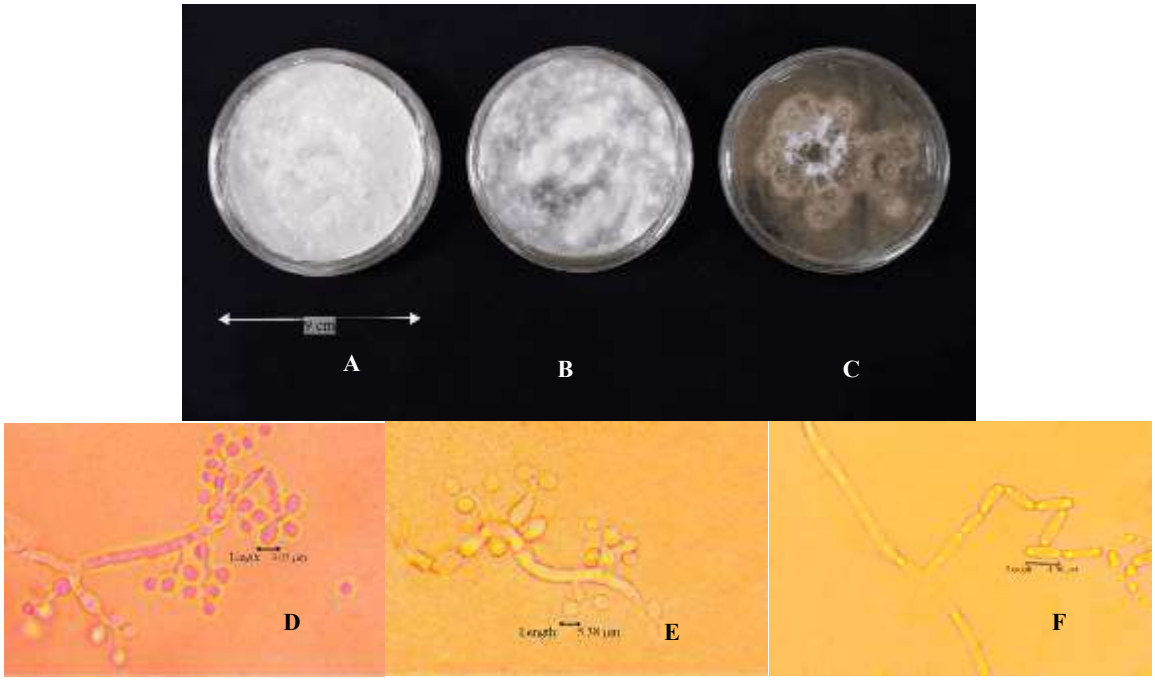


Figure 2. Colony morphology of endophytic fungi isolated from the cadavers, and cultured on SDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D) and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

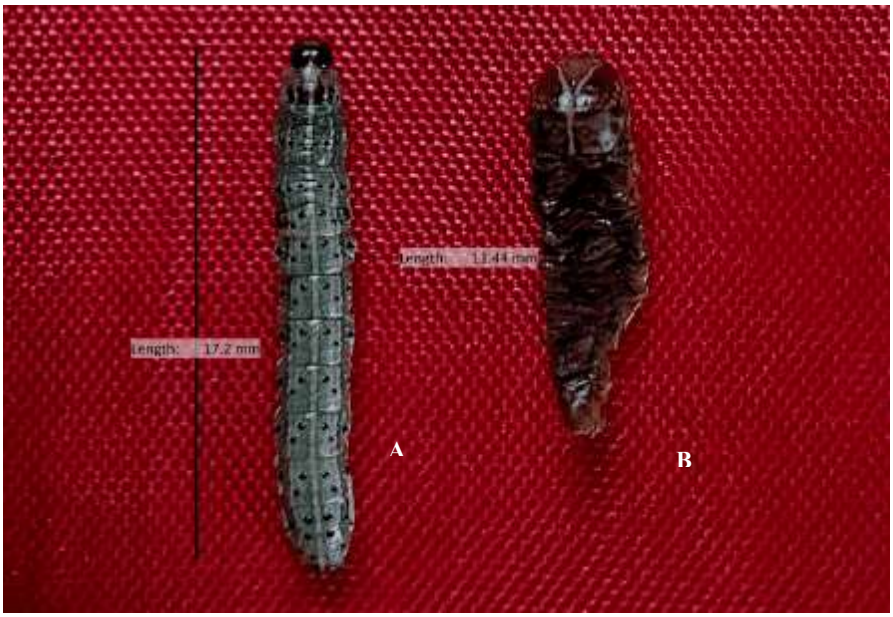


Figure 3. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

Figure 4. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 5. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia were able to colonize within the maize tissue including the leaves that were consumed by the neonate larvae. The fungal mycelia were not found whitin the leaves uninoculated by seed immersion treatment (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached 100% of leaves at 14 days after seed inoculation. The fungi, *B. bassiana* and *M. anisopliae* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo et al., 2020). The fungal endophytes remained to colonize more than 14 days after inoculation, the fungi were found within roots, stems, and leaves of tomato up to 30 days after inoculation (Carolina et al., 2020). *B. bassiana* could be detected within entire plant growth cycle (120–140 days after sowing) and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga et al., 2014). *B. bassiana* and *M. anisopliae* isolates in the present study could be detected within entire maize tissue and this obtained finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is very susceptible to be attacked by *S. frugiperda* larvae (Supartha et al., 2021), so the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effective to be controlled by using the endophytic fungi compared to the fungal topical application (Gustianingtyas et al., 2021; Herlinda et al., 2021a).

B. bassiana and *M. anisopliae* in the present study inoculated as seed treatments of maize seeds prolonged developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased, however the adult longevity decreased. The obtained finding was in accordance with some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword, 2015; Hussain et al., 2009) due to the fungi reduced the conversion of digested and ingested food that could stimulate the larvae to develop more slowly (Hussain et al., 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in current study caused negative effects on the *S. frugiperda* development. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs, and enhance the larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo et al., 2020) due to the fungi produced secondary metabolites and

caused mycosis on insect body (Vidal and Jaber, 2015). The fungal mycelia within the plant tissue fed on the larvae could produce blastospores in the larvae hemolymph, and the blastospores could produce secondary metabolites with their toxins that disrupted the normal cell metabolism and finally killed the insects (Mancillas-Paredes et al., 2019). The endophytic fungi also reduced the larvae appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas et al., 2021) because the fungi could produce secondary metabolites in planta resulting antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley, 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo et al., 2020). When, the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber, 2015). The current study showed that the mycosis occured on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The other previous study also showed that the mycosis could occur on the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo et al., 2020).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments, but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the *S. frugiperda* development. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda.*

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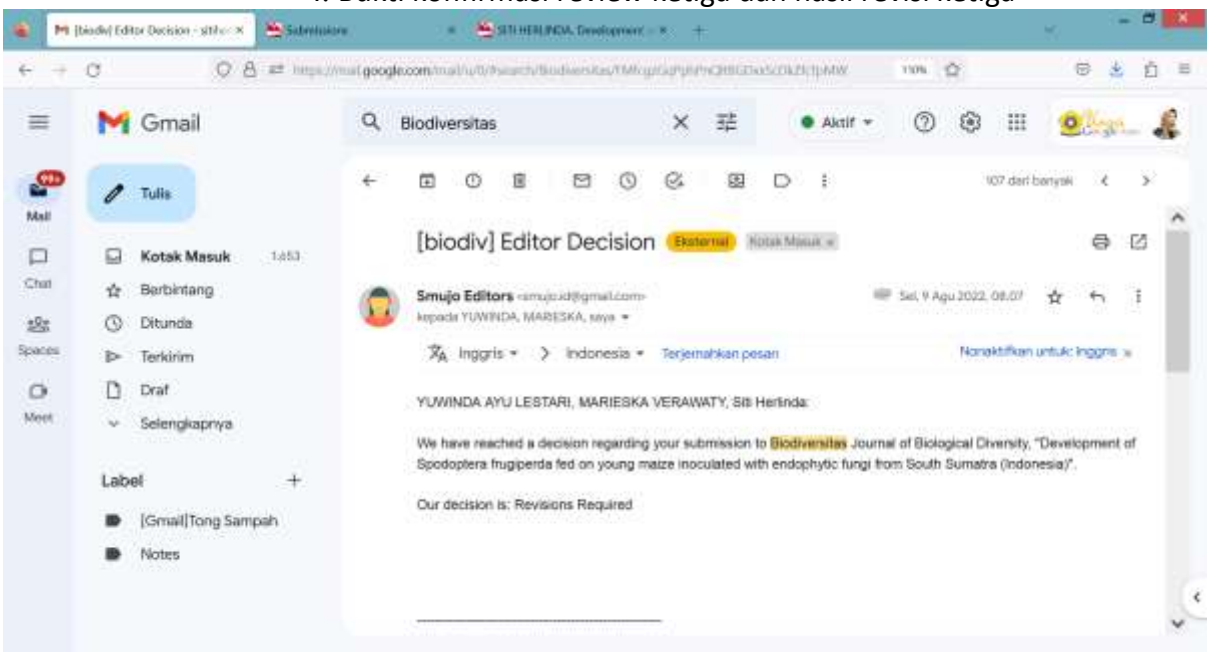
4. Bukti konfirmasi review ketiga dan hasil revisi ketiga



Development of *Spodoptera frugiperda* fed on young maize inoculated with endophytic fungi from South Sumatra (Indonesia)

Abstract. The endophytic entomopathogenic fungi need to control the hiding larvae of *Spodoptera frugiperda*. The objective of this research was to assess the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). The developmental time (eggs, larvae, pupae stages, and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

Keywords: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

Abbreviations (if any): -

Running title: Development of *Spodoptera frugiperda* treated with endophytic fungi

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano et al., 2018). This pest comes from South America (Otim et al., 2018). The FAW came into Africa in 2016 (Goergen et al., 2016) and crossed over to Europe in 2017 (Early et al., 2018). In Asia, the FAW was discovered for the first time in India in 2018 (Ganiger et al., 2018; Mahat et al., 2021), and on March 26, 2019, the pest came into Indonesia for the first time in West Sumatra (Sartiami et al., 2020). More recently, it has spread throughout Indonesia (Maharani et al., 2019; Ginting et al., 2020; Supartha et al., 2021) and has become a new invasive pest in Indonesia (Herlinda et al., 2021b) As a polyphagous insect the FAW 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). It causes about a third of the annual maize production losses, about 1 million tonnes in Kenya (De Groote et al., 2020) and 18 million tons/year in 12 African countries. The losses reach the US $ 13 million (Harrison et al., 2019). The most severely attacked crop in Indonesia is maize (*Zea mays* L.). It causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun et al., 2021) and 26.50% to 70% in Lampung (Lestari et al., 2020), and reaching 100% in South Sumatra (Herlinda et al., 2021b). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano et al., 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaf's midribs at daylight (Gustianingtyas et al., 2021), and this behavior makes the FAW larvae difficult to be controlled.

The FAW commonly controls using synthetic insecticides due to the fast action and easy application (Kumela et al., 2018). Still, the insecticide application has a negative effect on human health and the environment (Harrison et al., 2,019) and the resistance against the pest (Zhang et al., 2021). An alternative eco-friendly control for FAW is utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos, 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda et al. 2020). *Beauveria bassiana* (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam et al., 2020). The topical fungal application is less effective in the field (Gustianingtyas et al., 2021) because from daylight up to the night, the FAW larvae hide in the corn midribs (Herlinda et al., 2021a). To control the hiding larvae, the entomopathogenic fungi can colonize in plant tissues (endophytic fungi) (Gustianingtyas et al., 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their hosts (Lira et al., 2020), can stimulate plant growth, and depress insect growth (Russo et al., 2020).

The results of previous studies showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda et al., 2021a). However, there is no information on the development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. This study investigated the effect of young maize inoculated with endophytic fungi on S. frugiperda development. So, the objective of this research is to assess the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of *Spodoptera frugiperda*

Eggs of *S. frugiperda* were obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya, which have been mass-rearing since January 2020 (Herlinda et al., 2020) and were identified molecularly by (Herlinda et al., 2021b). The FAW was mass-reared in the laboratory at 29°C temperature, and 83% relative humidity (RH), and the lighting was set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda et al., 2021a). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage also placed fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the Laboratory of Entomology collection and were identified molecularly by Herlinda et al. (2021a). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073) (Table 1). The fungi were originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, assessing the ability of the fungi to colonize in maize tissue was carried out by maize seeds treated. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 days. Before being treated with the fungi, the 45 corn seeds were surface sterilized using Russo et al. (2020) method. Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas

the seed control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti et al. (2020). To confirm the fungi as endophytes, detecting the fungi colonizing the young maize tissues was done by cutting the tip leaves of 7 and 14-day-old young maize. Then the tip leaves were grown onto the SDA medium to detect the mycelia of the endophytic fungi within the leaves. The rest young maize leaves were used for bioassays. Before the leaves grew onto the SDA medium they were first surface-sterilized by immersion in 70% ethanol and sodium hypochlorite for 2 minutes and rinsed twice in sterile distilled water (Russo et al., 2020). Finally, the last rinse water was also grown onto the SDA medium. If no fungal growth was found on the last rinse water, it confirmed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungus growing on the treated medium were endophytes.

The bioassay for assessing the effect of young maize inoculated with endophytic fungi on the development of *Spodoptera frugiperda*

Assessing the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda* was conducted at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged from 28−29 °C and 82−83%, respectively.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia, used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	Herlinda et al. (2021a)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	Herlinda et al. (2021a)
Tanjung Payang. Pagar Alam	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	Herlinda et al. (2021a)

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on the development of *S. frugiperda* followed the method of Russo et al. (2019). The leaves of young maize colonized by the endophytic fungi were consumed by the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda*, whereas, for control, the non-treated leaves of young maize were consumed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves were eaten. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm), fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae, and replaced with new ones every day. This experiment consisted of three fungal isolates, and the control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae was recorded every day. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs, dead larvae, and pupae were grown in aSDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily, and their sex was recorded. The adults were placed in the wire mesh cage for copulation with fresh maize leaves to provide egg laying. Eggs laid by the adults were counted every day. Adult longevity was determined by counting the time (days) from emergence until death.

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's or Tukey's Honestly Significant Difference (HSD) test was applied to determine the significant differences among the isolates at p = 0.05. All data were calculated using the software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The fungi colonized the young maize leaves when they were grown onto the SDA medium, the fungus grew, and their mycelia covered the leaves. However, no mycelia were found on untreated maize leaves and in the final flushing water. This confirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophytes originating from the maize tissues. The results showed that a percentage of fungal colonization in leaves after being inoculated by seed immersion treatment increased from 7 to 14 days after seed inoculation, and at the 14 days after seed inoculation, all maize leaves were colonized by the fungi (100%) (Table 2).

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 fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00c	0.00b
JgSPK	*Beauveria bassiana*	73.33b	100.00a
JgCrJr	*Beauveria bassiana*	53.33b	100.00a
CaTpPga	*Metarhizium anisopliae*	100.00a	100.00a
F-value		145.02^{*}	143.40^{*}
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

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

Effect of young maize colonized and non-colonized by fungi on the development of *Spodoptera frugiperda*

The first instar neonate larvae fed on leaves of fungal colonized maize caused the next instar significantly shift their developmental time (P<0.0001) (Table 3). The developmental time of all instars fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time; however, the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the total lifespan of *S. frugiperda* to increase significantly (P<0.0001). The longest total lifespan or generation time of *S. frugiperda* was found in *M. anisopliae* (46.87 days), among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize was significantly longer (P<0.0001) compared to those fed on leaves of non-colonized maize.

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d
JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		$4.66x10^{-08}$	$3.31x10^{-08}$	$1.43x10^{-09}$	$3.99x10^{-07}$	$9.13x10^{-09}$	$3.61x10^{-09}$
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Table 4. Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		Prepupae	Pupae	Female adult	Male adult	Egg	Total of lifespan
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		$2.71x10^{-08}$	$6.29x10^{-06}$	$2.51x10^{-05}$	$1.02x10^{-07}$	$1.91x10^{-04}$	$1.33x10^{-09}$
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize (control) (Table 5). The mortality of the last larval instar fed on young maize colonized by *M. anisopliae* (57.67%) was the highest among other treatments. Still, it was not significantly different from those feds on maize colonized by *B. bassiana* of JgSPK isolate (51.33%). The mean percentage of pupae and adults' emergence of fungal treatments was significantly lower (P<0.0001) than those of the control (Table 6). The percentage of pupae and adults' emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize colonized with the fungi was not significantly different from those of the control. The maize colonized with fungi significantly decreased eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 5. The cumulative mortality of different instar larvae of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The cumulative mortality of different instar larvae (%)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*
P-value		0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value		-	5.98	4.70	3.83	5.06	6.42

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 percentage of pupae and adult emergence, sex ratio, an egg laid, and viable eggs of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08**	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and color change, such as no appetite and muddy body color. The dead larvae showed unique symptoms, specifically shrunken and hardened like a mummy; the larvae body was covered with fungal mycelia. It became white or green depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colors, respectively (Figure 1). The result indicated that the fungal isolate from re-isolation of the cadavers was the same as the fungal isolate used for seed treatment of maize seeds. The colony morphology of the fungi resulted from re-isolation from the cadavers with white and greenish white for *B. bassiana* and *M. anisopliae*, respectively (Figure 2). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae, mycelia, and globose conidia. Nevertheless, the morphology of *M. anisopliae* had green hyphae and mycelia and cylindrical conidia. The dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* could cause malformation (Figures 3, 4, and 5). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown, and their integuments were harder than the healthy ones. The sick larvae could produce abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and an inability to fly.

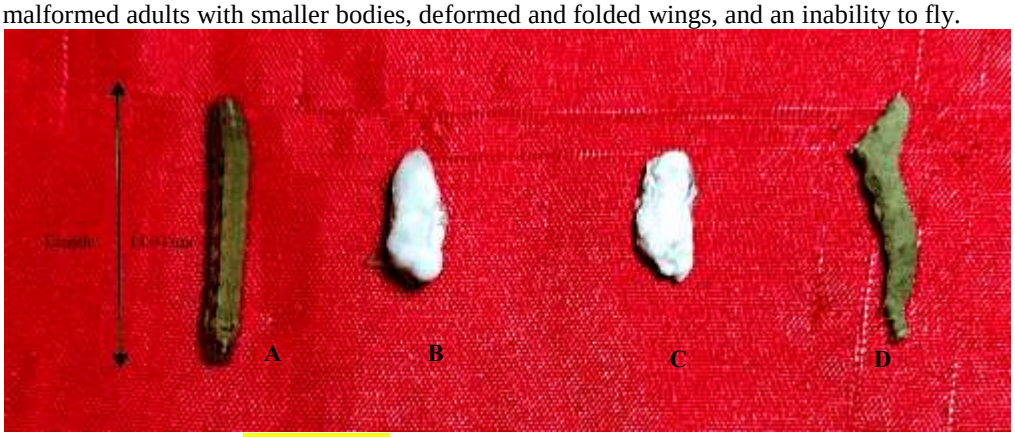


Figure 1. The cadavers from larvae fed on maize leave untreated with fungi or control (A), and the cadavers from larvae fed on maize leaves colonized by *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D)

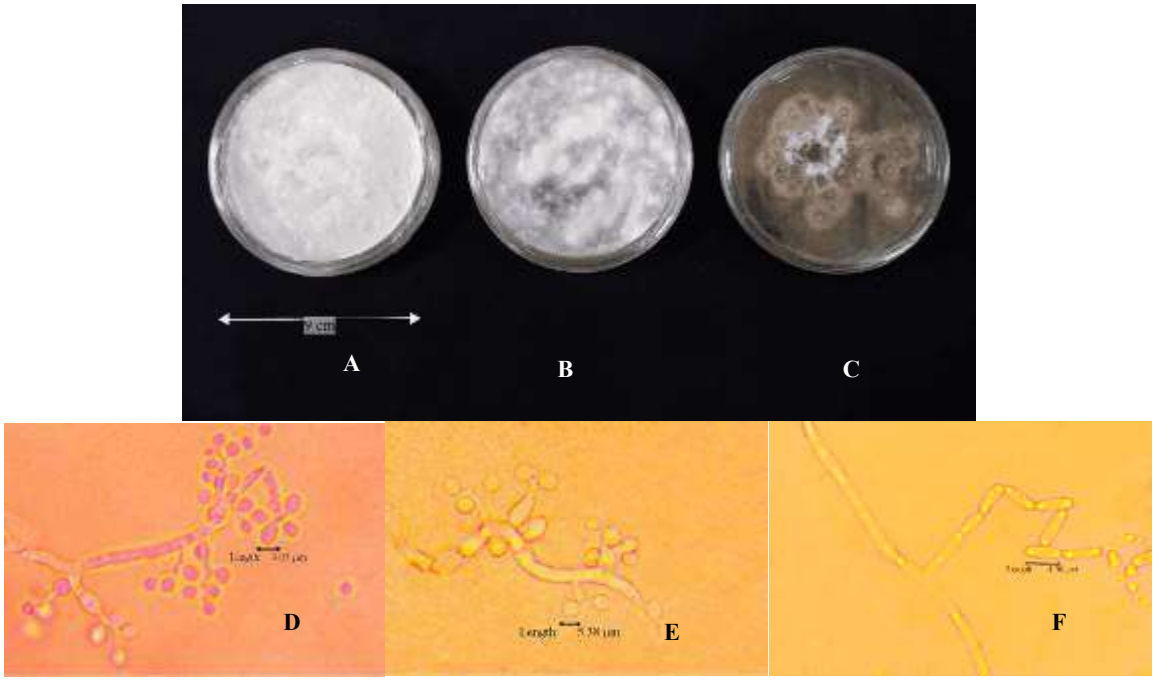


Figure 2. Colony morphology of endophytic fungi isolated from the cadavers and cultured on SDA media (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D), and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

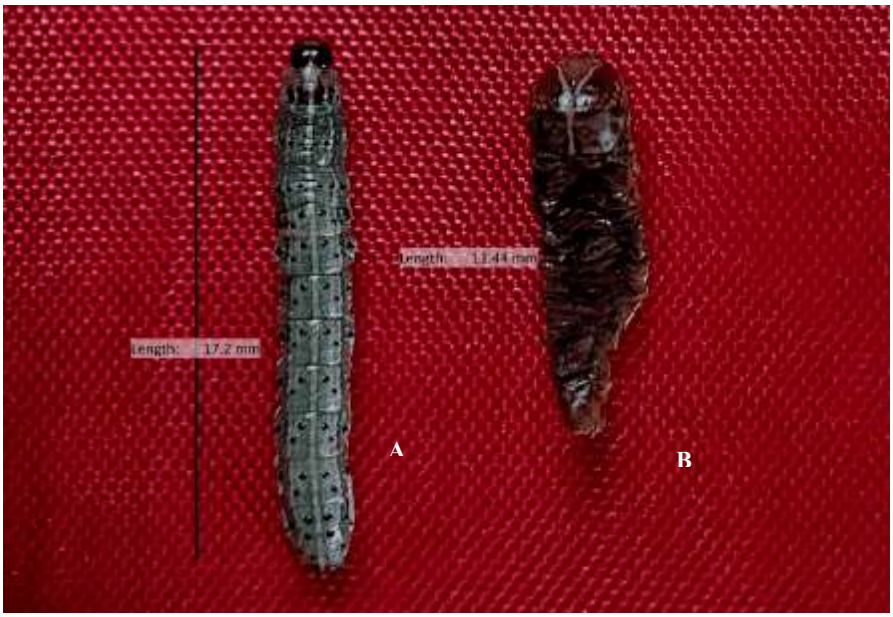


Figure 3. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

Figure 4. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 5. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study were confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia could colonize within the maize tissue, including the leaves consumed by the neonate larvae. The fungal mycelia were not found within the leaves uninoculated by seed immersion treatment (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached 100% of leaves 14 days after seed inoculation. The fungi, *B. bassiana*, and *M. anisopliae* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo et al., 2020). The fungal endophytes remained to colonize more than 14 days after inoculation; the fungi were found within the roots, stems, and leaves of tomatoes up to 30 days after inoculation (Carolina et al., 2020). *B. bassiana* could be detected within the entire plant growth cycle (120–140 days after sowing), and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga et al., 2014). In the present study, B. bassiana and M. anisopliae isolates could be detected within entire maize tissue. This finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is very susceptible to attack by S. frugiperda larvae (Supartha et al., 2021), the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant's defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effectively controlled by using the endophytic fungi than the topical fungal application (Gustianingtyas et al., 2021; Herlinda et al., 2021a).

In the present study, *B. bassiana and M. anisopliae* inoculated as maize seed treatments prolonged the developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased; however, the adult longevity decreased. Those finding follow some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword, 2015; Hussain et al., 2009) due to the fungi reduced the conversion of digested and ingested food which could stimulate the larvae to develop more slowly (Hussain et al., 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in the current study caused negative effects on the development of *S. frugiperda*. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs and enhance larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo et al., 2020) due to the fungi produced secondary metabolites.

The secondary metabolites caused mycosis in the insect body (Vidal and Jaber, 2015). The fungal mycelia within the plant tissue fed on the larvae could produce blastospores in the larvae hemolymph. The blastospores could produce secondary metabolites with toxins that disrupt the normal cell metabolism and kill the insects (Mancillas-Paredes et al., 2019). The endophytic fungi also reduced the larvae's appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas et al., 2021) because the fungi could produce secondary metabolites in planta resulting in antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley, 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo et al., 2020). When the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber, 2015). The current study showed that the mycosis occurred on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The previous study also showed that the mycosis could occur on the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo et al., 2020).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

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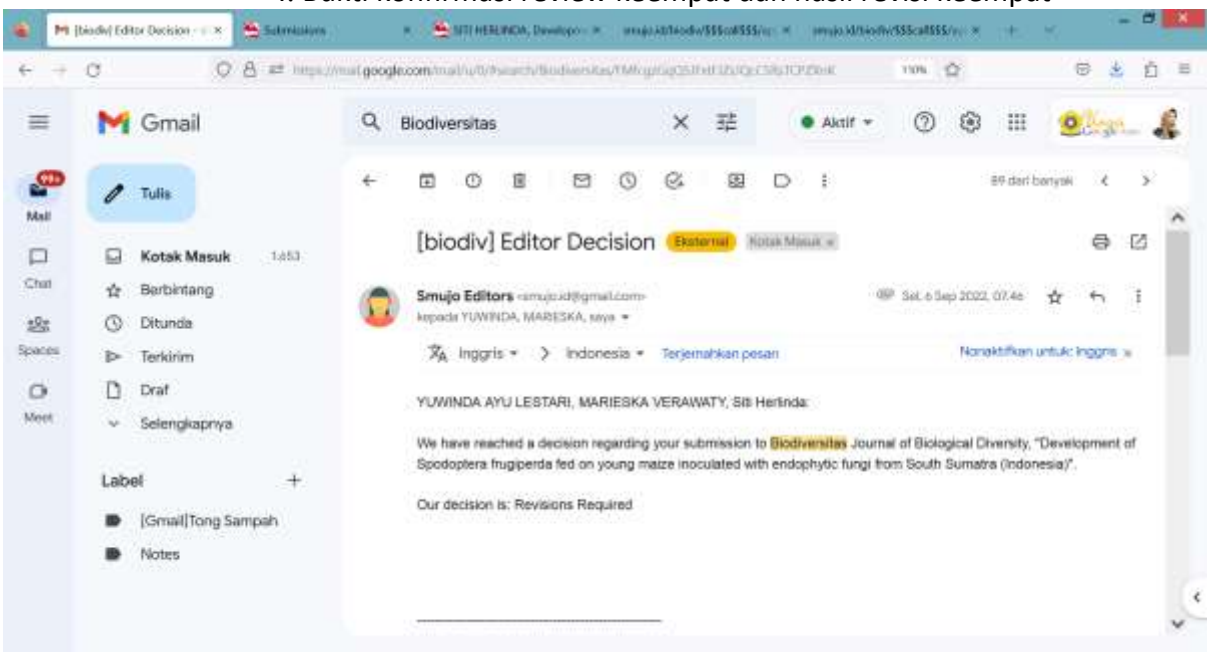
4. Bukti konfirmasi review keempat dan hasil revisi keempat



Development of *Spodoptera frugiperda* fed on young maize plant's fresh leaves inoculated with endophytic fungi from South Sumatra, Indonesia

Abstract. The endophytic entomopathogenic fungi is needed to control the *Spodoptera frugiperda* larvae hiding inside the maize plants. The objective of this research was to assess the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize plant significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs of *S. frugiperda*. The fungi also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). The developmental time (eggs, larvae, pupae stages, and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

Keywords: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

Abbreviations (if any): -

Running title: Development of *Spodoptera frugiperda* treated with endophytic fungi

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano et al., 2018). This pest comes from South America (Otim et al. 2018). The FAW came into Africa in 2016 (Goergen et al. 2016) and crossed over to Europe in 2017 (Early et al. 2018). In Asia, the FAW was discovered for the first time in India in 2018 (Ganiger et al. 2018; Mahat et al. 2021), and on March 26, 2019, the pest came into Indonesia for the first time in West Sumatra (Sartiami et al. 2020). More recently, it has spread throughout Indonesia (Maharani et al. 2019; Ginting et al., 2020; Supartha et al. 2021) and has become a new invasive pest in Indonesia (Herlinda et al. 2021b) As a polyphagous insect the FAW 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). It causes about a third of the annual maize production losses, about 1 million tonnes in Kenya (De Groote et al., 2020) and 18 million tons/year in 12 African countries. The losses reach the US $ 13 million (Harrison et al. 2019). The most severely attacked crop in Indonesia is maize (*Zea mays* L.). It causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun et al. 2021) and 26.50% to 70% in Lampung (Lestari et al. 2020), and reaching 100% in South Sumatra (Herlinda et al. 2021b). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano et al. 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaf"s midribs at daylight (Gustianingtyas et al.2021), and this behavior makes the FAW larvae difficult to be controlled.

The FAW is commonly controlled using synthetic insecticides due to the fast action and easy application (Kumela et al., 2018). Still, the iinsecticide application has a negative effect on human health and the environment (Harrison et al. 2,019) and the resistance against the pest (Zhang et al. 2021). An alternative eco-friendly control for FAW is utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda et al. 2020). *Beauveria bassiana* (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam et al. 2020). The topical fungal application is less effective in the field (Gustianingtyas et al. 2021) because from daylight up to the night, the FAW larvae hide in the corn midribs (Herlinda et al. 2021a). To control the hiding larvae, the entomopathogenic fungi can colonize the plant tissues (endophytic fungi) (Gustianingtyas et al. 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their hosts (Lira et al. 2020), can stimulate plant growth, and depress insect growth (Russo et al. 2020).

The results of previous studies showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda et al. 2021a). However, there is no information on the development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. This study investigated the effect of young maize plant inoculated with endophytic fungi on *S. frugiperda* development. So, the objective of this research was to assess the effect of young maize plants inoculated with endophytic fungi on the development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of *Spodoptera frugiperda*

Eggs of *S. frugiperda* were obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya, which have been mass-reared since January 2020 (Herlinda et al. 2020) and were identified molecularly (Herlinda et al. 2021b). The FAW was mass-reared in the laboratory at 29°C temperature, and 83% relative humidity (RH), and the lighting was set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda et al., 2021a). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage also placed fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the Laboratory of Entomology collection and were identified molecularly by Herlinda et al. (2021a). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolate (GenBank acc. no. MZ242073) (Table 1). The fungi were originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, assessing the ability of the fungi to colonize maize tissue was carried out by treating the maize seeds. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 days. Before being treated with the fungi, the 45 corn seeds were surface sterilized using Russo et al. (2020) method. Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas the control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti et al. (2020). To confirm the fungi as endophytes, detecting the fungi colonizing the young maize tissues was done by cutting the tip leaves of 7 and 14-day-old young maize. Then the tip leaves were grown onto the SDA medium to detect the mycelia of the endophytic fungi within the leaves. The rest young maize leaves were used for bioassays. Before the leaves colonized with the fungi grew onto the SDA medium they were first surface-sterilized by immersion in 70% ethanol and sodium hypochlorite for 2 minutes and rinsed twice in sterile distilled water (Russo et al., 2020). Finally, the last rinse water was also grown onto the SDA medium. If no fungal growth was found on the last rinse water, it confirmed that the surface-sterilization of maize tissues eliminated the epiphytic microorganisms and the fungus growing on the treated medium were endophytes.

The bioassay to assess the effect of young maize inoculated with endophytic fungi on *Spodoptera frugiperda* development

Assessing the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda* was conducted at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged from 28−29 °C and 82−83%, respectively.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia, used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	Herlinda et al. (2021a)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	Herlinda et al. (2021a)
Tanjung Payang. Pagar Alam	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	Herlinda et al. (2021a)

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on the development of *S. frugiperda* followed the method of Russo et al. (2019). The fresh leaves of young maize colonized by the endophytic fungi were provided to the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda* as feed, whereas, for control, the non-treated leaves of young maize were provided as feed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves were eaten. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm), fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae, and replaced with new ones every day. This experiment consisted of three fungal isolates, and the control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae was recorded every day. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs, dead larvae, and pupae were grown in a SDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily, and their sex was recorded. The adults were placed in the wire mesh cage for copulation with fresh maize leaves to provide egg laying. Eggs laid by the adults were counted every day. Adult longevity was determined by counting the time (days) from emergence until death.

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's or Tukey's Honestly Significant Difference (HSD) test was applied to determine the significant differences among means of the isolates at p = 0.05. All data were calculated using the software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates, and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The fungi colonized the young maize leaves when they were grown onto the SDA medium, the fungus grew, and their mycelia covered the leaves. However, no mycelia were found on untreated maize leaves and in the final flushing water. This confirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophytes originating from the maize tissues. The results showed that a percentage of fungal colonization in leaves after being inoculated by seed immersion treatment increased from 7 to 14 days after seed inoculation. At the 14 days after seed inoculation, all maize leaves were colonized by the fungi (100%)(Table 2). The percentage of fungal colonization was determined or calculated as number of sampled leave tissue showing fungal outgrowth divided by the total number of plated leave tissue samples x 100.

Table 2. The effect of endophytic-entomopathogenic fungal isolates on mean maize leaf colonization (%) at 7 and 14 days after inoculation

Isolate	Species	Mean fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00c	0.00b
JgSPK	*Beauveria bassiana*	73.33b	100.00a
JgCrJr	*Beauveria bassiana*	53.33b	100.00a
CaTpPga	*Metarhizium anisopliae*	100.00a	100.00a
F-value		145.02*	143.40*
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

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

Effect of colonized fresh leaf of young maize on *Spodoptera frugiperda* evelopment

The first instar neonate larvae fed on fungal colonized maize leaf caused the next instar significantly shift their developmental time (P<0.0001) (Table 3). The developmental time of all instars fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time; however, the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the total lifespan of *S. frugiperda* to increase significantly (P<0.0001). Longevity is the age of adult but lifespan is the length of time from egg stage up to adult of death. The longest total lifespan or generation time of *S. frugiperda* was found in *M. anisopliae* (46.87 days), among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize leaves was significantly longer (P<0.0001) compared to those fed on non-colonized maize leaves.

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d
JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		$4.66x10^{-08}$	$3.31x10^{-08}$	$1.43x10^{-09}$	$3.99x10^{-07}$	$9.13x10^{-09}$	$3.61x10^{-09}$
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Table 4. Length of different developmental stages of pupae and adults of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		Prepupae	Pupae	Female adult	Male adult	Egg	Total lifespan
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		$2.71x10^{-08}$	$6.29x10^{-06}$	$2.51x10^{-05}$	$1.02x10^{-07}$	$1.91x10^{-04}$	$1.33x10^{-09}$
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize leaves (control) (Table 5). The mortality of the last larval instar fed on young maize leaves colonized by *M. anisopliae* (57.67%) was the highest among other treatments. Still, it was not significantly different from those feds on maize leaves colonized by *B. bassiana* of JgSPK isolate (51.33%). The mean percentage of pupae and adults' emergence of fungal treatments was significantly lower (P<0.0001) than those of the control (Table 6). The percentage of pupae and adults' emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize leaves colonized with the fungi was not significantly different from those of the control. The maize leaves colonized with fungi significantly decreased the number of eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 5. The cumulative mortality of different larvae instars of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The cumulative mortality of different instar larvae (%)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*

P-value	0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value	-	5.98	4.70	3.83	5.06	6.42

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. Original data were transformed using Arcsin transformation prior to statistical analysis

Table 6. Mean percentage of pupae and adult emergence, sex ratio, an egg laid, and viable eggs of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	Fungal species	Pupae emergence (%)	Adult emergence (%)	Sex ratio	Eggs laid per female	Viable (hatched) eggs (%)
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08*	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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. Original data pupae and adult emergence of were transformed using Arcsin transformation prior to statistical analysis

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and color change, such as no appetite and muddy body color. The dead larvae showed unique symptoms, specifically shrunken and hardened like a mummy; the larvae body was covered with fungal mycelia and became white or green,depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colors, respectively (Figure 1). The result indicated that the fungal isolate from re-isolation of the cadavers was the same as the fungal isolate used for maize seed treatment. The colony morphology of the fungi was resulted from re-isolation from the cadavers with white and greenish white for *B. bassiana* and *M. anisopliae*, respectively (Figure 2). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae, mycelia, and globose conidia. Nevertheless, the morphology of *M. anisopliae* had green hyphae and mycelia and cylindrical conidia. The dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* could cause malformation (Figures 3, 4, and 5). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown, and their integuments were harder than the healthy ones. The sick larvae could produce abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and an inability to fly.

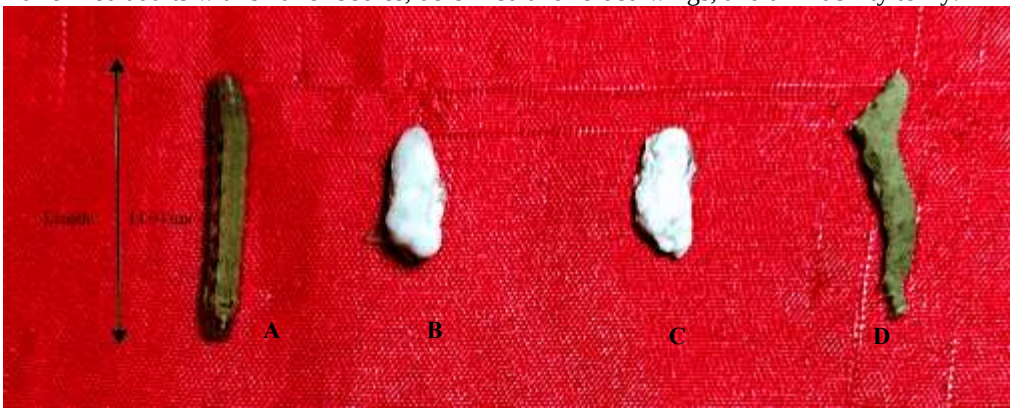


Figure 1. The cadavers from larvae fed on maize leave untreated with fungi or control (A), and the cadavers from larvae fed on maize leaves colonized by *Beauveria bassiana* of JgSPK isolate and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D) incubated for 14 days

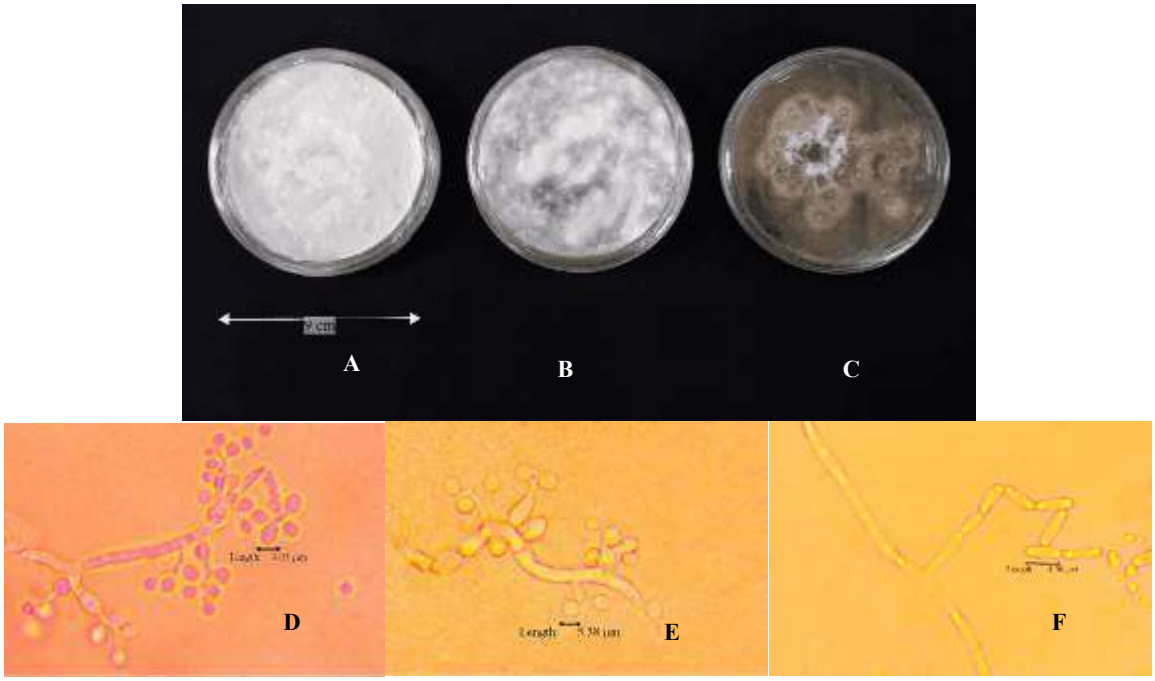


Figure 2. Colony morphology of endophytic fungi isolated from the cadavers and cultured on SDA media for 14 days (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D), and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

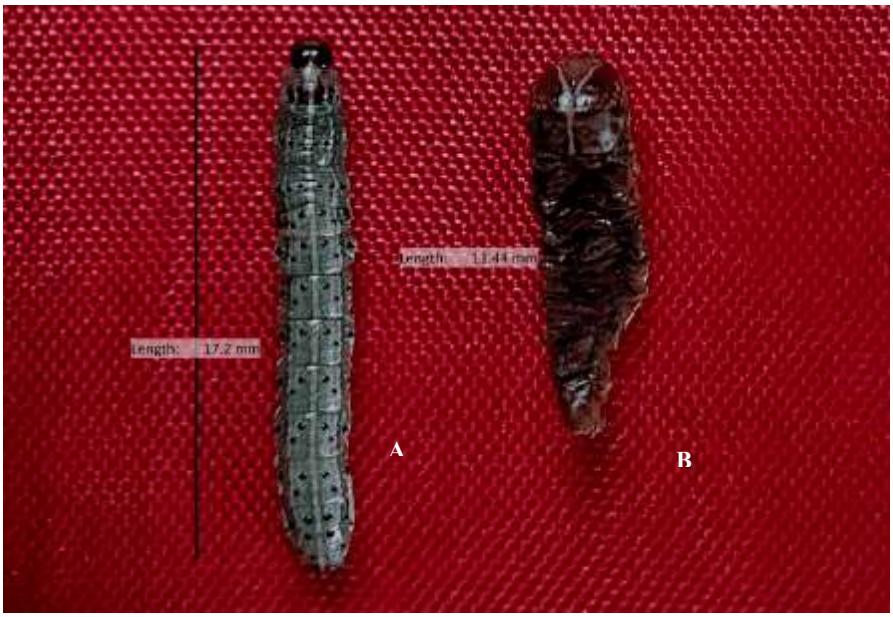


Figure 3. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

Figure 4. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 5. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study were confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia could colonize the maize tissue, including the leaves consumed by the neonate larvae. The fungal mycelia were not found within the uninoculated maize leaves (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached 100% of leaves 14 days after seed inoculation. The fungi, *B. bassiana*, and *M. anisopliae* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo et al. 2020). The fungal endophytes remained able to colonize after more than 14 days ; the fungi were found within the roots, stems, and leaves of tomatoes up to 30 days after inoculation (Carolina et al. 2020). *B. bassiana* could be detected within the entire plant growth cycle (120–140 days after sowing), and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga et al. 2014). In the present study, *B. bassiana* and *M. anisopliae* isolates could be detected within the entire maize tissue. This finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is highly susceptible *S. frugiperda* larvae (Supartha et al. 2021), the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant's defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effectively controlled by using the endophytic fungi than the topical fungal application (Gustianingtyas et al. 2021; Herlinda et al. 2021a).

In the present study, *B. bassiana and M. anisopliae* inoculated as maize seed treatments prolonged the developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased; however, the adult longevity decreased. Those finding follow some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword 2015; Hussain et al. 2009) because the fungi reduced the conversion of digested and ingested food, which could stimulate the larvae to develop more slowly (Hussain et al. 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in the current study caused negative effects on the development of *S. frugiperda*. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs and enhance larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo et al. 2020) due to the fungi produced secondary metabolites. The secondary metabolites caused mycosis in the insect body (Vidal and Jaber 2015). The fungal mycelia within the plant

tissue fed on the larvae could produce blastospores in the larvae hemolymph. The blastospores could produce secondary metabolites with toxins that disrupt the normal cell metabolism and kill the insects (Mancillas-Paredes et al. 2019). The endophytic fungi also reduced the larvae's appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas et al. 2021) because the fungi could produce secondary metabolites in planta resulting in antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo et al. 2020). When the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber 2015). The current study showed that the mycosis occurred on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The previous study also showed that the mycosis could occur on the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo et al. 2020).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

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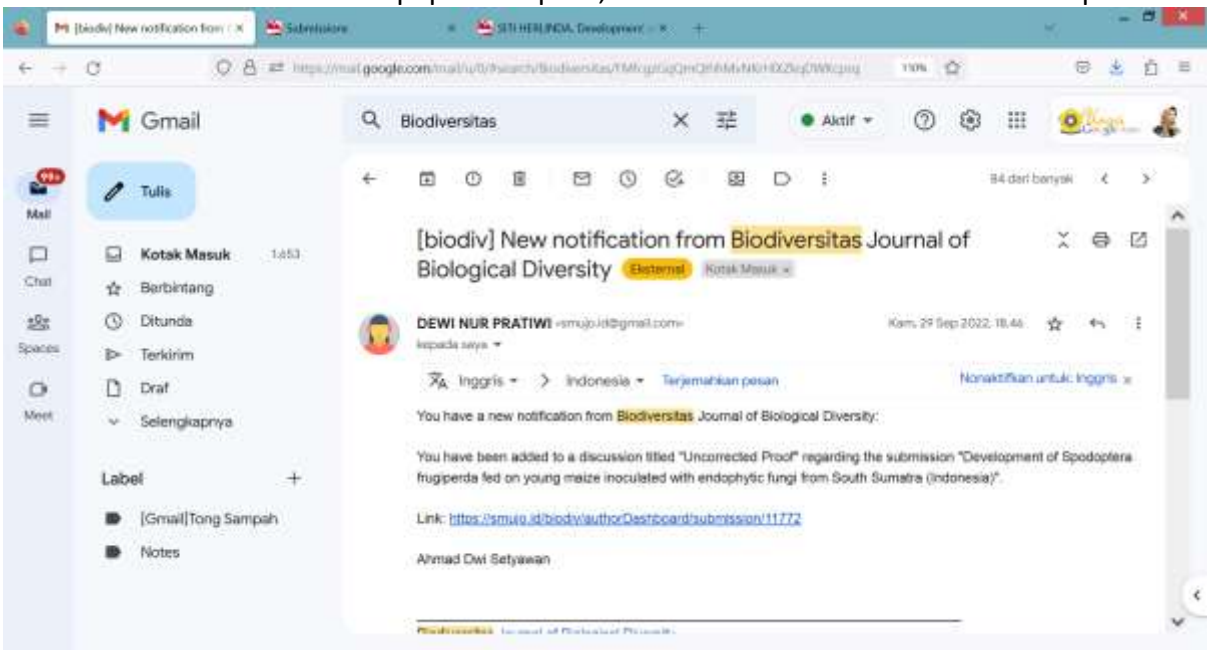

Development of *Spodoptera frugiperda* fed on young maize plant's fresh leaves inoculated with endophytic fungi from South Sumatra, Indonesia

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Abstract. *Lestari YA, Verawaty M, Herlinda S. 2022. Development of* Spodoptera frugiperda *fed on young maize plant's fresh leaves inoculated with endophytic fungi from South Sumatra, Indonesia. Biodiversitas 23: xxxx.* The endophytic entomopathogenic fungi are needed to control the *Spodoptera frugiperda* larvae hiding inside the maize plants. This research aimed to assess the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda*. The fungal isolates used for the bioassay were *Beauveria bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GenBank acc. no. MZ356497), and *Metarhizium anisopliae* CaTpPga isolates (GenBank acc. no. MZ242073). *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize plant significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs of *S. frugiperda*. The fungi also significantly enhanced larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate (57.67%) was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate (51.33%). Furthermore, the developmental time (eggs, larvae, pupae stages, and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda*.

Keywords: *Beauveria bassiana*, entomopathogen, fall armyworm, *Metarhizium anisopliae*, seed treatment

INTRODUCTION

The *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) or fall armyworm (FAW) is an important pest species in the world, attacking a wide range of plants (polyphagous) and migrating all over the whole world (Montezano et al. 2018). This pest comes from South America (Otim et al. 2018). The FAW came into Africa in 2016 (Goergen et al. 2016) and crossed over to Europe in 2017 (Early et al. 2018). In Asia, the FAW was discovered for the first time in India in 2018 (Ganiger et al. 2018; Mahat et al. 2021), and on March 26, 2019, the pest came into Indonesia for the first time in West Sumatra (Sartiami et al. 2020). More recently, it has spread throughout Indonesia (Maharani et al. 2019; Ginting et al. 2020; Supartha et al. 2021) and has become a new invasive pest in Indonesia (Herlinda et al. 2022). As a polyphagous insect, the FAW can attack 353 host plant species from 76 plant families (Montezano et al. 2018). The percentage 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). It causes about a third of the annual maize production losses, about 1 million tonnes in Kenya (De Groote et al., 2020) and 18 million tons/year in 12 African countries. The losses reach US $ 13 million (Harrison et al. 2019). The most severely attacked crop in Indonesia is maize (*Zea mays* L.). It causes damage ranging from 85% to 100% in East Nusa Tenggara (Mukkun et al. 2021) and 26.50% to 70% in Lampung (Lestari et al. 2020), and reaching 100% in South Sumatra (Herlinda et al. 2022). The larval stage of this pest eats leaves, stems, flowers, fruits, and growing points (Montezano et al. 2018). The larvae are found on the surface of maize leaves or stalks in the morning and then hide in the leaf's midribs at daylight (Gustianingtyas et al. 2021), and this behavior makes the FAW larvae difficult to be controlled.

The FAW is commonly controlled using synthetic insecticides due to its fast action and easy application (Kumela et al. 2018). Still, the insecticide application has a negative effect on human health and the environment (Harrison et al. 2,019) and the resistance against the pest (Zhang et al. 2021). An alternative eco-friendly control for FAW is utilizing biocontrol agents, such as entomopathogenic fungi (Mantzoukas and Eliopoulos 2020). Our previous study showed that *Metarhizium* spp. treated by topical application caused 78% mortality of the larval *S. frugiperda* (Herlinda et al. 2020). *Beauveria bassiana* (Balsamo) Vuillemin applied topically could kill more than 80% of the FAW larvae (Ramanujam et al. 2020). However, the topical fungal application is less effective in the field (Gustianingtyas et al. 2021) because from daylight up to night, the FAW larvae hide in the corn midribs (Herlinda et al. 2021). To control the hiding larvae, the entomopathogenic fungi can colonize the plant tissues (endophytic fungi) (Gustianingtyas et al. 2021). The endophytic fungi colonize the intercellular or intracellular spaces of host tissues and provide beneficial effects to their hosts (Lira et al. 2020), can stimulate plant growth, and depress insect growth (Russo et al. 2020).

The previous study results showed that the endophytic fungi from South Sumatra (Indonesia) could kill *S. frugiperda* larvae (Herlinda et al. 2021). However, there is no information on the development of *S. frugiperda* fed on young maize inoculated with endophytic fungi. So, the potential of the fungi isolated from plant tissues as endophytic entomopathogens needs to be evaluated. Therefore, this study investigated the effect of young maize plants inoculated with endophytic fungi on *S. frugiperda* development. So, this research aimed to assess the effect of

young maize plants inoculated with endophytic fungi on the development of *S. frugiperda*.

MATERIALS AND METHODS

Mass-rearing of Spodoptera frugiperda

S. frugiperda eggs were obtained from the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya, which have been mass-reared since January 2020 (Herlinda et al. 2020) and were identified molecularly (Herlinda et al. 2022). The FAW was mass-reared in the laboratory at 29°C temperature and 83% relative humidity (RH), and the lighting was set to photoperiod 12:12 (L:D) h. The larvae were maintained individually because the third up to the last instars are cannibal (Herlinda et al. 2021). The larvae were fed on the fresh corn leaves. The pupae were replaced in a transparent plastic cage (50 x 50 x 50 cm^3) containing more than 100 pupae per cage, and inside the cage also placed fresh corn leaves for the adults laying their eggs. The newly emerged larvae were used for bioassays as described below.

Assessing endophytic fungal colonization in the young maize tissue

The fungal isolates used for this bioassay were from the Laboratory of Entomology collection and were identified molecularly by Herlinda et al. (2021). The fungal species were *B. bassiana* JgSPK isolate (GenBank acc. no. MZ356494), *B. bassiana* JgCrJr isolate (GeneBank acc. no. MZ356497), and *M. anisopliae* CaTpPga isolates (GeneBank acc. no. MZ242073) (Table 1). The fungi originated from Simpang Padang Karet. Pagar Alam, South Sumatra (103°15'30.1788"E, 4°1'28.0308"S), Curup Jare, Pagar Alam, South Sumatra (103°13'17.0904"E, 4°0'58.7556"S), and Tanjung Payang, Pagar Alam, South Sumatra (103°14'28.0644"E, 4°2'20.8752"S), respectively.

To ensure the fungi used in this study were truly endophytic, the fungi's ability to colonize maize tissue was assessed by treating the maize seeds. The fungi were cultured on sabouraud dextrose agar (SDA) medium and incubated for 14 days. Before being treated with the fungi, the 45 corn seeds were surface sterilized using Russo et al. (2020) method. Then, the seeds were submerged in 10 ml of fungal suspension (1 x 10^8 conidia ml^{-1}) for 24 hours, whereas the control or untreated seeds were only immersed with 10 ml of sterilized water. After that, the seeds were grown in the hydroponic medium using the method of Novianti et al. (2020). To confirm the fungi as endophytes, detecting the fungi colonizing the young maize tissues was done by cutting the tip leaves of 7 and 14-day-old young maize. Then the tip leaves were grown onto the SDA medium to detect the mycelia of the endophytic fungi within the leaves. Finally, the rest young maize leaves were used for bioassays. Before the leaves colonized with the fungi grew onto the SDA medium, they were first surface-sterilized by immersion in 70% ethanol and sodium hypochlorite for 2 minutes and rinsed twice in sterile distilled water (Russo et al. 2020). Finally, the last rinse water was also grown onto the SDA medium. If no fungal growth was found on the last rinse water, it confirmed that the surface sterilization of maize tissues eliminated the epiphytic microorganisms, and the fungus growing on the treated medium were endophytes.

The bioassay to assess the effect of young maize inoculated with endophytic fungi on *Spodoptera frugiperda* development

Assessing the effect of young maize inoculated with endophytic fungi on the development of *S. frugiperda* was conducted at the Laboratory of Entomology, Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. The laboratory temperature and RH ranged from 28−29 °C and 82−83%, respectively.

The bioassay for assessing the effect of young maize colonized with endophytic fungi (after seed treatment) on the development of *S. frugiperda* followed the method of Russo et al. (2019). The fresh leaves of young maize colonized by the endophytic fungi were provided to the first instar neonate larvae (hatching within 24 hours) of *S. frugiperda* as feed, whereas, for control, the non-treated leaves of young maize were provided as feed by the larvae. The 100 neonate larvae for each isolate were provided with the treated young maize and untreated ones (control) for 6−12 hours or until the leaves were eaten. After that, the larvae were individually maintained in a porous plastic cup (Ø 6.5 cm), fed on fresh non-treated leaves (2 cm x 5 cm) per day per larvae, and replaced with new ones every day. This experiment consisted of three fungal isolates, and the control (water) was repeated three times using a completely randomized block design.

The variables recorded were the egg, larval, pupal, and adult developmental times and the mortality of each stage. The mortality of larvae and pupae was recorded every day. The morphology of the dead egg (unhatched), larvae, and pupae and the behavior of unhealthy larvae were recorded daily. The unhatched eggs, dead larvae, and pupae were grown in an SDA medium to confirm the microorganism that infected them. The adults emerging were monitored daily, and their sex was recorded. The *Spodoptera frugiperda* adults were placed in the wire mesh cage for copulation with fresh maize leaves to provide egg laying. Eggs laid by the adults were counted every day. Adult longevity was determined by counting the time (days) from emergence until death.

Table 1. Isolates of endophytic-entomopathogenic fungi from South Sumatra, Indonesia, used in this research

Location (village, district/city)	Isolate origin	Fungal species	Fungal isolate code	GenBank Acc. No.	References
Simpang Padang Karet. Pagar Alam	Maize	*Beauveria bassiana*	JgSPK	MZ356494	Herlinda et al. (2021)
Curup Jare. Pagar Alam	Maize	*Beauveria bassiana*	JgCrJr	MZ356497	Herlinda et al. (2021)

Tanjung Alam	Payang. Pagar	Red pepper	*Metarhizium anisopliae*	CaTpPga	MZ242073	Herlinda et al. (2021)

Data analysis

The differences in the egg, larval, pupal, and adult developmental times and the mortality of each stage, the adult longevity, the eggs laid, and the sex ratio of each isolate were analyzed using analysis of variance (ANOVA). Tukey's or Tukey's Honestly Significant Difference (HSD) test was applied to determine the significant differences among means of the isolates at p = 0.05. All data were calculated using the software of SAS University Edition 2.7 9.4 M5.

RESULTS AND DISCUSSION

The results of assessing endophytic fungal colonization in the young maize tissue

All fungal isolates (*B. bassiana* JgSPK and JgCrJr isolates, and *M. anisopliae* CaTpPga isolate) used in this study were endophytic fungi because the fungal mycelia were able to colonize within young maize tissue after being inoculated by seed immersion treatment. The fungi colonized the young maize leaves when they were grown onto the SDA medium, the fungus grew, and their mycelia covered the leaves. However, no mycelia were found on untreated maize leaves and in the final flushing water. This confirmed that the surface-sterilization of maize tissues eradicated the epiphytic microorganisms so that the fungus growing out of the leaf surface were endophytes originating from the maize tissues. The results showed that the percentage of fungal colonization in leaves after being inoculated by seed immersion treatment increased from 7 to 14 days after seed inoculation. After 14 days after seed inoculation, all maize leaves were colonized by the fungi (100%)(Table 2). The percentage of fungal colonization was determined or calculated as the number of sampled leave tissue showing fungal outgrowth divided by the total number of plated leave tissue samples x 100.

Effect of the colonized fresh leaf of young maize on *Spodoptera frugiperda* development

The first instar neonate larvae fed on fungal colonized maize leaf caused the next instar significantly shift their developmental time (P<0.0001) (Table 3). The developmental time of all instars fed on the treated maize was longer than those fed on the untreated ones (control). The treated maize colonized by the fungi also increased egg, prepupae, and pupal developmental time; however, the longevity of female and male adults decreased significantly (P<0.0001) (Table 4). Fungal colonized maize caused the total lifespan of *S. frugiperda* to increase significantly (P<0.0001). Longevity is the age of an adult but lifespan is the length of time from the egg stage up to adult death. The longest total lifespan or generation time of *S. frugiperda* was found in *M. anisopliae* (46.87 days), among other treatments. The lifespan of *S. frugiperda* caused by feeding on the fungal colonized maize leaves was significantly longer (P<0.0001) than those fed on non-colonized maize leaves.

Larval mortality of all instar fed on the fungal treatment leaves was significantly higher (P<0.0001) than those of larvae fed on untreated maize leaves (control) (Table 5). The mortality of the last larval instar fed on young maize leaves colonized by *M. anisopliae* (57.67%) was the highest among other treatments. Still, it was not significantly different from those feds on maize leaves colonized by *B. bassiana* of JgSPK isolate (51.33%). The mean percentage of pupae and adults' emergence of fungal treatments was significantly lower (P<0.0001) than those of the control (Table 6). The percentage of pupae and adults' emergence of *M. anisopliae* treatment was the lowest among other treatments. The sex ratio of *S. frugiperda* fed on maize leaves colonized with the fungi was not significantly different from those of the control. However, the maize leaves colonized with fungi significantly decreased the number of eggs laid and viable eggs of *S. frugiperda* compared to the non-colonized one.

Table 2. The effect of endophytic-entomopathogenic fungal isolates on mean maize leaf colonization (%) at 7 and 14 days after inoculation

Isolate	Species	Mean fungal colonization on maize leaves (%)	
		Seven days after inoculation	Fourteen days after inoculation
Control	-	0.00c	0.00b
JgSPK	*Beauveria bassiana*	73.33b	100.00a
JgCrJr	*Beauveria bassiana*	53.33b	100.00a
CaTpPga	*Metarhizium anisopliae*	100.00a	100.00a
F-value		145.02*	143.40*
P-value		5.48×10^{-06}	2×10^{-16}
HSD value		15.09	5.78

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

Table 3. The developmental time of instar larvae of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	Species	The developmental time (days)					
		1st larvae	2nd larvae	3rd larvae	4th larvae	5th larvae	6th larvae
Control	-	3.12d	3.02d	2.99d	2.99c	2.99d	3.01d

JgSPK	*Beauveria bassiana*	4.55b	4.48b	4.41b	4.33b	4.28b	4.49b
JgCrJr	*Beauveria bassiana*	4.18c	4.09c	4.12c	4.22b	4.16c	4.16c
CaTpPga	*Metarhizium anisopliae*	4.87a	4.96a	5.01a	4.05a	4.93a	4.81a
F-value		719.41*	806.67*	2301.21*	350.41*	1240*	1689.74*
P-value		$4.66x10^{-08}$	$3.31x10^{-08}$	$1.43x10^{-09}$	$3.99x10^{-07}$	$9.13x10^{-09}$	$3.61x10^{-09}$
HSD value		0.03	0.03	0.02	0.05	0.03	0.02

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

Table 4. Length of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates and *Metarhizium anisoplae* CaTpPga isolate

Isolate	**Species**	**The developmental time (days)**					
		Prepupae	**Pupae**	**Female adult**	**Male adult**	**Egg**	**Total lifespan**
Control	-	3.11d	6.15d	4.46a	4.52a	3.14c	31.90d
JgSPK	*Beauveria bassiana*	4.33b	8.00b	3.42bc	3.51c	3.60b	42.37b
JgCrJr	*Beauveria bassiana*	4.06c	7.21c	3.57bc	3.69b	3.47b	39.90c
CaTpPga	*Metarhizium anisopliae*	4.95a	9.05a	3.25c	3.35d	3.94a	46.87a
F-value		862.15*	138.38*	86.38*	553.52*	42.81*	2361.20*
P-value		$2.71x10^{-08}$	$6.29x10^{-06}$	$2.51x10^{-05}$	$1.02x10^{-07}$	$1.91x10^{-04}$	$1.33x10^{-09}$
HSD value		0.03	0.09	0.07	0.03	0.06	0.05

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

Table 5. The cumulative mortality of different larvae instars of *Spodoptera frugiperda* fed on young maize leaves treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolate	**Species**	**The cumulative mortality of different instar larvae (%)**					
		1st larvae	**2nd larvae**	**3rd larvae**	**4th larvae**	**5th larvae**	**6th larvae**
Control	-	3.00	7.00c	7.67c	7.67d	7.67c	7.67c
JgSPK	*Beauveria bassiana*	6.67	29.33a	37.67ab	47.00b	49.67ab	51.33ab
JgCrJr	*Beauveria bassiana*	4.00	14.33b	32.67b	39.67c	42.00b	43.67b
CaTpPga	*Metarhizium anisopliae*	5.33	29.67a	43.33a	54.00a	57.33a	57.67a
F-value		1.85ns	50.53*	138.96*	322.14*	206.97*	133.34*
P-value		0.24	$1.19x10^{-04}$	$6.21x10^{-06}$	$5.13x10^{-07}$	$1.91x10^{-06}$	$7.02x10^{-06}$
HSD value		-	5.98	4.70	3.83	5.06	6.42

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. Original data were transformed using Arcsin transformation prior to statistical analysis

Table 6. Mean percentage of pupae and adult emergence, sex ratio, an egg laid, and viable eggs of *Spodoptera frugiperda* fed on young maize treated with *Beauveria bassiana* of JgSPK and JgCrJr isolates, and *Metarhizium anisoplae* CaTpPga isolate

Isolates	**Fungal species**	**Pupae emergence (%)**	**Adult emergence (%)**	**Sex ratio**	**Eggs laid per female**	**Viable (hatched) eggs (%)**
Control		92.67a	92.67a	0.60	34.04a	98.85a
JgSPK	*Beauveria bassiana*	37.67c	33.33c	0.71	17.30b	83.42b
JgCrJr	*Beauveria bassiana*	47.67b	44.67b	0.72	15.33b	85.49b
CaTpPga	*Metarhizium anisopliae*	29.00d	23.67d	0.70	11.25b	81.65b
F-value		355.81*	335.72*	0.84ns	36.08*	63.44*
P-value		$3.81x10^{-07}$	$4.53x10^{-07}$	0.52	$3.11x10^{-04}$	$6.17x10^{-05}$
HSD value		4.86	5.39	-	0.85	5.51

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. Original data pupae and adult emergence were transformed using Arcsin transformation before statistical analysis

Mycosis of each stage (larvae, pupae, and adult) of *Spodoptera frugiperda*

The FAW larvae fed on the fungal colonized maize leaves underwent behavior and color change, such as no appetite and muddy body color. The dead larvae showed unique symptoms, specifically shrunken and hardened like a mummy; the larvae body was covered with fungal mycelia and became white or green, depending on the fungal species that infected and killed them. The larvae fed on leaves colonized by *B. bassiana* and *M. anisopliae* produced the cadavers with white and green colors, respectively (Figure 1). The result indicated that the fungal isolate from the re-isolation of the cadavers was the same as the fungal isolate used for maize seed treatment. The colony morphology of the fungi resulted from re-isolation from the cadavers with white and greenish white for *B.*

bassiana and *M. anisopliae*, respectively (Figure 2). The conidial and hyphal morphology of *B. bassiana* produced from the cadavers had hyaline hyphae, mycelia, and globose conidia. Nevertheless, the morphology of *M. anisopliae* had green hyphae and mycelia, and cylindrical conidia. Therefore, the dead larvae or other stages of *S. frugiperda* that produced fungal conidia and hyphae were considered to have died from mycosis.

The fungi that colonized the maize leaves could produce mycosis at each stage of *S. frugiperda*, but also they could cause the *S. frugiperda* could cause malformation (Figures 3, 4, and 5). The sick larvae caused by consuming the fungal colonized maize leaves underwent body shrinkage and dark brown, and their integuments were harder than the healthy ones. The sick larvae could produce abnormal and malformed pupae. The sick pupae infected by fungi could produce abnormal and malformed adults with smaller bodies, deformed and folded wings, and an inability to fly.

Figure 1. The cadavers from larvae fed on maize leave untreated with fungi or control (A), and the cadavers from larvae fed on maize leave colonized by *Beauveria bassiana* of JgSPK isolate, and JgCrJr isolate (B and C), *Metarhizium anisoplae* CaTpPga isolate (D) incubated for 14 days

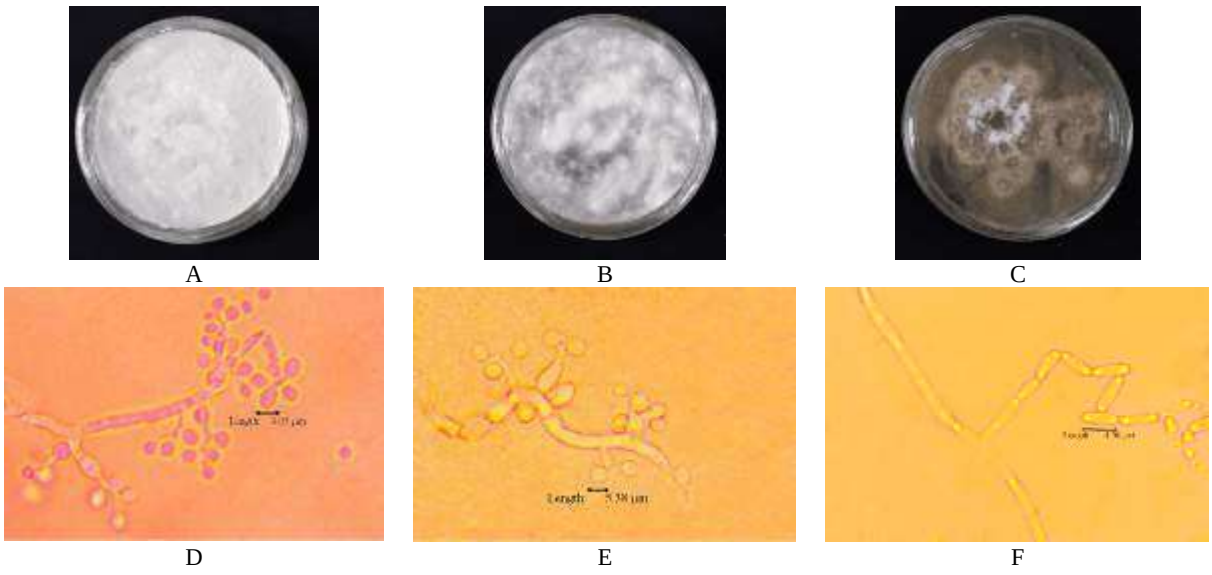

Figure 2. Colony morphology of endophytic fungi isolated from the cadavers and cultured on SDA media for 14 days (above) and the conidial and hyphal morphology (below) of the fungi: *Beauveria bassiana* of JgSPK isolate (A and D), and JgCrJr isolate (B and E), *Metarhizium anisoplae* CaTpPga isolate (C and F)

Figure 3. Larval *Spodoptera frugiperda*: healthy larvae of control (A) and larvae infected by endophytic fungi (B)

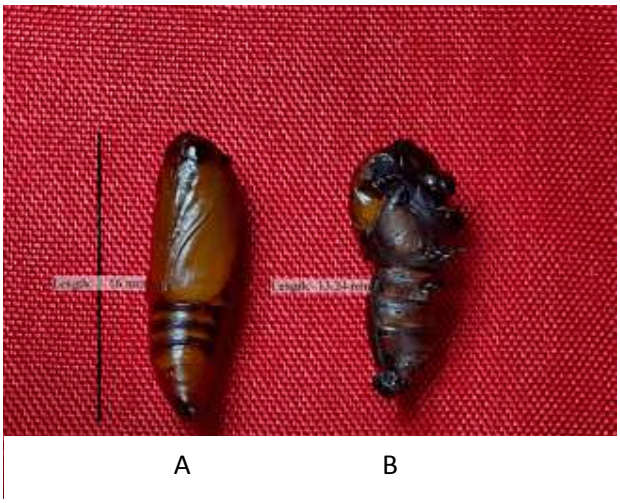

Figure 4. Pupal *Spodoptera frugiperda*: healthy pupae of control (A) and pupae infected by endophytic fungi (B)

Figure 5. *Spodoptera frugiperda* adults: healthy adult of control (A) and adult infected by endophytic fungi (B)

Discussion

The three fungal isolates used in the current study were confirmed as endophytic fungi. The obtained finding showed that the fungal mycelia could colonize the maize tissue, including the leaves consumed by the neonate larvae. The fungal mycelia were not found within the uninoculated maize leaves (control). In the current study, the ability of *B. bassiana* and *M. anisopliae* to colonize the young maize through seed treatment reached 100% of leaves 14 days after seed inoculation. The fungi, *B. bassiana,* and *M. anisopliae,* were also able to colonize the maize when inoculated by foliar spray and root dipping, and at seven days after foliar spray, the fungi could colonize 100% of leaves, 80% of stems, and 60% of roots (Russo et al. 2020). The fungal endophytes could colonize after more than 14 days; the fungi were found within the roots, stems, and leaves of tomatoes up to 30 days after inoculation (Carolina et al. 2020). *B. bassiana* could be detected within the entire plant growth cycle (120–140 days after sowing), and it was also detected in seeds of opium poppy plants (*Papaver somniferum*) (Quesada-Moraga et al. 2014). In the present study, *B. bassiana* and *M. anisopliae* isolates could be detected within the entire maize tissue. This finding showed that *B. bassiana* and *M. anisopliae* isolates from South Sumatra could cause high mortality of the larvae fed on the colonized young maize leaves. Because the young maize is highly susceptible *to S. frugiperda* larvae (Supartha et al. 2021), the early prevention with seed treatment by using the endophytic *B. bassiana* and *M. anisopliae* may increase the young corn plant's defense against *S. frugiperda* larvae. Moreover, the hiding larvae of *S. frugiperda* in the corn midribs were more effectively controlled by using the endophytic fungi than the topical fungal application (Gustianingtyas et al. 2021; Herlinda et al. 2021).

In the present study, *B. bassiana* and *M. anisopliae* inoculated as maize seed treatments prolonged the developmental time of *S. frugiperda*. The developmental time (eggs, larvae, and pupae stages and lifespan) of *S. frugiperda* fed on leaves colonized with endophytic fungi increased; however, the adult longevity decreased. Those findings follow some previous findings that the fungi enhanced the developmental time of insects (Lopez and Sword 2015; Hussain et al. 2009) because the fungi reduced the conversion of digested and ingested food, which could stimulate the larvae to develop more slowly (Hussain et al. 2009).

B. bassiana and *M. anisopliae* inoculated as seed treatments in the current study caused negative effects on the development of *S. frugiperda*. The fungi could reduce the pupae and adult emergence, eggs laid, and viable eggs and enhance larval mortality. *B. bassiana* and *M. anisopliae* in seed immersion, foliar spray, and root dipping caused adverse effects on *S. frugiperda* development and survival (Russo et al. 2020) due to the fungi producing secondary metabolites. The secondary metabolites caused mycosis in the insect body (Vidal and Jaber 2015). In addition, the fungal mycelia within the plant tissue fed on the larvae could produce blastospores in the larvae's hemolymph. The blastospores could produce secondary metabolites with toxins that disrupt the normal cell metabolism and kill the insects (Mancillas-Paredes et al. 2019). The endophytic fungi also reduced the larvae's appetite for consuming the plant leaves and increased the larval mortality (Gustianingtyas et al. 2021) because the fungi could produce secondary metabolites in planta resulting in antifeedant or deterrent and antibiosis for the larvae of *S. frugiperda* (Jaber and Ownley 2018) and also could increase the levels of terpenoid defense compounds against *S. frugiperda* (Russo et al. 2020). When the insects died, the endophytic fungi kept growing and caused mycosis characterized by fungal mycelia and spores emerging from the cadaver body (Vidal and Jaber 2015). The current study showed that the mycosis occurred on the larvae of *S. frugiperda* fed on the leaves colonized by the fungi, and no mycosis was found on the untreated larvae (control). The previous study also showed that mycosis could occur in the *S. frugiperda* larvae fed on fungal-endophytically colonized plants (Russo et al. 2020; Sari et al. 2022).

Finally, *B. bassiana* (JgSPK and JgCrJr isolates) and *M. anisopliae* (CaTpPga isolate) colonized young maize fed on the neonate larvae significantly reduced the pupae and adult emergence, adult longevity, eggs laid, and viable eggs, and also significantly enhanced the larval mortality compared to non-colonized ones. The larval mortality caused by *M. anisopliae* CaTpPga isolate was highest among other treatments but not significantly different from those treated with *B. bassiana* JgSPK isolate. The developmental time (eggs, larvae, and pupae stages) of *S. frugiperda* fed on leaves colonized with endophytic fungi significantly increased compared to non-colonized ones. So, *B. bassiana* and *M. anisopliae* inoculated as seed treatments caused negative effects on the development of *S. frugiperda*. These findings highlight the potential of endophytic *B. bassiana* and *M. anisopliae* from South Sumatra to protect maize against *S. frugiperda.*

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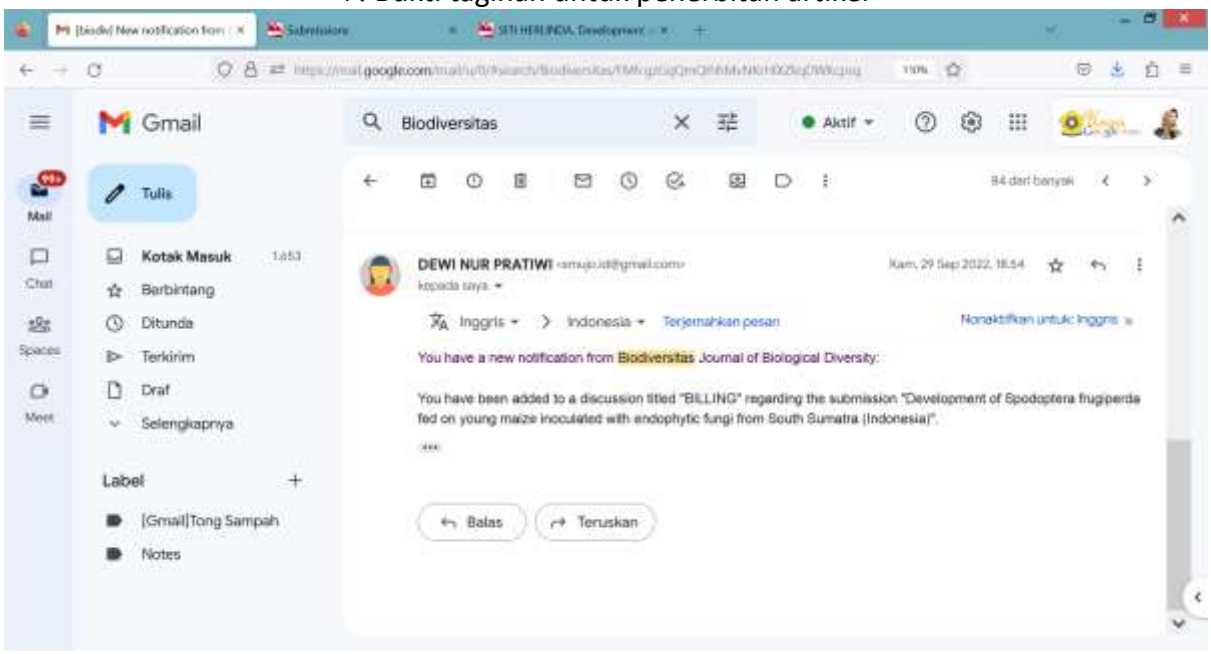

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