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Selection, Isolation, and Identification of Entomopathogenic Bacteria and Fungi Against *Spodoptera frugiperda* J.E. Smith in Maize

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Abstract: *Spodoptera frugiperda* is a highly destructive bug that causes significant damage to maize crops. The control of *S. frugiperda* so far primarily relies on the use of synthetic pesticides, which can cause deleterious effects on the environments, particularly as the insects develop resistance. Entomopathogenic bacteria and/or fungi offer ecologically sustainable method for controlling *S. frugiperda*. The process of selection began by collecting dead *S. frugiperda* larvae from multiple locations. These specimens were cultured in PDA and NA media. The isolated bacteria and fungi were cleansed and examined separately on second instar S. frugiperda larvae to determine the rates of mortality and feed consumption. This study employed a fully randomized design, with trials being replicated three times. Results showed that Lia and Lib bacterial isolates caused mortality rates of 50% and 23.33%, respectively, on *S. frugiperda* larvae. Fungal isolates P1, P2, K2, and K3 caused mortality rates ranging from 10 to 16.67% on *S. frugiperda* larvae. The K3 isolate achieved the greatest decrease in feed consumption, with a drop of 34.16%. The Lia, Lib, P1, P2, and K2 isolates decreased feed consumption at 14.77%, 26.87%, 24.02%, 33.18, and 31.14%, respectively. The molecular identifications showed that the Lia and Lib isolates were the Aeromonas hydrophila strain DUCC5728HX-3 and Acinetobacter soli strain GFJ2, respectively. This study represents the initial findings on the presence of entomopathogenic bacteria in *S. frugiperda* larvae. The fungal isolates K2, K3, P1, and P2 were identified to be Penicillium citrinum strain DUCC5728, Metarhizium rileyi strain 936, and Aspergillus flavus strain KU20018.4, respectively.

Keywords: Entomopathogenic bacteria, entomopathogenic fungi, *Spodoptera frugiperda*

1. Introduction

Fall armyworm (FAW) is the larval form of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), which is a major pest of over 350 plants, including maize (Montezano et al., 2018). *S. frugiperda* originated from north America (Montezano et al., 2018) has expanded to other continents between 2016 and 2019 (Goergen et al. 2016; Early et al., 2018; Ganiger et al., 2018). The initial documented infestation of *S. frugiperda* in Indonesia occurred in April 2019 in West Sumatra (Lestari et al., 2020) and subsequently spread to Java within the same year (Maharani et al., 2019).

According to a report by FAO and CABI (2019), the annual losses caused by *S. frugiperda* resulted in a loss of 8.3-20.6 million tons, valued at US$2.5-6.2 billion. This pest poses a significant danger to maize due to its rapid dissemination, wide range of hosts, and substantial economic losses. The prevalence of *S. frugiperda* is more

severe in young plants, and the number of infections decreases as plants mature (Trisyono et al., 2019). This pest affects both local and hybrid varieties of maize in Indonesia (Ginting et al., 2020).

The control of *S. frugiperda* so far depends on the use of synthetic pesticides (Day et al., 2017). It can create negative impacts on the ecosystem, as certain pests have evolved resistance to specific pesticides (Wu et al., 2016; Yang et al., 2017). Entomopathogenic fungi and bacteria offer environmentally safe solutions for pest management. Fungi, including *Beauveria bassiana*, *Metarhizium anisopliae*, and *Nomuraea rileyi* have been reported to effectively control the population of *S. frugiperda* (Rivero-Borja et al., 2018; Ngangambe and Mwatawala, 2020; Shylesha et al., 2018). *M. anisopliae* MA, *Cladosporium* sp. BM-8, *Penicillium citrinum* CTD-28, and *Penicillium* sp. CTD-2 have demonstrated lethality in *S. frugiperda* larvae at rates of 54.0%, 48.0%, 44.0%, and 42.0% respectively (Idrees et al., 2023). A study conducted in South Sumatra showed that *Aspergillus* sp., *Beauveria* sp., *Chaetomium* sp., and *Curvularia* sp. possess the ability to exterminate *S. frugiperda* (Gustianingtyas et al., 2021). The use of bacteria for controlling *S. frugiperda* has not been extensively investigated. Bacillus thuringiensis, a type of bacteria, has been identified to be capable of killing *S. frugiperda* larvae (Patel et al., 2020; Polanczyk et al., 2000).

Due to its adaptability to new environments and the presence of compatible hosts, *S. frugiperda* has the potential to turn from being an invading pest to being an endemic one across Asia and Africa. There is a need to conduct investigations to find local bacteria and fungi that may kill insects, as this would offer environmentally beneficial options. This study aimed to isolate, select, and identify bacteria and fungi with the potential to act as entomopathogenic agents for controlling the population of *S. frugiperda* larvae. Explorations were necessary to assess the performance of local isolates, which have adapted to the specific location and are less likely to become invasive species (Zhang et al., 2004; Ahmad et al., 2013).

2. Methodology

2.1 Exploration and Isolation of Entomopathogenic Bacteria and Fungi

Preliminary surveys were conducted prior to the commencement of exploration. In March-September 2021, samples were collected randomly from selected locations in the regencies of Banyumas and Purbalingga, located in Central Java. *S. frugiperda* larvae exhibiting signs of mortality were collected and transferred to PDA (Lee et al., 2015) and NA media. The bacteria and fungi that had been purified were used to conduct experiments on *S. frugiperda* larvae.

The tests were performed using the leaf dipping method (Balfas & Wilis, 2009). Bacterial cultures were cultivated in nutritional broth until they reached the density of 108 CFU/ml, whereas fungal cultures were cultivated in potato dextrose broth until they reached the density of 106 CFU/ml. The maize leaves used in the experiments were soaked in either bacterial or fungal cultures for 10 minutes and subsequently dried by exposure to air. The leaves were placed in test tubes, with each test tube containing a single larva at instar 2 (Krishanti et al., 2017). Data on the mortality rate of larvae and the extent of leaf consumption collected daily were analyzed using the Duncan Multiple Range Test (DMRT) with α set of 5%.

Hypersensitivity tests were performed by injecting bacterial suspensions with a concentration of 1 × 106 CFU/ml into tobacco leaf tissues. The observations were carried out for four days. The appearance of hypersensitive reaction indicated that the tested isolates were pathogenic bacteria.

2.2 Identification of Entomopathogenic Bacteria and Fungi

The bacteria and fungi were identified at the molecular level using the following steps: DNA extraction, PCR amplification, DNA sequencing, and data analysis. The modified GES method (Pitcher et al. 1989) followed by amplification was used to extract bacterial isolates (16S rDNA). The fungal isolates (18S rDNA) were subjected to DNA extractions using the i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology). The amplifications of bacterial DNA were conducted using Taq Master Mix (Promega) with the following primers: 27 F: 5`-- AGA GTT TGA TCC TGG CTC AG – 3` and 1492R: 5`-- TAC GGY TAC CTT GTT ACG ACT T –-3`, while that of fungal isolates used the primers ITS 4: 5`-- TCC TCC GCT TAT TGA TAT GC – 3` and ITS 5: 5`-- GGA AGT AAA AGT CGT AAC AAG G –-3`. The process of sanger sequencing was carried out in the Genetic Analyzer ABI 3130 XL, subsequently analyzed in the BioEdit program. The findings were deposited in the GeneBank of National Center for Biotechnology International (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) using BLAST (Basic Local Alignment Tools).

3. Results and Discussion

The explorations for dead S. frugiperda larvae on maize plants occurred in the regencies of Banyumas and Purbalingga, located in Central Java, Indonesia. There are two distinct indicators of dead larvae: stiff, mummified, and covered in a white layer (Figure 1), and wet rot (Figure 2). The symptoms observed in larvae infected with fungi included mummification (stiffness), the mycelium formation in the form of white layer covering the larvae,

and the presence of conidial mass with dark green coloration (Sridhar & Devacfsxprasad 1996). The symptoms observed in larvae infected with bacteria were being soft, excreting liquid, and emitting odor (Krishanti et al., 2017).

Figure 1. Dead larva with symptoms of stiffness and mummification

Figure 2. Dead larva with symptoms of wet rot

Thirteen bacterial isolates and eighteen fungal isolates were obtained from dead larvae. These isolates had the potential to impact the mortality and feed consumption of S. frugiperda larvae. The in vitro treatments of bacterial isolates were found to be efficient in killing S. frugiperda larvae at a significant level of α=5%. The use of Lia isolate resulted in the highest percentage of larval mortality, reaching 50% followed by that of Lib isolate at 23.3% (Table 1). The efficacy of these therapies was substantially greater than that of the control. Figure 3 illustrates an additional instance of dead larva caused by bacterial infection.

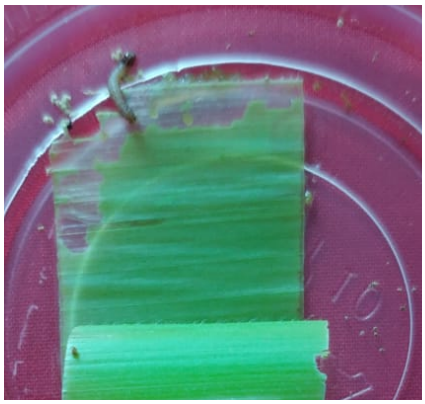

Figure 3. Dead *S. frugiperda* larva after being treated with bacterial isolates

Table 1. Effects of treatments with bacterial isolates on the mortality of *S. frugiperda* larvae

Treatment	Isolate origin	Mortality (%)
Control		0.00 e
L14x	Banyumas	13.33 bcde
Lia	Banyumas	50.00 a
Lib	Banyumas	23.33 b
L2	Banyumas	13.33 bcde
L3	Banyumas	3.33 de
L3x	Banyumas	16.67 bcd
L4	Banyumas	3.33 de
L4x	Banyumas	16.67 bcd
L5	Banyumas	6.67 cde
L6	Banyumas	20.00 bc
L6x	Banyumas	6.67 cde
L7x	Banyumas	20.00 bc
S3	Purbalingga	3.33 de

Note: values followed by the same letters in the Mortality column indicate that those results are not significantly different based on DMRT at α = 5%.

Bacterial treatments began to affect the mortality rate of *S. frugiperda* larvae within 24 hours. Larvae exhibited signs of lethargy and eventually died, displaying symptoms of wet rot. The effects became more evident on the second day (Figure 4).

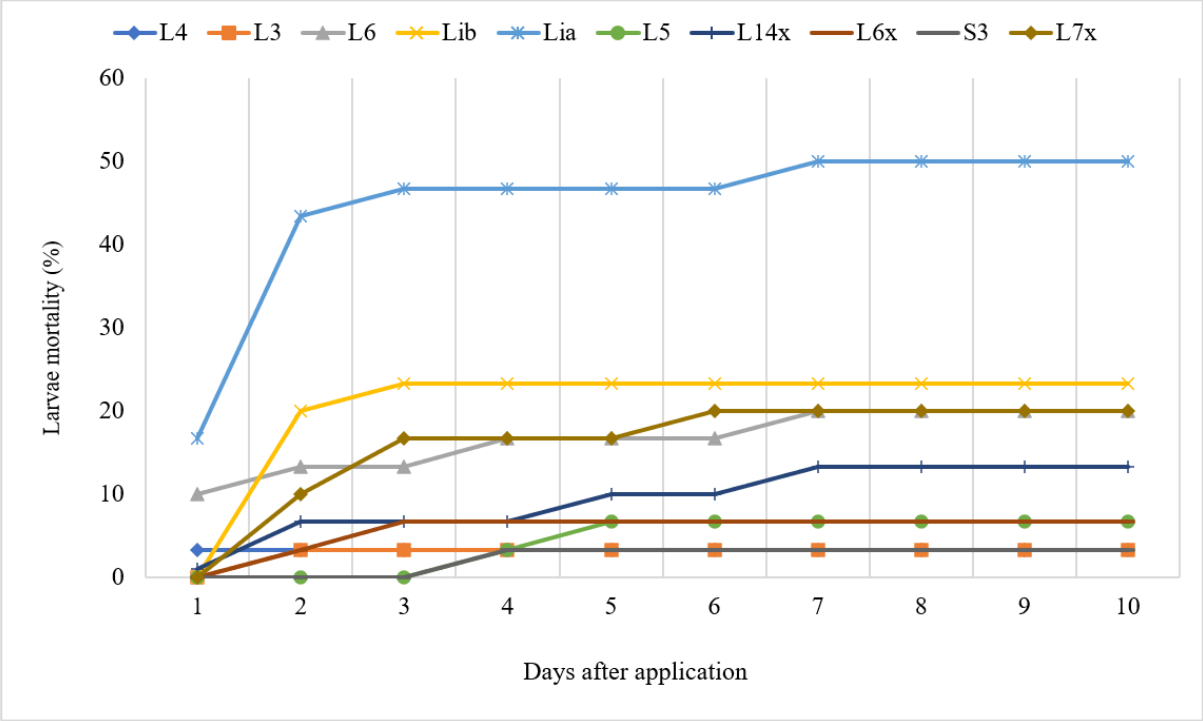

Figure 4. Dead *S. frugiperda* larva two days after being infected with bacteria

Treatments involving certain bacterial isolates considerably affected the feed consumption of *S. frugiperda* larvae. Lib, L3, and L5 isolates reduced the feed consumption by 26.87%, 22.07%, and 25% correspondingly. The L3 and L5 isolates did not significantly increase the mortality rate; however, they did cause a decrease in feed consumption. On the other hand, the Lia isolate exhibited the highest mortality rate while at the same time reducing feed consumption by 14.77% (Table 2).

Table 2. Effects of treatments with bacterial isolates on the feed consumption of *S. frugiperda* larvae

Isolate	Average daily feed consumption (gr)	Inhibitory rate (%)
Control	0.31	0.00 bc
L14x	0.27	12.23 ab
Lia	0.26	14.77 ab
Lib	0.22	26.87 a
L2	0.26	15.33 ab
L3	0.24	22.07 a
L3x	0.33	-7.77 c
L4	0.26	13.85 ab
L4x	0.27	12.81 ab
L5	0.23	25.00 a
L6	0.26	14.12 ab
L6x	0.26	15.84 ab
L7x	0.32	-5.71 c
S3	0.26	15.34 ab

Note: values followed by the same letters in the Inhibitory rate column indicate that those results are not significantly different based on DMRT at $\alpha = 5\%$

Treatments of fungal isolates K2, PI, P2, and K3 resulted in mortality rates of 20%, 16.67%, 16.67%, and 10% respectively in *S. frugiperda* larvae (Table 3). These results demonstrated a substantial increase compared to the control group, with p-value of less than 5%. Dead *S. frugiperda* larvae treated with fungal isolates showed symptoms of stiffness and mummification (Figure 5).

Table 3. Effects of treatments with fungal isolates on *S. frugiperda* larval mortality

Isolate	Isolate origin	Mortality (%)
Control		0c
K2	Banyumas	20.00 a
K3	Banyumas	10.00 abc
KO	Banyumas	0.00 c
L13	Banyumas	0.00 c
LA	Banyumas	0.00 c
LA44	Banyumas	0.00 c
P1	Purbalingga	16.67 ab
P2	Purbalingga	16.67 ab

PB1	Purbalingga	0.00 c
PB3a	Purbalingga	6.67 bc
PB3b	Purbalingga	10.00 abc
PK2	Purbalingga	6.67 bc
PK3	Purbalingga	3.33 c
S1	Purbalingga	0.00 c
S10	Purbalingga	3.33 c
S2	Purbalingga	3.33 c
S5	Purbalingga	0.00 c
S7	Purbalingga	6.67 bc

Note: values followed by the same letters in the Mortality column indicate that those results are not significantly different based on DMRT at α = 5%

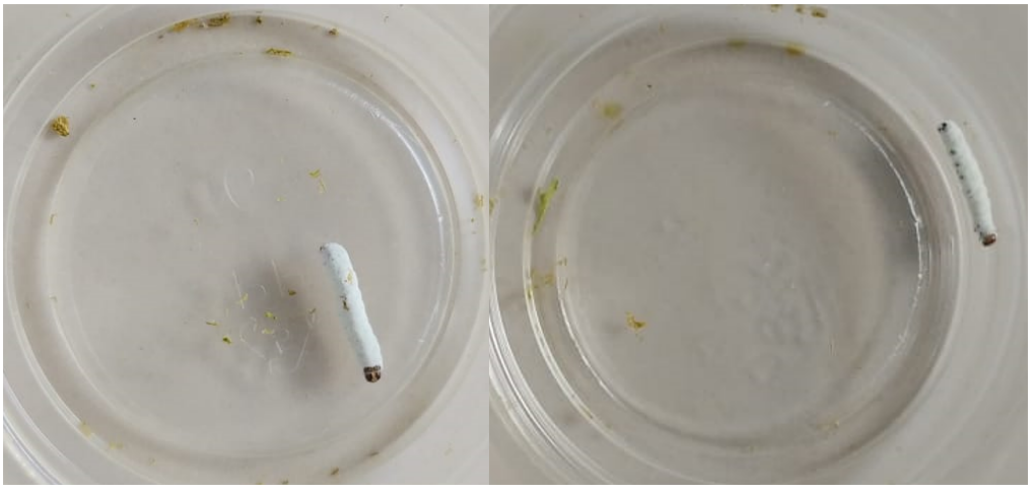

Figure 5. Dead *S. frugiperda* larvae treated with fungal isolates showing the signs of stiffness and mummification

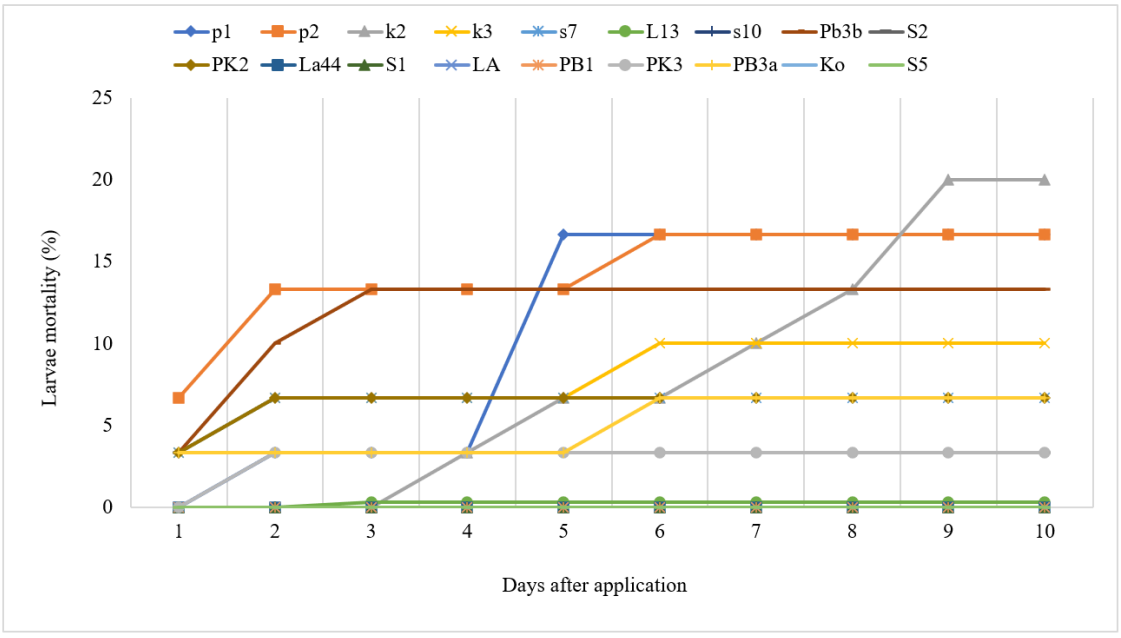

Figure 6. Mortality rate of *S. frugiferda* larvae treated with fungal isolates

The treatment of fungal isolates began to take effects on the mortality rate of *S. frugiferda* larvae after 24 hours and showed a considerable increase after the fifth day (Figure 6). Initially, conidia attached to the cuticle or entered through the mouth and started to appear on the fifth day. The conidial infestation through the integument proceeds by the conidial attachment on the cuticle followed by conidial expansion to form tubes that grow towards the integument and invade the cuticle (Fernandes et al., 2007). Infection commences when hyphae are able to puncture the insect's cuticle and the capacity to infect is a determining feature of fungal virulence (Altre & Vandenberg, 2001). Fungi initiate a targeted hyphal infection from appressoria after the hyphae penetrate the cuticle and enter the hemocoel (El-Ghany, 2015).

Table 4. Effects of treatments with fungal isolates on the feed consumption of *S. frugiperda* larvae

Isolate	Average daily feed consumption (gr)	Inhibitory rate (%)
K2	0.21	31.14 a
K3	0.20	34.16 a
KO	0.30	3.14 cd
L13	0.30	2.28 cd
LA	0.29	6.47 cd
LA44	0.28	7.70 cd
P1	0.23	24.02 ab
P2	0.20	33.18 a
PB1	0.30	0.56 cd
PB3a	0.31	-1.22 cd
PB3b	0.30	3.24 cd
PK2	0.29	6.51 cd
PK3	0.30	3.43 cd
S1	0.27	13.41 bc
S10	0.28	9.78 bc
S2	0.32	-2.93 cd
S5	0.30	3.20 cd
S7	0.33	-6.92 d
Control	0.31	0.00 cd

Note: values followed by the same letters in the Inhibitory rate column indicate that those results are not significantly different based on DMRT at $\alpha = 5\%$

Treatments using fungal isolates K1, K2, P1, and P2 significantly reduced the feed consumption of S. frugiperda larvae by 31.14%, 34.16%, 24.02%, and 33.18%, respectively, compared to that of Control (p-value<0.05). Although the effects on mortality rate varied between 10 and 20%, these isolates successfully reduced the feed consumption of S. frugiperda by 24.02% to 33.18%.

Hypersensitivity tests were performed on Lia and Lib bacterial isolates based on their death rates. The experiments were conducted on tobacco leaves. The findings indicated that these isolates did not induce necrosis and, thus, they were not pathogenic on plants (Figure 7).

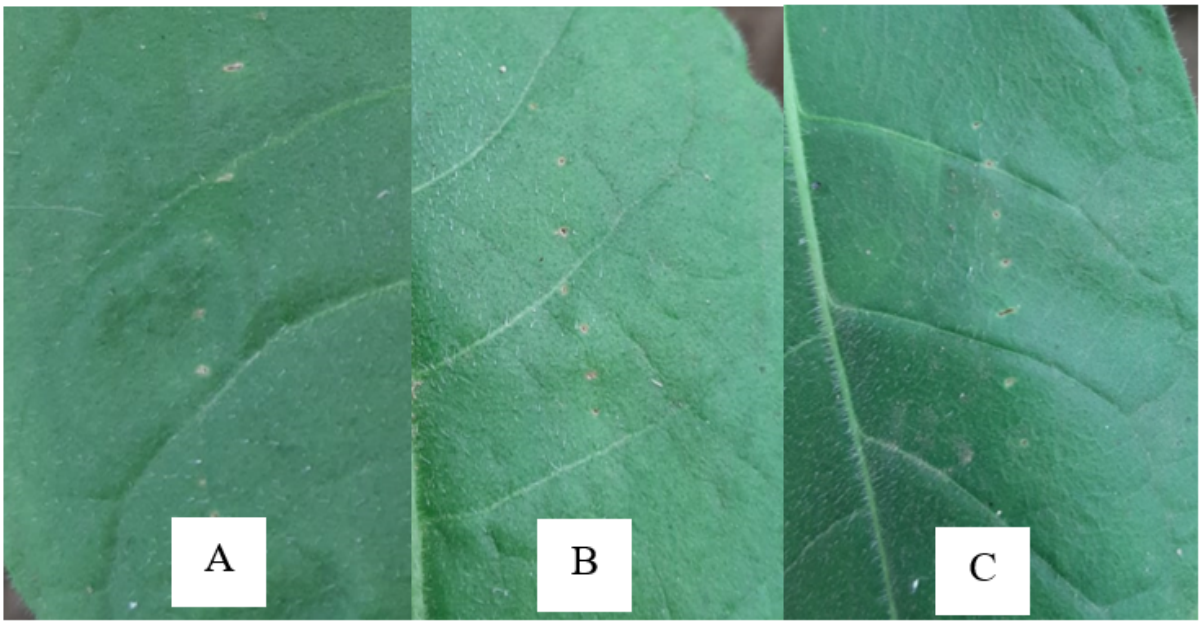

Figure 7. Hypersensitive test: Lia isolate (A), Lib (B) and Control (C)

Table 5. Results of BLAST analysis

Isolate code	Description	Scientific Name	Percentage Identification	Accession
Lia	*Aeromonas hydrophila* strain HX-3	*Aeromonas hydrophila*	100.00%	CP046954.1
Lib	*Acinetobacter soli* strain GFJ2	*Acinetobacter soli*	99.40%	CP016896.1
P1	*Penicillium citrinum* strain DUCC5728	*Penicillium citrinum*	99.80%	MT582768.1
P2	*Metarhizium rileyi* strain 936	*Metarhizium rileyi*	100.00%	LR792766.1
K2	*Aspergillus flavus* strain KU20018.4	*Aspergillus flavus*	100.00%	MT487825.1
K3	*Metarhizium rileyi* strain 936	*Metarhizium rileyi*	99.84%	LR792766.1

Molecular identifications were performed on two bacterial isolates (Lia and Lib) and four fungal isolates (K2, K3, P1, and P2). The results are shown in Table 5. The BLAST analysis showed that Lia and Lib isolates were Aeromonas hydrophila strain DUCC5728HX-3 (100% homology) and Acinetobacter soli strain GFJ2 (99.40% homology). Additionally, P1 was identified as *Penicillium citrinum* strain DUCC5728 (99.8% homology), P2, K2, and K3 were identified as, respectively, *Metarhizium rileyi* strain 936 (100% homology), *Aspergillus flavus* strain KU20018.4 (100% homology), and *Metarhizium rileyi* strain 936 (99.84% homology).

Aeromonas hydrophila are aquatic gram-negative bacteria (Noonin et al., 2010). These bacteria are known to be pathogenic towards fish and have the potential to infect humans and animals (Noonin et al., 2010; Ivey et al., 2016; Citterio & Biavasco, 2015). This pathogen has the capacity to cause economic loss in freshwater fishery

(Kristianingrum et al., 2018).

There has been limited research on the utilization of *A. hydrophila* as an entomopathogen. Noonin et al. (2010) demonstrated that certain strains of *A. hydrophila* caused complete mortality in Tenebrio molitor larvae (mealworm) within 16 hours of exposure. In their study, Korany et al. (2019) discovered that *A. hydrophila* could produce chitinase enzyme at 295.57 ± 1.03 U/mg protein. Treatments of crude chitinase produced by *A. hydrophila* (295 U/mg protein) resulted in a death rate of up to 90% in instar 1 larvae of *Galleria mellonella* L. (a moth belonging to the Pyralidae family under the order Lepidoptera). The potential of *A. hydrophila* to produce chitinase can be used for the development of a biocontrol method against insect pests. Nevertheless, further research is needed to investigate the pathogenicity of *A. hydrophila* strain DUCC5728HX-3 on both humans and animals.

Acinetobacter soli is a newly identified bacterial species, initially found in forest soil in Korea and classified as strain B1(T) (= KCTC 22184(T)= JCM 15062(T)) (Kim et al., 2008). The first documented cases of blood flow infection caused by the *A. soli* clone were reported in 2011 (Pellegrino et al., 2011). Studies of *A. soli* in the field of agriculture cited by Susilowati et al. (2016) demonstrated that *A. soli* had the potential to become Plant Growth Promoting Rhizobacteria (PGPR) due to its ability to fix Nitrogen, dissolve Phosphate, and create Indole Acetic Acid (IAA) hormone. *A. soli*, isolated from saline soil, had the ability to dissolve potassium, accounting for 68% of their dry cell weight (Bhattacharya et al., 2016). It has also been found in a significant rice pest and suspected to serve as an indicator of infestation and be associated with the plants' defensive mechanism through the emission of volatile chemicals (Wari et al., 2018). Another strain of bacteria belonging to the same genus, *A. calcoaceticus*, is known to be entomopathogenic to a nematode, *Steinernema* sp. (Reghunath et al., 2017).

A treatment of 1x 106 conidia/ml of *Penicillium citrinum* CTD-24 resulted in a mortality rate of 7.9% on *S. frugiperda* larvae (Idress et al., 2021) and 98.67% on S. litura larvae (Herlinda et al., 2020a). Russel et al. (2001) showed that *P. citrinum* produced a mycotoxin capable of impeding the egg development of *Aedes aegpti* mosquitoes. In a laboratory study, it was found that *P. citrinium* was able to completely eliminate instar 3 larvae of Culex quinquefasciatus mosquitoes with the mortality rate of 100% within two hours after the treatment at the dose of 1× 106 conidia/mL. *P. citrinum* has the capability to produce gibberellins hormone, which makes it a promising candidate to be developed as PGPR (Khan et al., 2008).

The genus Metarhizium is renowned for its ability to infect and kill insects, making it a highly recognized group of entomopathogens. In their study, Herlinda et al. (2020b) isolated entomopathogenic fungi from agricultural soils and discovered 14 fungal isolates belonging to the genus Metarhizium. These isolates shown varying levels of effectiveness in controlling *S. frugiperda* larvae, with mortality rates reaching as high as 78.67%. Visalakshi et al. (2020) shown that *Metarhizium rileyi* effectively regulates the population of *S. frugiperda*. Barros et al. (2020) demonstrated the impact of *M. rileyi* on *S. frugiperda* larvae, resulting in mortality rates ranging from 74% to 84%. The pathogenesis of *M. rileyi* is characterized by the development of conidia on conidiophores and its airborne infective spread. Conidia attach to the surface of hosts, sprout, and produce germ tubes infiltrating the cuticle and proliferating in the haemocoel until the pests die (Srisukchayaku et al., 2004).

Treatments with *Apsergillus* sp. at a concentration of 1x 106 conidia/ml on *S. frugiperda* larvae resulted in mortality rates ranging from 2.3% to 3.4%. The mortality rate can be elevated by augmenting the density of Apsergillus sp.; 1x 107 conidia/ml and 1x 108 conidia/ml resulted in mortality rates of 3.4-4.7% and 7.5-8.7%, respectively (Idress et al., 2021).

4. Conclusions

The process of selecting, isolating, and identifying entomopathogenic bacteria and fungi resulted in the discovery of bacterial and fungal isolates that significantly affected the mortality and feed consumption rates of *S. frugiperda* larvae. Bacteria strains DUCC5728HX-3 of *Aeromonas hydrophila* and GFJ2 of *Acinetobacter soli* caused mortality rates of 50% and 23.33%, respectively, on S. frugiperda larvae. Concurrently, treatments with

fungi *Penicillium citrinum* strain DUCC5728, *Metarhizium rileyi* strain 936, *Aspergillus flavus* strain KU20018.4 resulted in larval mortality rates ranging from 10% to 16.67%. The larvae treated with various bacterial and fungal isolates exhibited a decrease in feed consumption, ranging from 14.77% to 33.18%.

Author Contributions

Contributions: Conceptualization, L.S. and N.W.A.L.; methodology, N.W.A.L. and A.S.; software, N.W.A.L. and A.S; validation, N.W.A.L..; formal analysis, L.S. and N.W.A.L.; investigation, N.W.A.L.; resources, N.W.A.L.; data curation, N.W.A.L.; writing—original draft preparation, L.S.; N.W.A.L. and A.S.; writing—review and editing, N.W.A.L.; visualization, N.W.A.L..; supervision, L.S.; project administration, N.W.A.L.; funding acquisition, L.S. and N.W.A.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare there no conflict of interest

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