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Effect of endophytic entomopathogenic fungal conidia and blastospores induced in maize plants by seed inoculation on *Spodoptera frugiperda* immune response and mortality

JELLY MILINIA PUSPITA SARI[1], SITI HERLINDA[2,3,♥], SUWANDI SUWANDI[2,3], ELFITA[4]

[1]Doctoral Program of Agriculture Sciences, Faculty of Agriculture, Universitas Sriwijaya. Jl. Padang Selasa 524, Palembang 30139, South Sumatra, Indonesia

[2]Department of Plant Protection, Faculty of Agriculture, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km 32, Ogan Ilir 30662, South Sumatra, Indonesia. Tel.: +62-711-580059, Fax.: +62-711-580276, ♥email: sitiherlinda@unsri.ac.id

[3]Research Center for Sub-optimal Lands (PUR-PLSO), Universitas Sriwijaya. Jl. Padang Selasa 524, Palembang 30139, South Sumatra, Indonesia

[4]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km 32, Ogan Ilir 30662, South Sumatra, Indonesia

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Abstract. *Sari JMP, Herlinda S, Suwandi S, Elfita. 2023. Effect of endophytic entomopathogenic fungal conidia and blastospores induced in maize plants by seed inoculation on* Spodoptera frugiperda *immune response and mortality. Biodiversitas 24: 5709-5717.* Endophytic entomopathogenic fungi (EPF) can produce conidia and blastospores; however, the pathogenicity of conidia and blastospores inoculated in plants on *Spodoptera frugiperda* larvae is limited. This research aimed to detect the effect of the endophytic entomopathogenic fungal conidia and blastospores induced in maize plants by seed inoculation on *S. frugiperda's* immune response and mortality. A total of 10 isolates of endophytic EPF were used in this experiment. The study revealed that 9 days after treatments, *S. frugiperda* larvae consuming maize leaves inoculated with blastospores of endophytic entomopathogenic fungi; their hemocyte concentration did not show any significant differences between the fungal treatments and control. However, 1 up to 7 days after treatments, the concentration significantly differed from the control. Furthermore, *B. bassiana* JgSPK, JaGiP, and JaSpkPGA(2) isolates tended to be the most pathogenic compared to other isolates. Feeding on leaves colonized by endophytic EPF could reduce the larval and pupal weight of *S. frugiperda*. The percentage of *S. frugiperda* non-emergence pupae from larvae-eating maize leaves colonized with *B. bassiana* JgSPK and JaGiP isolates was significantly higher than other fungal treatments and the control. The endophytic entomopathogenic fungal conidia and blastospores inoculated in maize plants by seed inoculation have a lethal effect on *S. frugiperda* larvae. However, exposure to conidia and blastospores did not reduce or increase the hemocyte concentration in the larvae hemolymph of *S. frugiperda*.

Keywords: *Beauveria bassiana*, *Chaetomium* sp., *Curvularia lunata*, *Metarhizium anisopliae*, *Penicillium citrinum*

Abbreviations: EPF: entomopathogenic fungi

INTRODUCTION

Fall armyworm (FAW) or *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is the most dangerous pest in the world nowadays because of its high spreading ability and voracity. FAW is an invasive species that entered Indonesia in March 2019 in West Sumatra (Sartiami et al. 2020). *Spodoptera frugiperda* originated in South America (Goergen et al. 2016); however, the pest has spread to several continents, including Europe (Early et al. 2018) and Africa (Goergen et al. 2016). The voracious nature of this pest has caused 80-100% damage to maize and other crops in Ethiopia and Kenya (Sisay et al. 2019). In Indonesia, it damages the maize up to 100% in Bali (Supartha et al. 2021), East Nusa Tenggara (Mukkun et al. 2021), and South Sumatra (Herlinda et al. 2022). FAW attacks many species of plants, reaching 353 species from 76 families of plants (Montezano et al. 2018). Two strains of this pest have been found in Indonesia: rice and corn (Herlinda et al. 2022), potentially threatening Indonesia's food security. Financially, the losses caused by *S. frugiperda* globally reach US$ 13 million annually (Harrison et al. 2019).

FAW is generally controlled using synthetic insecticides (Kumela et al. 2018). However, the chemical control has more disadvantages than advantages. The advantages are fast and easy to use, while the disadvantages include causing environmental damage and agricultural products contaminated with toxic residues of synthetic insecticides (Harrison et al. 2019). In addition, spraying of synthetic insecticides causes resistant or immune strains of *S. frugiperda* (Zhang et al. 2021). Currently, conventional chemical control is beginning to be abandoned, and biological control is more developed rapidly due to being environmentally friendly (Mantzoukas and Eliopoulos 2020). Biological control of *S. frugiperda* using entomopathogenic fungi (EPF) has been widely studied. For example, *Beauveria bassiana*, which was tested in the laboratory by spraying topically (direct contact), can kill up to 98.3% of *S. frugiperda* larvae (Ramirez-Rodriguez and Sánchez-Peña 2016). Likewise,

Metarhizium anisopliae can topically kill up to 100% of FAW larvae in the laboratory (Gutiérrez-Cárdenas et al. 2019). However, topical application of EPF has several obstacles, including that *S. frugiperda* larvae appear only when the sun rises until 08.00 am, after which the larvae hide in the leaf midrib (Gustianingtyas et al. 2021), thus the potential for contact with fungal conidia is low. In addition, fungal conidia applied to the leaf surface are more difficult to survive extreme weather (sunlight, wind, rain) (Boomsma et al. 2014). Spraying conidia on maize also has a high potential for exposure to natural enemy arthropods (for example, egg parasitoids and predatory arthropods). To overcome this obstacle, the ideal position of EPF is within the host plant tissue so that they can target the phytophagous insects (*S. frugiperda*) which attacks only that host plant. EPF that have such properties are called endophytic fungi.

Endophytic fungi can colonize plant tissue intercellularly and intracellularly and have a mutualistic symbiotic relationship with their host plants (Lira et al. 2020). Endophytic fungi have the advantage of living within host plant tissue but can increase their hosts' growth and protect them from phytophagous attacks (Russo et al. 2020). From previous explorations in 2021, 20 isolates of endophytic EPF isolated from plants in South Sumatra were confirmed as endophytes (Herlinda et al. 2021) and have the potential to kill *S. frugiperda* larvae (Herlinda et al. 2022). The endophytic EPF can produce conidia and blastospores when cultured in broth medium (Sari et al. 2023); however, the pathogenicity of the conidia and blastospores inoculated in plants on *S. frugiperda* larvae is unknown. There is no information about the response of *S. frugiperda* larvae that consume leaves inoculated with the endophytic EPF from South Sumatra. Nevertheless, much information shows insect immune responses to EPF that are applied topically (Enríquez-Vara et al. 2012; Bitencourt et al. 2023). This research's novelty was to detect the effect of the endophytic entomopathogenic fungal conidia and blastospores induced in maize plants by seed inoculation on *S. frugiperda's* immune response and mortality.

MATERIALS AND METHODS

Preparation of endophytic entomopathogenic fungi

A total of 10 isolates of endophytic EPF were used in this experiment. The isolates were maintained in the Entomology Laboratory, Faculty of Agriculture, Universitas Sriwijaya. The fungal isolates, consisting of 5 species, were identified molecularly and deposited in the GenBank (Herlinda et al. 2021). The fungal species were *B. bassiana*, *Chaetomium* sp., *Curvularia lunata*, *Penicillium citrinum*, and *M. anisopliae*. *B. bassiana* consisted of JgSPK isolate (acc. no. MZ356494), JaGiP isolate (acc. no. MZ356495), JaSpkPGA(2) isolate (acc. no. MZ356496), JgCrJr isolate (acc. no. MZ356497), and JaTpOi (1) isolate (acc. no. MZ356498). *Chaetomium* sp. comprises PiCrPga isolate (acc. no. MZ359735). *C. lunata* had JaMsBys isolate (acc. no. MZ359819), JaSpkPga(3) isolate (acc. no. MZ359818). *Penicillium citrinum* had JaTpOi(2) isolate (acc. no. MZ359812). *Metarhizium anisopliae* consisted of CaTpPga isolate (acc. no. MZ242073). All the fungal isolates were confirmed as endophytes and entomopathogens because they can colonize plants as endophytes and kill host insects as entomopathogens (Herlinda et al. 2021) and referred to as endophytic EPF (Mantzoukas and Eliopoulos 2020).

Mass-rearing of *Spodotera frugiperda*

Spodoptera frugiperda was mass-reared in the laboratory according to the method of Herlinda et al. (2020). The *S. frugiperda* colonies used in the present study have been maintained in the laboratory for generations and identified molecularly by Herlinda et al. (2022). Temperature and relative humidity during rearing were 28-29°C and 82-83%, respectively. Lighting was adjusted to a 12:12 (L:D) photoperiod. Due to their cannibalistic behavior, the larvae of *S. frugiperda* were kept individually. The larvae were fed an artificial diet and replaced every two days. The emerging prepupae and pupae were placed in a wire mesh cage (30×30×30 cm^3) containing sterile soil and maize seedlings for adult insects to lay their eggs. FAW mass rearing was carried out for more than 10 generations to obtain homogeneous test insects for bioassays. Eggs were collected daily; after hatching, first instar larvae were used for bioassay.

Fungal conidia and blastospores induced in maize plants and bioassay

Fungal inoculation in corn by seed treatment was conducted by following the method of Herlinda et al. (2021). All the 10 isolates were first cultured in Sabouraud Dextrose Agar (SDA) incubated for 14 days at 28-29°C. The fungal cultures from the SDA medium were transferred to the Sabouraud Dextrose Broth (SDB) using the method of Gustianingtyas et al. (2020). The blastospores were produced when the fungi were cultured in broth (liquid) medium (SDB) for 14 days and fermented in a shaker, programmed at 120 rpm for 7 days and 7 days without shaking, modifying the method of Moslim and Kamarudin (2014). The conidia and blastospores were separated from hyphae by filtering the suspension through a sterile cloth into a new centrifuge tube and then estimated using a hemocytometer. The blastospores and conidia concentrations were estimated using a hemocytometer. Corn seeds for treatment (N=125) were surface sterilized following the method of Russo et al. (2020). The seeds were soaked in 10 mL of the fungal suspension (1×10^{10} conidia mL^{-1}) for 12 hours, while the control (the untreated seeds) were immersed in 10 mL of sterilized water for 12 hours. Finally, seeds were grown in a hydroponic medium and incubated for 14 days (Novianti et al. 2020). The tip leaves of 14-day-old maize were cut to 5×5 mm^2 to be grown onto the SDA medium to detect the mycelia of the endophytic EPF colonized the maize leaves, following the Sari et al. (2023) method.

Bioassay was performed in a controlled room at a constant temperature and relative humidity of 25°C and 97%. Leaves of 14-day-old maize inoculated with the fungi by seed treatments were fed to first instar neonates

hatching within 24 hours of FAW larvae (N=60), while untreated larvae (the control) were fed maize leaves from untreated seeds. First instar larvae were allowed to feed on fungus-inoculated and non-inoculated maize (the control) for 6 hours. Treated and untreated larvae were kept individually in a container (Ø 6.5 cm, height 4.6 cm). Then, they were fed an artificial diet and replaced it with a fresh diet daily. This treatment was repeated three times for each isolate. The research was designed with a completely randomized block design. Every two days, larval growth and mortality were recorded. Therefore, to confirm that the fungal isolate from the corpses was the same as the fungal isolate used for the maize seed treatment, the conidia from the corpses were grown onto the SDA medium.

Observation of immune response of *Spodoptera frugiperda* larvae on the fungal conidia and blastospores exposure

The immune response of *S. frugiperda* to endophytic EPF was evaluated using the hemocyte concentrations according to Jiang et al. (2020). Therefore, to evaluate the effects of the fungal blastospores and conidia on the hemocyte concentrations, *S. frugiperda* larvae individuals were selected for examination after consuming the inoculated corn leaves. After eating inoculated corn leaves (and the control), the *S. frugiperda* larvae were sampled for hemolymph. They were previously surface-sterilized with 70% ethanol and then rinsed twice using sterile distilled water using the method of Elfita et al. (2019). Hemolymph samples were obtained by cutting the third thoracic leg using a needle following the method Enríquez-Vara et al. (2012), and three drops (approximate 20 µL) of hemolymph were placed in microtubes following the method Fiorotti et al. (2019). The hemolymph was put on a hemocytometer; then, hemocyte density was quantified at × 400 under a microscope following the method of Bitencourt et al. (2023). Hemocyte's photographs were taken using an Olympus Binocular Microscope CX23 with 1000x m with an Optilab Advance Plus Sony IMX577

Data analysis

The differences in hemocyte concentrations, larval weight, percentage of non-emergence pupae from larvae, normal and abnormal pupae, pupal weight and length, the mean percentage of non-emergence adults from pupae, normal and abnormal adult, and egg laying of each treatment were analyzed by analysis of variance (ANOVA). Tukey's test (HSD) was applied to determine the differences among the isolates) at p = 5%. SAS University Edition 2.7 9.4 M5 software was used to calculate the data. In addition, the data on cumulative larval mortality were presented in a graph, and photomicrography of hemocytes was also presented in this research.

RESULTS AND DISCUSSION

Immune response of *Spodoptera frugiperda* larvae to the fungal conidia and blastospores or conidia

The total hemocyte concentrations of *S. frugiperda* larvae after 24 hours (a day) up to 7 days after consuming maize leaves colonized with the endophytic EPF (1 x 10^{10} conidia mL^{-1}) were significantly different ($p < 0.01$) from the control. The highest hemocyte concentration was shown by the larvae that consumed maize leaves colonized with *C. lunata* (Table 1). To detect the leaves were colonized by the fungi applied to seeds by growing the tip leaves of 14-day-old maize onto the SDA medium. However, three days after consuming maize leaves colonized with *B. bassiana* JaSpkPGA(2) isolate, their hemocyte concentration was the highest ($p < 0.01$) among other treatments.

Table 1. Hemocyte concentrations of *Spodoptera frugiperda* larvae after consuming maize leaves colonized with endophytic entomopathogenic fungi (1×10^{10} conidia mL^{-1})

Isolates	Species	Mean of hemocyte concentrations (1×10^6 cells.mL^{-1}) after eating inoculated leaves				
		1 day	3 days	5 days	7 days	9 days
Control	-	5.86abc	5.58ab	5.85a	5.85a	5.35
JgSPK	*Beauveria bassiana*	4.99c	4.86ab	4.24bcd	3.99bcd	3.39
JaGiP	*Beauveria bassiana*	4.89c	4.24b	3.99cd	3.74cd	4.13
PiCrPga	*Chaetomium sp,*	5.83abc	4.14b	5.08abc	4.83abc	4.34
JaMsBys	*Curvularia lunata*	6.84a	4.83ab	6.09a	5.84a	4.32
JaSpkPGA(2)	*Beauveria bassiana*	5.18bc	5.84a	3.18de	2.93de	2.12
JgCrJr	*Beauveria bassiana*	5.43abc	4.18b	2.89e	2.64e	2.31
JaTpOi	*Beauveria bassiana*	5.14c	4.43ab	2.86e	2.61e	3.05
JaSpkPga(3)	*Curvularia lunata*	6.60ab	4.14b	6.05a	5.80a	5.10
JaTpOi(2)	*Penicillium citrinum*	6.15abc	5.60ab	5.40abc	5.15ab	4.68
CaTpPga	*Metarhizium anisopliae*	6.14abc	5.15ab	5.39ab	5.14ab	3.40
F-value		5.93^{**}	4.66^{**}	23.65^{**}	25.98^{**}	1.96^{ns}
p-value		2.49×10^{-4}	1.26×10^{-3}	1.68×10^{-9}	6.65×10^{-10}	0.08
HSD value		0.10	0.13	0.13	0.14	-

Notes: *: significantly different; data labeled by the different letters in a column were significantly different at $p < 0.05$ according to Tukey's test (HSD)

The hemocyte concentration tended to decrease further with increasing age of the *S. frugiperda* larvae. Fungal conidia and blastospores had a more significant effect on the reduction of hemocyte density ($P < 0.01$) than the control. Nine days after the larvae eating continuously maize leaves from seeds treated, their hemocyte concentration did not show any significant differences ($p > 0.05$) between treatments. Therefore, exposure to conidia and blastospores did not reduce or increase the hemocyte concentration in the larvae hemolymph of *S. frugiperda*. Hemocytes of *S. frugiperda* larvae were observed and identified, namely prohemocytes, plasmatocytes, oenocytoids, coagulocytes, and granulocytes (Figure 1). However, this study could not detect propagules (conidia and blastospores) of the endophytic EPF invading the hemocoel or penetrating the integument of the *S. frugiperda* larvae.

Lethal effect of the endophytic entomopathogenic fungal blastospores induced in maize plants

Cumulative mortality of *S. frugiperda* larvae treated with the endophytic EPF (1×10^{10} conidia mL^{-1}) during 14 days of observation resulted in *B. bassiana* JgSPK, JaGiP, and JaSpkPGA(2) isolates were the most pathogenic compared to other isolates within 13 to 25% cumulative mortality (Figure 2). On the contrary, untreated larvae's cumulative mortality (control) was 0%.

The body weight of *S. frugiperda* larvae after eating maize leaves colonized with endophytic EPF continued to increase during 11 days of observation (Table 2). Moreover, 3 to 11 days after treatment, the larvae weight was significantly lower ($p < 0.01$) than the control. Furthermore, 11 days after consuming leaves colonized with *Chaetomium* sp. PiCrPga Isolate and *B. bassiana* JaSpkPGA(2) isolate, the larvae weights were decreased significantly compared to other fungal treatments and control. Furthermore, the body weight of fungal-treated pupae was significantly lower ($p < 0.01$) compared to the control as well pupae length of fungal-treated pupae (Table 3). Therefore, feeding on leaves colonized by endophytic EPF could reduce the larval and pupal weight of *S. frugiperda*.

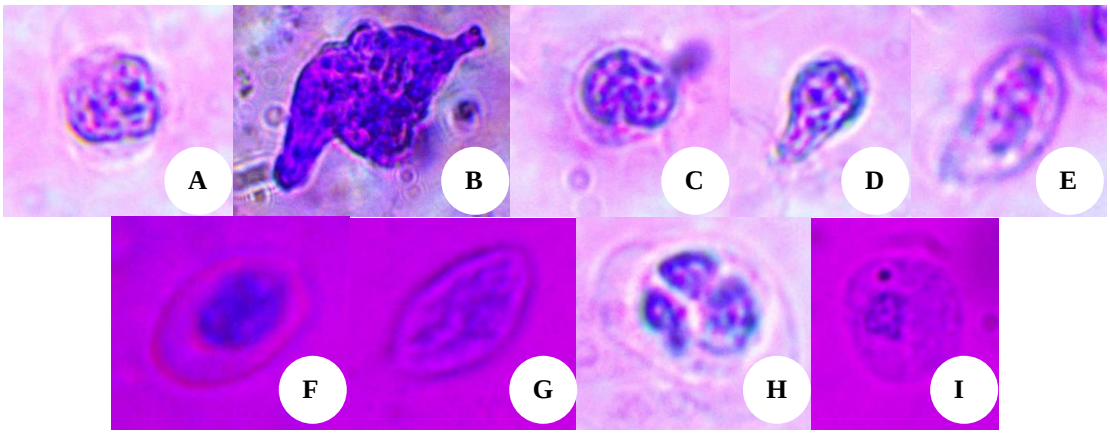

Figure 1. Photomicrography of hemocytes of *Spodoptera frugiperda* larvae: prohemocytes (A-E), plasmatocytes (E), oenocytoids (G), coagulocytes (H), granulocytes (I), cell length 70-119 µm

Table 2. Body weight of *Spodoptera frugiperda* larvae after consuming maize leaves colonized with endophytic entomopathogenic fungi

Isolates	Species	Mean of larvae body weight (mg) after eating inoculated leaves					
		1 day	3 days	5 days	7 days	9 days	11 days
Control	-	15.60	41.17a	50.41	72.06a	111.40a	139.17a
JgSPK	*Beauveria bassiana*	12.50	23.47ab	29.60	46.82abcd	62.40cd	88.00bc
JaGiP	*Beauveria bassiana*	13.73	24.13ab	45.20	60.93ab	70.40bc	81.92bc
PiCrPga	*Chaetomium* sp.	12.43	17.60b	22.67	27.43d	31.84e	46.31e
JaMsBys	*Curvularia lunata*	14.23	22.53b	32.27	50.82abc	66.80bc	86.93bc
JaSpkPGA(2)	*Beauveria bassiana*	12.67	26.26ab	21.57	36.00cd	45.47de	52.67de
JgCrJr	*Beauveria bassiana*	14.40	24.67ab	31.20	44.67bcd	60.04cd	72.67cd
JaTpOi	*Beauveria bassiana*	14.97	24.27ab	36.00	59.59ab	83.33b	107.20ab
JaSpkPga(3)	*Curvularia lunata*	16.00	25.87ab	37.88	56.93abc	72.80bc	90.00bc
JaTpOi(2)	*Penicillium citrinum*	14.13	21.47b	30.00	56.27abc	65.60bc	93.98bc
CaTpPga	*Metarhizium anisopliae*	15.47	25.07ab	31.07	53.87abc	71.87bc	88.00bc
F-value		1.17^{ns}	2.90^{*}	1.74^{ns}	7.53^{**}	28.34^{**}	17.39^{**}
p-value		0.36	0.02	0.13	4.28×10^{-5}	2.82×10^{-10}	3.17×10^{-8}
HSD value		-	1.53	-	1.64	1.17	1.64

Notes: ns: not significantly different*: significantly different; data labeled by the different letters in a column were significantly different at $p < 0.05$ according to o Tukey's test (HSD)

Table 3. Weight and length of *Spodoptera frugiperda* pupae treated with endophytic EPF

Isolates	Species	Mean of pupae weight (g)	Mean of pupae length (cm)
Control	-	0.15a	1.49a
JgSPK	*Beauveria bassiana*	0.14b	1.36ab
JaGiP	*Beauveria bassiana*	0.14b	1.39ab
PiCrPga	*Chaetomium* sp.	0.14b	1.35ab
JaMsBys	*Curvularia lunata*	0.14b	1.39ab
JaSpkPGA(2)	*Beauveria bassiana*	0.14b	1.41ab
JgCrJr	*Beauveria bassiana*	0.14b	1.49a
JaTpOi	*Beauveria bassiana*	0.14b	1.31b
JaSpkPga(3)	*Curvularia lunata*	0.14b	1.34ab
JaTpOi(2)	*Penicillium citrinum*	0.13b	1.34ab
CaTpPga	*Metarhizium anisopliae*	0.14b	1.35ab
F-value		6.00^{**}	2.74^{*}
p-value		2.30×10^{-4}	0.02
HSD value		0.01	0.18

Notes: *: significantly different; data labeled the different letters in a column were significantly different at $p < 0.05$ according to Tukey's test (HSD)

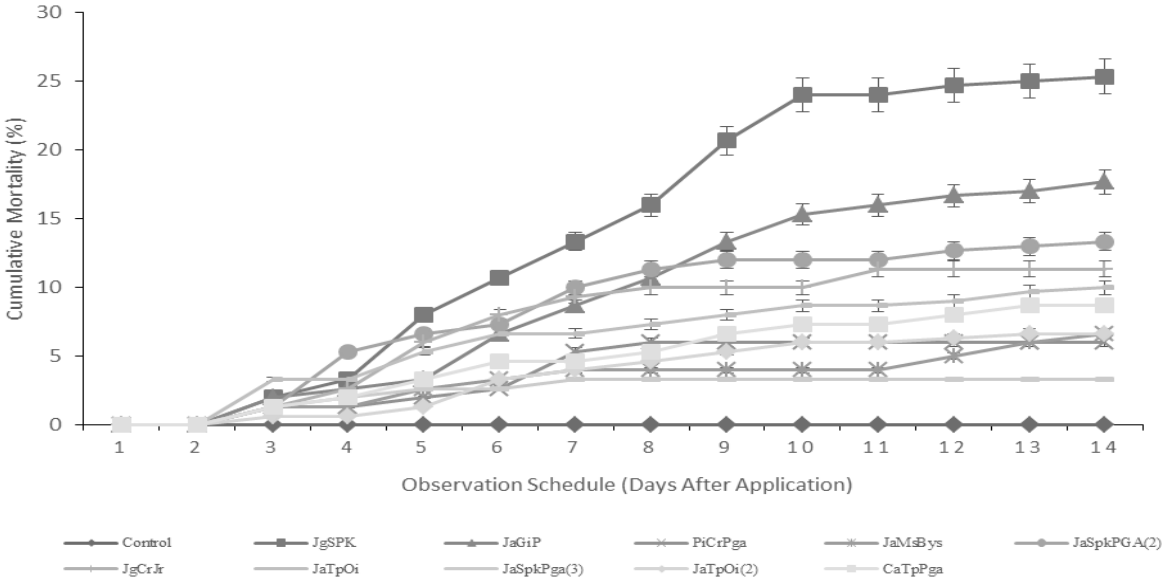

Figure 2. Cumulative mortality of *Spodoptera frugiperda* larvae treated with endophytic EPF (1×10^{10} conidia mL^{-1})

The percentage of *S. frugiperda* pupae non-emergence from larvae eating maize leaves colonized with *B. bassiana* JgSPK and JaGiP isolates was significantly higher ($p < 0.01$) compared to other fungal treatments and the control (Table 4). Pupae that were able to emerge from larvae consuming maize leaves colonized with endophytic EPF consisted of normal and abnormal pupae. The highest percentage of abnormal pupae occurred in the treatment of *B. bassiana* JgSPK and JgCrJr isolates, and the lowest was found in the treatment of *C. lunata* JaSpkPga(3) isolate and the control. The percentage of *S. frugiperda* adult non-emergence treated with *B. bassiana* JgSPK and JaGiP isolates was significantly higher ($p < 0.01$) than other fungal treatments and the control (Table 5). Adults that were able to emerge from pupae treated with endophytic EPF consisted of normal and abnormal adults. The highest percentage of abnormal adults was found in treating *B. bassiana* JgSPK, JaGiP, JaSpkPGA(2), JgCrJr and JaTpOi isolates. In addition, the normal adults from endophytic EPF treatments could still produce eggs, but their egg number is significantly lower ($p < 0.0001$) than controls (Table 5).

Spodoptera frugiperda larvae fed on maize leaves from seeds inoculated with endophytic EPF showed behavioral and color changes such as lack of appetite and muddy body color. The dead larvae showed unique symptoms, likely stiffening, drying out, shrinking, and hardening like a mummy. Depending on the infected fungal species, the larval body was mostly covered with fungal mycelia and became white, green, or brown. The larvae consumed by the leaves colonized by endophytic EPF will produce white, green, and brown corpses (Figure 3). The fungal isolate from the re-isolation of the corpses confirmed that it was the same as the fungal isolate used for the maize seed treatment. This could happen because the fungal isolate infected the larvae. The fungi that colonized the maize leaves could cause mycosis at any stage of *S. frugiperda*, but it could affect *S. frugiperda* to develop abnormal morphology or malformation. The infected and diseased larvae could produce abnormal or malformed pupae (Figure 4) and adults. The infected adults had smaller bodies, deformed and folded wings (Figure 5), and an inability to fly.

Table 4. Percentage of pupae non-emergence from larvae, normal and abnormal pupae of *Spodoptera frugiperda* treated with endophytic EPF (1×10^{10} conidia mL^{-1})

Isolates	Species	Mean of pupae non-emergence from larvae (%)	Mean of normal pupae (%)	Mean of abnormal Pupae (%)
Control	-	0.00f	100.00a	0.00e
JgSPK	*Beauveria bassiana*	54.00a	32.67g	13.33a
JaGiP	*Beauveria bassiana*	51.33ab	38.00fg	10.67ab
PiCrPga	*Chaetomium* sp.	12.67de	85.33b	2.00de
JaMsBys	*Curvularia lunata*	14.67de	83.33bc	2.00cde
JaSpkPGA(2)	*Beauveria bassiana*	34.00c	57.33de	8.67abc
JgCrJr	*Beauveria bassiana*	36.00bc	52.00ef	12.00ab
JaTpOi	*Beauveria bassiana*	30.00c	64.00de	6.00abcd
JaSpkPga(3)	*Curvularia lunata*	8.67e	90.67b	0.67e
JaTpOi(2)	*Penicillium citrinum*	16.00de	80.67bc	3.33bcde
CaTpPga	*Metarhizium anisopliae*	23.33cd	70.00cd	6.67abcd
F-value		5.10^{**}	57.23^{**}	12.47^{**}
p-value		6.02×10^{-13}	2.13×10^{-13}	6.52×10^{-7}
HSD value		9.52	10.72	10.12

Notes: ns: not significantly different*: significantly different; data labeled the different letters in a column were significantly different at $p < 0.05$ according to Tukey's test (HSD)

Table 5. The mean percentage of adult non-emergence from pupae, normal and abnormal adults, and eggs laid by *Spodoptera frugiperda* treated with endophytic EPF (1×10^{10} conidia mL^{-1})

Isolates	Species	Mean of adult non-emergence from Pupae (%)	Mean of normal adult (%)	Mean of abnormal adult (%)	Mean of eggs laid/female
Control	-	0.00f	100.00a	0.00e	29.30a
JgSPK	*Beauveria bassiana*	67.33a	26.67g	6.00a	4.93b
JaGiP	*Beauveria bassiana*	62.00ab	30.00fg	8.00ab	12.22b
PiCrPga	*Chaetomium* sp.	14.67de	82.67b	2.67de	8.11b
JaMsBys	*Curvularia lunata*	16.67de	82.00bc	1.33cde	8.09b
JaSpkPGA(2)	*Beauveria bassiana*	42.67c	49.33de	8.00abc	1.97b
JgCrJr	*Beauveria bassiana*	48.00bc	41.33ef	10.67ab	8.34b
JaTpOi	*Beauveria bassiana*	36.00c	58.00de	6.00abcd	5.45b
JaSpkPga(3)	*Curvularia lunata*	9.33e	90.00b	0.67e	11.80b
JaTpOi(2)	*Penicillium citrinum*	19.33de	76.67bc	4.00bcde	9.02b
CaTpPga	*Metarhizium anisopliae*	30.00cd	65.33cd	4.67abcd	5.18b
F-value		51.80^{**}	57.23	12.47	9.10^{**}
p-value		6.02×10^{-13}	2.13×10^{-13}	6.52×10^{-7}	9.49
HSD value		9.52	10.72	10.12	12.11

Notes: ns: not significantly different, *: significantly different; data labeled the different letters in a column were significantly different at $p < 0.05$ according to Tukey's test (HSD)

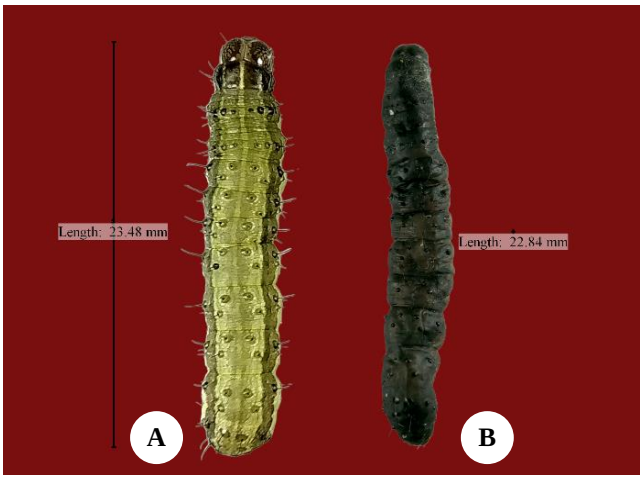

Figure 3. Morphology of healthy larvae (untreated) (A) and corpses of treatment with endophytic EPF (B)

Figure 4. Morphology of healthy pupae from untreated larvae (A) and abnormal unhealthy pupae from treated insects (B)

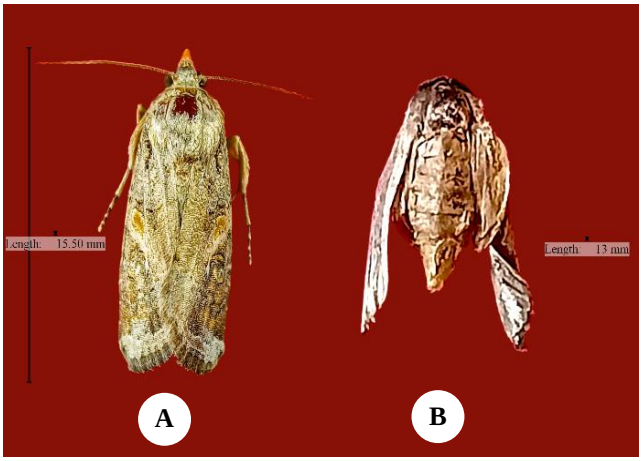

Figure 5. Morphology of healthy adults from untreated (A) and abnormal, unhealthy adults from treated insects (B)

Discussion

In general, the total hemocyte concentrations of *S. frugiperda* larvae consuming maize leaves colonized with the endophytic EPF were significantly different from the control in which the larvae hemocyte concentration was the highest after consuming maize leaves colonized with *B. bassiana*, *Chaetomium* sp., *C. lunata*, *P. citrinum*, and *M. anisopliae*. The hemocyte concentration tended to decrease further with increasing age of the *S. frugiperda* larvae. The study found hemocyte concentration differentiation after 3 days of exposure to larvae with the endophytic EPF. Although the endophytic EPF did not increase abundantly in the *S. frugiperda* hemolymph, the fungal presence in the larval gut could raise hemocyte differentiation. At last day observation (9 days after the larvae exposure), exposure to conidia and blastospores of the endophytic EPF in the present study did not have an effect in reducing or increasing the hemocyte concentration in the larvae hemolymph of *S. frugiperda*. The endophytic EPF with 1 x 1010 conidia mL-1 propagules did not cause an immune response in *S. frugiperda* larvae. The immune response in insects is indicated by increasing the hemocyte concentration in the larvae hemolymph of insect hosts (Bitencourt et al. 2023). This study could not detect propagules of the endophytic EPF invading the hemocoel or penetrating the integument of the *S. frugiperda* larvae. However, Bitencourt et al. (2023) reported that mosquitos (Diptera) exposed directly to a conidial or blastospore suspension caused the fungal propagules to invade the mosquito's midgut. In the present study, the blastospores or conidia could not be observed in the hemocoel of *S. frugiperda* larvae because the larvae were not exposed directly to a conidial or blastospores suspensión. Hemocytes of *S. frugiperda* larvae were observed and identified, namely prohemocytes, plasmatocytes, oenocytoids, coagulocytes, and granulocytes. However, we did not observe and count each hemocyte, except a mixture suspension of all types of hemocytes. Granulocytes and oenocytes act to initiate infection, and they secrete antimicrobial peptides (AMPs) into the gut lumen (Butt et al. 2016). Plasmatocytes and granulocytes are mainly involved in encapsulation and phagocytosis. At the same time, oenocytes are related to nodulation and melanization, and granulocytes and oenocytoids adhere to the fungus, demonstrating both hemocytes' involvement in stopping the fungal infection (Bitencourt et al. 2023).

Cumulative mortality of *S. frugiperda* larvae treated by the endophytic EPF demonstrated *B. bassiana* JgSPK, JaGiP isolates proved to be the most pathogenic compared to other isolates. The *B. bassiana* JgSPK and JaGiP isolates also decrease the percentage of *S. frugiperda* pupae and adult non-emergence (mortality). Larvae feeding on leaves colonized by endophytic EPF could reduce the larval and pupal weight of *S. frugiperda*. The highest percentage of abnormal adults was found in treating *B. bassiana* JgSPK, JaGiP, JaSpkPGA(2), and JaTpOi isolates. Therefore, four isolates of *B. bassiana* could be considered pathogenic to *S. frugiperda*. The present study found that larvae eating the maize leaves colonized with the endophytic EPF conidia and blastospores could kill larvae, pupae, and adults and cause their bodies to abnormal or malformation. The larvae began to die after three days of fungal exposure.

The previous study showed that the larvae began to die 3-4 days after the neonate larvae treated with endophytic *B. bassiana* and *M. anisopliae* (Sari et al. 2023). The fungal spores cause dead larvae through hyphae of the endophytic fungus to enter orally, and the fungi infect through the intestinal epithelium (Boomsma et al. 2014). The hyphae and spore grow and produce conidia and blastospores in the hemolymph, producing secondary metabolites that could kill larvae (Mancillas-Paredes et al. 2019). In the present study, the exposure of endophytic EPF conidia and blastospores did not affect the immune response in *S. frugiperda* larvae because the hemocyte concentration in the larvae hemolymph of *S. frugiperda* did not decrease or increase. If the larvae have no immune response to the endophytic EPF, the conidia and blastospores could produce secondary metabolites that kill the larvae (Mancillas-Paredes et al. 2019). The fungi continue to grow saprophytically by absorbing the body fluids of the corpse (Gabarty et al. 2014). The corpse's body can grow and appear as sexual ascospores and asexual conidia (Boomsma et al. 2014), and the fungi can induce mycosis (Russo et al. 2020). The symptoms of mycosis in the corpse's body in this present study were similar to the symptoms from the previous studies; the corpses became hardened, stiffened, dry, shriveled, and covered with mycelium and spores of fungus (Herlinda et al. 2022; Lestari et al. 2022; Sari et al. 2023).

The abnormal pupae and adults were resulted in this study could increase insect mortality. The abnormal pupae could produce abnormal adults due to asymmetrical, fold, or small wings that obstacle the imago from flying, spreading, and copulating. Therefore, the abnormal pupae and adults caused by endophytic EPF could contribute to decreasing insect pest population densities in the field. These findings highlighted that the endophytic EPF conidia and blastospores could protect maize plants against *S. frugiperda* by seed treatment.

Finally, the endophytic entomopathogenic fungal conidia and blastospores inoculated in maize plants by seed

inoculation have a lethal effect on *S. frugiperda* larvae. However, exposure to conidia and blastospores did not reduce or increase the hemocyte concentration in the larvae hemolymph of *S. frugiperda*. Furthermore, the endophytic EPF with 1 x 10^{10} mL^{-1} conidia propagules did not cause an immune response in *S. frugiperda* larvae.

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