

Laboratory Evaluation of The Selected Entomopathogenic Fungi and Bacteria Against Larval and Pupal Stages of *Spodoptera litura* L

A Zulfitri¹, A. S Lestari¹, N. P. R. A Krishanti¹ and D Zulfiana¹

¹Research Center for Biomaterials, Indonesian Institute of Sciences (LIPI)
Jl. Raya Bogor Km 46 Cibinong 16911, Indonesia

E-mail: apriwi.zulfitri@lipi.go.id

Abstract. In the present study, efficacy of selected entomopathogenic fungi and bacteria against *Spodoptera litura* were evaluated. The study used two life phase of the insect, third to fourth larval and pupal stages. Evaluation on larval stage used larvae-dipping method while soil premixing method was used in efficacy test against *S. litura* pupae. Spore solution of five entomopathogenic fungals (*Paecilomyces* sp, *Beauveria bassiana*, T2B, B2 and T4B), cell suspension of 1 entomopathogenic bacteria (*Serratia* sp.) and suspension solution of consortium between T2B and *Serratia* sp. were used. Evaluation on larval stage showed the highest mortality was found in B2 treatment (70%), followed by *Serratia* sp. (60%) and consortium (50%) treatment. While on *S. litura* pupal stage evaluation, consortium treatment resulted in 62,5% of tested pupae failed to become adult moth. Other treatments on pupal stage evaluation showed under 40% failure transformation to adult stage.

1. Introduction

Managing *Spodoptera litura* is complicated because they are actively feeding at nighttime while hiding under the plant-host leaves at daytime. Since regular usage of pesticide causes rapid resistance in *S. litura*, more effective and safer biological ways to control the pest are needed. Controlling insect pest using entomopathogenic microbes including fungi and bacteria has played key role in sustainable pest management program. Entomopathogenic microbes usage are lower in cost, higher efficiency and safer for beneficial organisms than chemical pesticide [1]. During their life cycle, the fungi occur as infections in living insect only for short period while may remain dormant as conidia in the soil surrounding host cadaver [2]. Therefore, the soil environment is an important source for isolating entomopathogenic fungi and soil-inhabiting entomopathogenic fungi are an important and wide spread component of most terrestrial ecosystems [3].

Mechanism of fungal infection is different than those of bacteria and viruses. Both microbes are need to be ingested first by the host while fungal invades their host using mycelium through cuticle [1]. Zulfiana [4] reported that based on *Scanning Electronic Microscope* (SEM) observation on fungal infected termites larvae, infective process might be started by invasion of formed mycelium to larvae body via cuticle, then mycelium were germinated, proliferating and take over the larvae body.



Therefore, entomopathogenic fungi are promising biocontrol for non feeding insect life stages such as eggs and pupae [5].

During last decades, *S. litura* have been reported to show increase resistance to commonly used and several newer insecticide [6][7]. The studies showed that generally newer insecticides exhibited low and very low resistance may due to different mechanism of action used to targeting *S. litura* than those of conventional insecticides. However, not many farmers can afford these insecticides due to their high prices [7], thus resulted in low application. In the future, this might change since other insecticides company will come up with similar composition with lower price that will lead to increasing application. Continuous use of insecticides has resulted in negative impacts such as: increasing the amount of residues that are harmful to the environment, the killing of natural enemies (parasitoids and predators), and health hazards for users [8]. Utilizing biocontrol agents to control agricultural insect pests is an alternative strategy that is more environmentally friendly than the use of chemical pesticide [9].

In the study we applied indigenous entomopathogenic fungi resulted from soil isolation using bait method and entomopathogenic bacteria isolated from undeveloped *S. litura* pupae against larval and pupal stages of *S. litura*. In addition, we also compared efficacy of those entomopathogenic microbes with other several strains of entomopathogenic fungi. In order to increase the efficacy of entomopathogenic microbes, we tried to apply co-inoculation between entomopathogenic bacteria and fungal againsts pest target. Since bacterial and fungal biocontrol agents utilize different mechanisms of action to control pest on crops [10], it is important to investigate antagonistic activity of those microbes. Therefore we did a microbial compatibility assessment on potato dextrose agar (PDA) plate before applied the entomopathogens consortiums to test.

2 Materials and Methods

2.1 Qualitative compatibility assessment antagonistic activity between entomopathogenic fungal and bacteria isolates

Entomopathogenic fungal isolates used in the antagonistic study were isolates from Sumba island and West Sulawesi soil samples (T2B, B2, T4). The fungal were obtained using bait system method by incubating *Tenebriomolitor* larvae on soil sample. If any of the larvae showing surface mycosis, then it will be isolated and the colony formed was purified on PDA [2]. Beside those three isolates, we also applied other entomopathogenic fungal isolates which are collection of Research Center for Biomaterials (*Beauveria bassiana*, *Paecilomyces* sp. dan *Metarhizium* M1570)

Entomopathogenic bacterial used was BLSP4 isolated from dead *S. litura* pupae [9] and a known entomopathogenic bacteria, *Bacillus thuringiensis* (BLBt- IPBCC collection), as comparison was used. *B. thuringiensis* is one of the entomopathogenic bacteria that has been widely used as bioinsecticide especially to cotton plant pests. Therefore, there were twelve-combinations for antagonistic activity assay.

Microbial compatibility assay was carried out using dual culture on 4 cm PDA plate according to [11, modified]. Fungal inoculum ($\varnothing$: 5mm) was placed 1 cm from one side and bacteria inoculums was streaked 1 cm from the other side (Figure 1). The plates were then incubated at room temperature for 72 hours. The isolates growth was evaluated whether they have inhibiting or supporting relationship. Bacteria and fungi that can grow together on a plate will be used for consortium treatment.

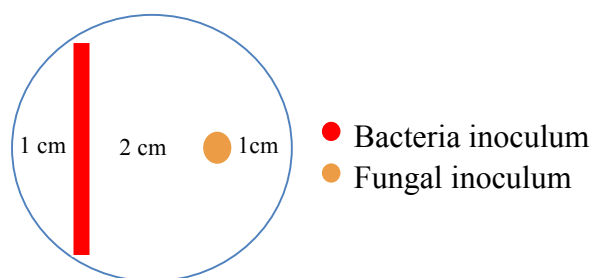


Figure 1. Illustration of qualitative compatibility assessment between entomopathogenic fungal and bacteria isolates

2.2 Entomopathogenic fungus and bacteria treatment before bioassay against *S. litura*

Isolates of entomopathogenic fungus used in this study were *B. bassiana*, *Paecilomyces* sp. (IPBCC collection), and 3 isolates from exploration of Sumba and Sulbar's soil (T2B, B2, T4B). The fungus are cultured on *potato dextrose agar* (PDA) at 25-27°C for 10 days. Next, into the media, 10-20 ml of sterile 0.01% Tween solution was added as surfactant. The conidia was harvested by gently scraping the surface of media using inoculation needle so the conidia suspended in the Tween solution. The hyphal debris were removed by filtering the mixture through autoclaved fine mesh cloth. Conidial concentration was counted using haemocytometer under 100x magnification.

Entomopathogenic bacteria isolates (*Serratia* sp.) was subcultured on *nutrient agar* (NA). In efficacy test, bacteria cell was harvested from a 24 hours old culture in nutrient broth (NB). Centrifugation (10 mins, 8000 rpm) was carried out to harvest bacteria cells then the cells were dissolved in 10 ml of sterile 0.85% NaCl solution and vortexed.

Potato dextrose broth (PDB) was used as fungal growth media in consortium treatment. Entomopathogenic fungal isolate was inoculated into 100 mL of PDB and incubated with shaking at 120 rpm, room temperature. On day-7, a 24 hours old of *Serratia* sp. grown in NB (1% v/v) was added into the fungal culture. The consortium culture was then incubated for 24 hours before applied.

2.3 Rearing of *S. litura*

Larvae of *S. litura* were reared in plastic containers (34×25×7 cm) provided with freshly picked taro leaf for food supply. The containers were kept clean and the food was changed daily. Fifth and sixth instar were kept in plastic cup individually to avoid crowding. The larvae were reared until pupal stage. Two to three days old of pupae were collected to be used further for bioassay. Before used, the pupae were dipped with sterile 0.5% NaCl solution for 5sec, rinsed with sterile aquadest and air dried.

2.4 Efficacy test of entomopathogenic microbes against *S. litura*

Efficacy test was conducted against two life stages, larva and pupae. Sterile 0.01% Tween solution was used as control treatment.

2.4.1 Efficacy test of entomopathogenic microbes against *S. litura* pupae.

Treatments applied were fungal entomopathogen (*B. bassiana*, *Paecilomyces* sp. T2B, T4B), *Serratia* sp and consortium. Method used was premixing using soil as media and zip plastic bag, referred to Anand, R, Prasad, B. & Tiwary, B. N. [5]. Before used, soil was sieved and weighed as much as 30 g for each replication. The conidial suspension (13 mL) was inoculated to the soil and mixed well. Premixed soil was transferred to plastic cup and 2 pupae were transferred in the middle of the soil. The set of experiment were kept in the dark at 22-23°C and examined for emergence of adults up to 14 days post inoculation. Unemerged pupae were considered dead.

2.4.2 Efficacy test of entomopathogenic microbes against *S. litura* larvae.

Treatments applied were fungal entomopathogen (*B. bassiana*, *Paecilomyces* sp., B2, T2B, T4B), *Serratia* sp and consortium. Third to fourth instar *S. litura* larvae were dipped in the conidial solution for 2-3 seconds and air dried. The larvae were then incubated for two weeks in plastic cup lined with

filter paper. During incubation, larvae were fed with fresh taro leaf every day. Mortality rate was recorded everyday or until all larvae dead [3, modified].

3 Results and Discussion

3.1 Compatibility assessment between entomopathogenic fungal and bacteria

The results of the compatibility test are shown in Table 1. Based on growth patterns on PDA media, from 12 test pairs, only T2B isolates when paired with BLBt or BLSP4 are not inhibited by the bacteria growth (**Figures 2a and b**)

Table 1 Results of compatibility tests between bacteria and entomopathogenic fungi on PDA media.

Fungal isolates	Bacteria	
	BLBt	BLSP4
<i>Paecilomyces</i> sp.	X	X
<i>B. bassiana</i>	X	X
M1570	X	X
T4B	X	X
T2B	O	O
B2	X	X

X: growth inhibition, O: no growth inhibition shown

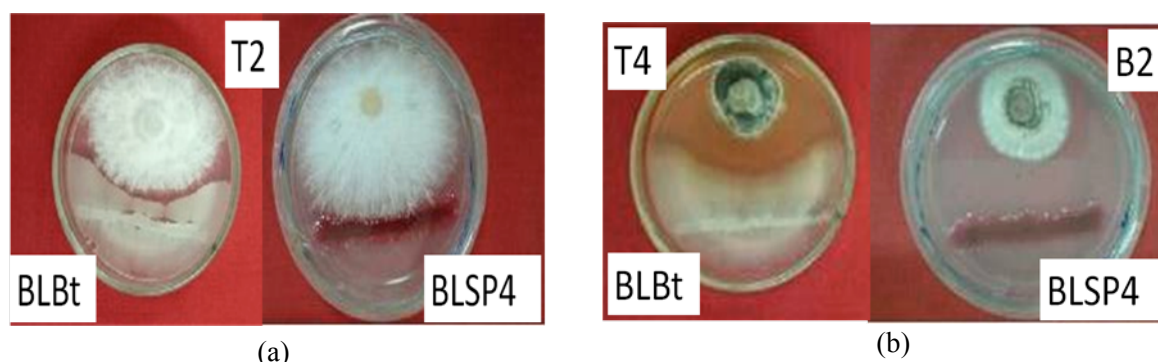


Figure 2. Illustration of compatibility test of fungi and entomopathogenic bacteria on PDA media. a. The growth of T2 fungi and entomopathogenic bacteria did not show any inhibitory effect in one PDA plate. b. Growth of fungal isolates (T4 and B2) and entomopathogenic bacteria inhibit each other.

Visual observation of bacterial growth and entomopathogenic fungus on PDA plate showed that there was interaction between fungi and entomopathogenic bacteria. Of the 6 fungal isolates tested, only T2 isolates showed normal mycelium growth and almost covered the plate when it was grown together between entomopathogenic bacteria BLBt and BLSP4. Meanwhile other fungal isolates showed growth inhibition when was grown together with entomopathogenic bacteria. The growth of this fungus appears to be clearly inhibited when compared to the control, ie isolates of fungi grown on individual PDAs in the same incubation period. Therefore, based on qualitative test, it can be concluded that T2 isolate can be applied together as consortium with entomopathogen bacterium BLBt or BLSP4.

Generally, the selection of isolates to be combined is based on the similarity of target pathogens possessed, for the control of pests or plant diseases [12]. But, in this experiment, the consortium is consist of two biocontrol agents with different mechanism of action This compatibility may allow simultaneous mechanism to increase pathogenicity against *S. litura*. Next, consortium between T2 and BLSP4 was used in the bioassay against larval and pupal stage of *S. litura*.

3.2 Efficacy test of entomopathogenic microbes against *S. litura* pupae

The conidiospores in the suspension were calculated using haemasitometer (**Table 2**) as follows:

Table 2 Spore concentration in suspension of 0.01% tween 80

Isolates	Spore concentration (spora/mL)
<i>B. bassiana</i>	$0,9 \times 10^7$
<i>Paecilomyces sp.</i>	$0,2 \times 10^7$
T2B	$1,6 \times 10^7$
T4B	$4,1 \times 10^7$

While the cell density of *Serratia sp.* in 10 mL 0.85% NaCl was equal to 10^9 cells/mL

Efficacy results (**Figure 3**) showed that the highest mortality (62.5%) in pupae *S. litura* was obtained in the suspension treatment of T2B and *Serratia sp.* This may indicate that dual inoculation may be able to increase pathogenicity compared to single inoculation. In some treatments, pupae turn into moths and lay eggs however, the eggs did not hatch into larvae. From the observation result, no mycosis was found, so dead pupae was inoculated on PDA and incubated for further test of cause of death of pupae. After 8 days, symptoms of mycosis in pupae (**Figure 4**) were obtained. The mycelium that grows enveloping pupae exhibited morphological characteristics similar to the inoculated isolates. In the consortium treatment's PDA plates *Serratia sp* growth was also found.

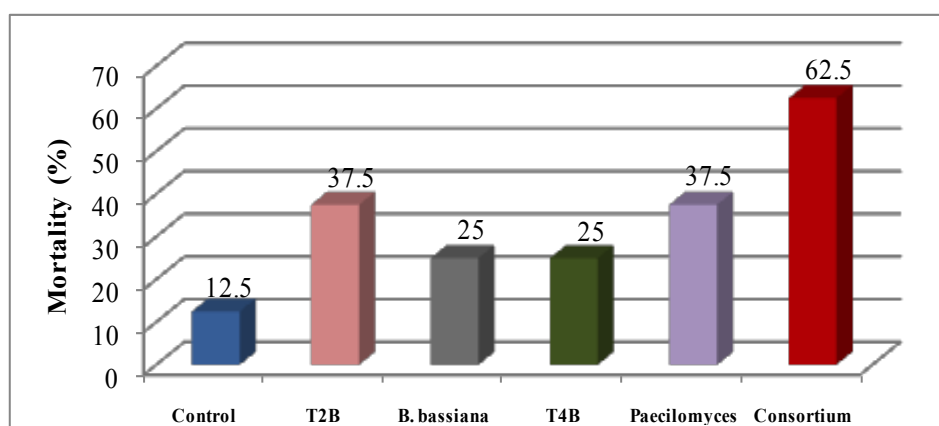


Figure 3. Mortality percentage of *S. litura* pupae 8 days post inoculation with conidial suspension of entomopathogen fungi and bacteria. Values given are mean of 4 replicates

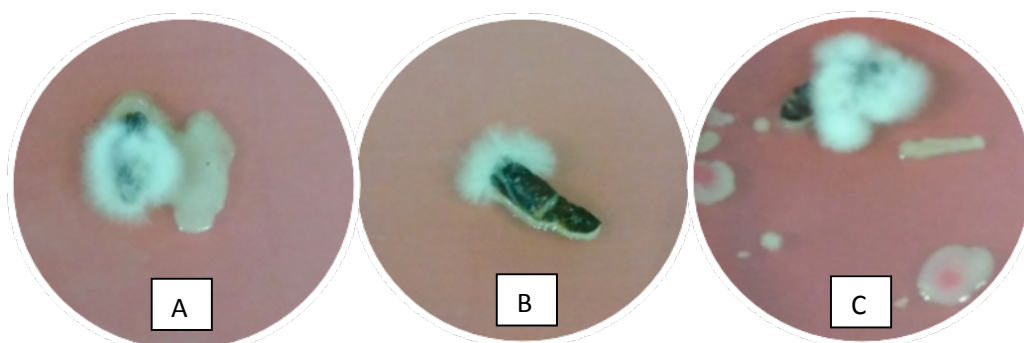


Figure 4. Growth of mycelium in dead pupa isolated with PDA after treatment with A. *B. bassiana*, B. T2B, and C. consortium showed growth characteristics of mycelium and colony that similar to the inoculated isolates

3.3 Efficacy test of entomopathogenic microbes against *S. litura* pupae

The conidiospores in the suspension (**Figure 5**) were calculated using haemasitometer as follows:

Table 3 Spore concentration in 0.01% tween 80 suspension

Isolates	Spore concentration (spora/mL)
<i>B. bassiana</i>	5.6×10^7
<i>Paecilomyces</i> sp.	3.9×10^7
B2	1.4×10^7
T2B	0.6×10^7
T4B	0.5×10^7

While the cell density of *Serratia* sp. in 10 mL 0.85% NaCl was equal to 10^9 cells/mL

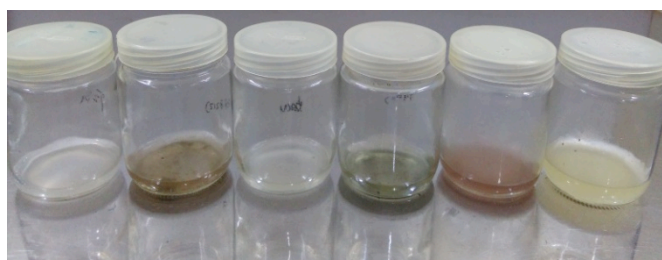


Figure 5. Conidiaspora suspension, from left to right: T2B, B2, *B. bassiana*, T4B, *Paecilomyces*, and consortium

Pathogenicity test (**Figure 6**) of microbial entomopathogen isolates showed that the highest mortality (70%) in larvae *S. litura* was obtained in the treatment of isolate B2. Treatment of consortium and entomopathogenic bacteria (*Serratia* sp) showed the next highest percentage of mortality, 60 and 50%, respectively. While larval mortality was not found in control treatment, isolate T4B and *Paecilomyces* sp. as found in the pupae test, the consortium treatment was shown to exhibit a sufficiently good pathogenic effect on the third to fourth instar larvae of *S. litura*.

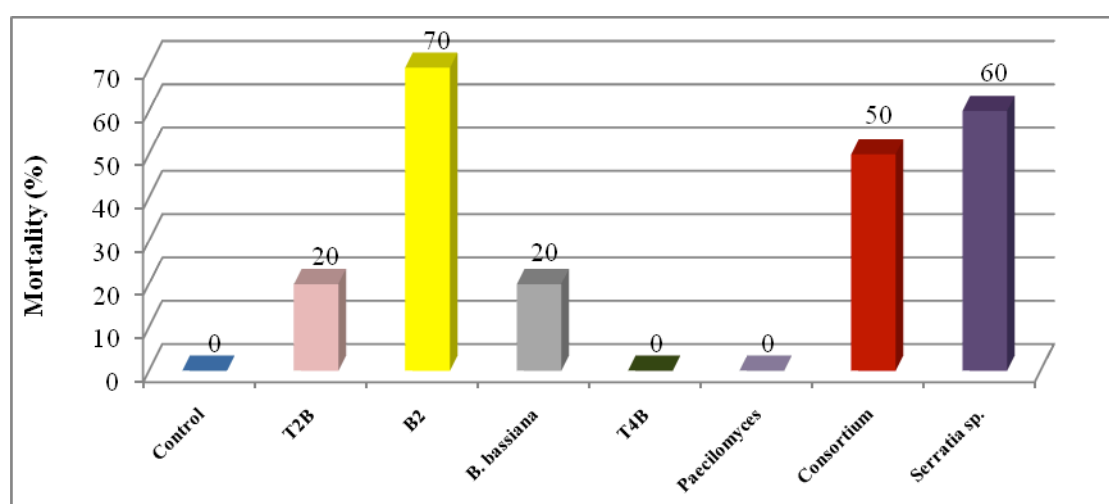


Figure 6 Mortality percentage of *S. litura* larvae 8 days post inoculation with conidial suspension of entomopathogen fungi and bacteria. Values given are mean of 5 replicates

The dead larvae from entomopathogenic fungal treatments showed uniform characteristics of dry and rigid larval bodies whereas in dead larvae due to consortium and *Serratia* sp tend to be wet and reddish (**Figure 7**). From the observation, there were no symptoms of mycosis on all treatments

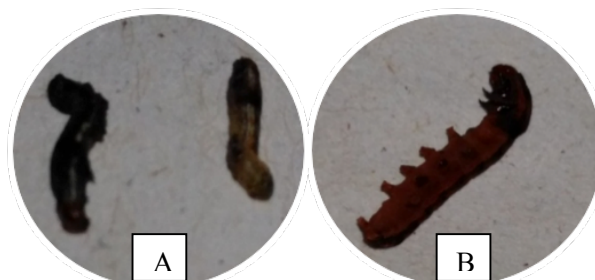


Figure 7. Third to fourth instar larvae of *S. litura* treated with A. suspension of conidia of fungus and B. *Serratia* sp. cell suspension. No symptom of mycosis was found on the surface of the larvae at all treatments

The efficacy results showed that each isolate had different levels of effect on the insect stages tested. At the pupae stage, all treatments demonstrated effect on the mortality of the pupae or the failure of the pupae to grow into moths. While in the larval stage there were some isolates that did not show the effect of pathogenicity. This is in contrast to the results of studies suggesting that the lower the instar stage, the more susceptible to pathogenicity of entomopathogenic microbes [1][7]. Differences in results may be due to the condition of the pupae was not too good because there were 12.5% of the control larvae failed to turn into a moth.

The consortium treatment showed a sufficiently good pathogenic effect on the larval and pupae stages. *Serratia* sp. used in this study was isolated from dead *S. litura* pupae and has been identified molecularly showed 94% similarity with *Serratia marcescens* [9]. The authors also reported that the symptoms of larval mortality due to this isolate is the softened and reddish larval bodies and released unpleasant odor when squashed. These symptoms were not found in larvae that were infected with entomopathogenic fungi. Otherwise the larval bodies were shrank and dried. Symptoms of mycosis were not found at the time of observation but when dead pupae that did not develop into moths were grown on PDAs, pupae was overgrown with mycelium that had characteristics and growth patterns similar to those applied. This indicates that the isolates used have pathogenicity effect to *S. litura*.

The use of biopesticides derived from entomopathogenic microbes needs to be accompanied by adequate mass application. The selection of easy to found and inexpensive carrier needs to be further developed for the sustainability of sustainable agriculture.

4. Conclusion

Consortium treatment consisting of T2B and *Serratia* sp. showed the highest effectiveness of pupae mortality. The results of dead pupae isolation on PDA media showed similar mycelium growth characteristics to those of isolates used in the test. While on pathogenicity test to larvae stadia, B2 fungi showed the highest effectivity, followed by *Serratia* sp and consortium treatment. In general, the consortium has a pretty good antagonistic effect on the two stages of *S. litura* life stages.

5. Reference

- [1] Asi MR, Bashir MH, Afzal M, Zia Kand Akram 2013 Potential of entomopathogenic fungi for biocontrol of *Spodoptera litura* fabricius (Lepidoptera: Noctuidae) *Journal of Animal and Plant Sciences* **23** 913-918.
- [2] Meyling, NV 2007 Methods for Isolation of Entomopathogenic Fungi from The Soil Environment - laboratory manual Deliverable 5.1 of VegQure under the research programme FØJO III.
- [3] Sevim A, Demir I, Hofte M, Humber RA, and Demirbag Z 2010 Isolation and Characterization of Entomopathogenic Fungi from Hazelnut-Growing Region of Turkey *Biocontrol* **55** 279-297.

- [4] Zulfiana D, Tarmadi D, Ismayati M and Yusuf S 2010. Pathogenicity of *Metarhiziumanisopliae* to Subterranean Termites *Coptotermes* sp. Proceeding of The Seventh Conference of The Pacific-Rim Termite Research Group 7-11.
- [5] Anand R, Prasad B, and Tiwary, BN 2009 Relative Susceptibility of *Spodoptera litura* Pupae to Selected Entomopathogenic Fungi *Biocontrol* **54** 84-92.
- [6] Ahmad M andMehmood R 2015 Monitoring of Resistance to New Chemistry Insecticides in *Spodoptera litura*(Lepidoptera: Noctuidae) in Pakistan *Journal of Economic Entomology* **108(3)**, 1279–1288.
- [7] Tong, H, Su, Q, Zhou, X, Zhou X and Bai L 2013 Field Resistance of *Spodoptera litura* (Lepidoptera: Noctuidae) to Organophosphates, Pyrethroids, Carbamates and Four Newer Chemistry insecticides in Hunan, China *J Pest Sci* **86 (3)** 599–609
- [8] Direktorat Perlindungan Tanaman Hortikultura 2008. *Pengenalan dan Pengendalian Hama Tanaman Sayuran Prioritas* Jakarta : Direktorat Perlindungan Tanaman Hortikultura.
- [9] Krishanti NPRA, Wikantyoso B, Zulfitri A, and Zulfiana D 2017 Bakteri Entomopatogen Sebagai Agen biokontrol terhadap larva *Spodoptera litura* (f.) *Berita Biologi* **16 (1)** 13-21.
- [10] Mishra D.S., Kumar A., Prajapati C.R., Singh A.K.,Sharma S.D. 2013 Identification of Compatible Bacterialand Fungal Isolate and Their Effectiveness Against Plant Disease. *Journal of Environmental Biology* **34** 183-189
- [11] Islam MR, Jeong YT, Ryu YJ, Song CH, and Lee YS 2009 Isolation, Identification and Optimal Culture Conditions of *Streptomyces albidoflavus* C247 Producing Antifungal Agents against *Rhizoctonia solani* AG2-2 *Mycobiology* **37 (2)** 114-120.
- [12] Siciua O. A., Dinu S., Dinu M., Fătu C., Vălimareanu D., Mincea C., and Constantinescu F 2014 Pests and Diseases Management Using Compatible Biocontrol Bacteria and Entomopathogenic Fungal Strains Scientific Bulletin Series F. Biotechnologies **18** 66-72.