The Formulation of Biopesticide Combination (SpltMNPV Virus, Beauveria bassiana and Neem Seed (Azadirachta indica A. Juss.) as Biocontrol Agents Against Soybean Pest

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The Formulation of Biopesticide Combination (*Splt*MNPV Virus, *Beauveria bassiana* and Neem Seed (*Azadirachta indica* A. Juss.) as Biocontrol Agents Against Soybean Pest

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Control of pests in soybean farmers commonly use a chemical synthetic pesticide that has a negative impact both on people and the agroecosystem. Another biological control methods are effective and safe against agroecosystem is the use of microorganisms such as viruses *Spodoptera litura* Multiple Nucleopolyhedrosis Virus (*Splt*MNPV), fungus (*Beauveria bassiana*), Neem Seed (*Azadirachta indica*), they are not resistance to the solar radiation. The study aim was to formulate the combination of solar radiation-resistant bioinsecticides that have ability against *Spodoptera litura*, *Nezara viridula* and *Riptortus* (non-target pest) and *Menochilus sexmaculatus* (major pest predator). *Splt*MNPV was multiplied in the epithelial cell culture. Neem seed was extracted and *B. bassiana* was isolated for biopesticide samples. The various combination of biopesticide used to treat pest (target and non-target organisms) in laboratory and greenhouse. The mortality was observed for 17 days. The morphological and histological analysis was conducted to evaluate the effect of biopesticide formulation. The results showed that formulation of photoprotectant and vegetable microbial biopesticide was *Splt*MNPV + *B. bassiana* + NSE + Kaolin + EPMS 15% (1:1:1:4:15%) resulted in 93.5% mortality. This biopesticide formula was compatible to control target pest indicated by morphological and histological features by the presence of three symptoms in reaction with active ingredients, those were dead with aqueous body, followed by fungal hyphae growth, hardened body, the presence of PIBs virus and fungal hyphae as seen from histological images, as well as reduced epithelia in some cells.

Keywords: Biopesticide, Photo-Protectant, Soybean Pest.

1. INTRODUCTION

Common pests on soybean plants include leaf-eating pests and exploiters and pod borer. Soybean leaf-eating pests are leaf beetle, leaf roller caterpillars, and armyworm (*Spodoptera litura*).[1] Armyworm is a dangerous pest because its control is difficult and its resistance to certain bio-insecticide e.g., chlorpyrifos, methomyl, cyhalothrin, endosulfan, and monocrotophos. Additionally, these pests can cause a decrease in soybean harvest as much as 20–85%, and even can cause crop failure.

Pest management in soybeans agroecosystem on the farmers generally use a synthetic chemical insecticide that has a number of negative effects, so that it began to shift to bioinsecticide like virus (*Splt*MNPV/*Spodoptera litura* Multiple Nucleopolyhedrosis Virus) and fungus (*Beauveria bassiana*) and Neem (*Azadirachta indica*) seed extract[2] which is effective against armyworm.[3]

Biopesticides that have a complementary assault mechanism, the virus for poisoning stomach, the fungus as a contact poison and the neem seeds extract as a poison on haemolymph that cause an effect repellence, discontinuation of the development and growth of insects and ecdysis, growth retardation, fertility and reduced fecundity, behavioural-physiological changes that lead to death,[2] a change in the hormonal system that causes developmental disorders, deformation and even infertility. The extent of the impact and the reaction time is the effect of the used dose and the time exposure.[4]

Several studies of virus biopesticide (*Splt*MNPV) revealed that it has a specific host range to *S. litura* which has clathrin as specialized receptors that match to glycoprotein GP 64, the fusion protein virus which is inserted into the cell membrane.[4–6] Fungus *B. bassiana* and Neem seeds extract (NSE) have an adverse effect on more than 430 species of insect pests in several countries.[2] *B. bassiana* has chitinase enzymes that break

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down chitin compounds in insect integument. Neem leaf extract has nimbin compounds that are toxic and spread by insects haemolymph if these toxins are ingested through the leaves of plants sprayed with this bioinsecticide physiological effects of pests. *Splt*MNPV and *B. bassiana* biopesticide are known to be prone to solar radiation. Thus, in this study, the biopesticide is protected with photo-protectant compounds. Previous research revealed that ethyl-p-methoxycinnamate (EPMS) and kaolin compound protected *Splt*MNPV from the sun radiation for 12 hours.[7] *B. bassiana* fungus is sensitive to solar radiation. Insecticidal activity of this fungus can be protected with the addition of cassava dregs flour 2% at 10^8 conidia/ml *B. bassiana*.[10]

This research aimed to examine the compatibility of some biopesticide the target pest and also examines the effects that can be caused by non-target organisms thus obtained the proper formulation to overcome the pest in soybean plants. From the results of this study are expected to be found biopesticide effectively control pests of soybean plants and safe for non-target organisms as at soybean agroecosystem.

2. MATERIALS AND METHODS

2.1. Biopesticide Multiplication

2.1.1. SpltMNPV Multiplication

Midgut epithelial cells of infected *S. litura* instar larvae 5 were aseptically taken and grown in Grace's medium enriched with fetal bovine serum (FBS) and added with antibacterial (penicillin, streptomycin and gentamicin) and antifungal (amphotericin-B). These epithelial cell cultures are incubated at room temperature for 5 days, then adapted to *S. litura*.

2.1.2. Neem Seeds Extraction (NSE)

Neem seeds simplicia (50–75 gr) macerated with ethanol solvent for 3×24 hours (v/v 1:3) and allowed to sit for 24 hours and filtered into ethanol filtrate. The sediment was re-macerated in the ratio of 1:2 and allowed to stand for 24 hours. Then filtering back into the filtrate ethanol. This is done two times. Results of maceration were concentrated using a Rotary evaporator. The solution is referred to as the extracts from neem seeds. NSE doses used in this study was 20 g/litre.[9]

2.1.3. B. bassiana Multiplication

B. bassiana was inoculated on Potato Dextrose Agar and incubated at room temperature for 8 to 18 days. The spores were harvested and the concentration was calculated using haemocytometer. 10^8 spores/ml was used dose.[10]

2.2. Bio-Insecticide Formulation

Viruses, pure mould spores pure and neem extract were formulated with photo-protectant compounds Kaolin (4× volume of virus) and EPMS 15% on a variety of compound dose photoprotectant and biopesticide.

2.3. Laboratory and Green House Experiment

2.3.1. Laboratory Experiment Step 1

*Splt*MNPV was propagated on midgut epithelial cells of *S. litura* larvae and have been stored for 20 months. *Splt*MNPV concentration was 5.95×10^8 PIBs/ml, 5.95×10^7 PIBs/ml, 5.95×10^6 PIBs/ml, infected through feeding method. The treatment was repeated three times and each tested group consists of 10 larvae. The parameters were observed as the percentage of mortality, abnormalities, and normality on infected *S. litura* at day 17th post infection.

2.3.2. Laboratory Experiment Step 2

*Splt*MNPV propagated *in vitro* adapted to the original host is *S. litura* 2 times, the multiplication of the virus *Splt*MNPV 2 to be used for testing. Concentration bio-insecticide with photo-protectant used is $A =$ *Splt*MNPV + *B. bassiana* + NSE + Kaolin (1:1:1:4), $B =$ *Splt*MNPV + *B. bassiana* + NSE + EPMS 15% (1:1:1:15%), $C =$ *Splt*MNPV + *B. bassiana* + NSE + EPMS + Kaolin 15% (1:1:1:4: 15%), $D =$ *Splt*MNPV + *B. bassiana* + NSE-photo-protectant (positive control).

2.3.3. Green House Experiment

This virus was also tested on the non-target pest (*Nezara viridula*, *Ryptortus linearis* and *Menocillus sexmaculatus*) in a controlled environment and the given treatment were: $A = B.$ *bassiana* spores + *Splt*MNPV + NSE + kaolin (1:1:1:4), $B = B.$ *bassiana* + *Splt*MNPV + NSE + EPMS (1:1:1:15%), $C =$ *Splt*MNPV + *B. bassiana* + NSE + kaolin + EPMS (1:1:1:4:15%), $D = B.$ *bassiana* + *Splt*MNPV + NSE-photo-protectants.

2.4. Observed Parameters

Mortality was observed for 17 days, the morphology and histology characteristic using paraffin methods[7] either on targeted and non-targeted organisms which were exposed to various formulations bioinsecticide treatment.

3. EXPERIMENTAL RESULT

3.1. *In Vitro Splt*MNPV Pathogenicity Test That Has Been Stored for 20 Months Against Second Instar Larvae of *S. litura* in Laboratory

The highest percentage of mortality, abnormalities and normality of *S. litura* imago infected by *Splt*MNPV were achieved with doses of 5.95×10^8 PIBs/ml were 44%, 14% and 42% on day 17. Compared with previous research[8] which had 100% mortality on day 5 by infecting new culture of *Splt*MNVPV on *S. litura* larvae instar 2, this result was still lower than the new cultured virus since the virus used in this study had been in storage for 20 months. To make the biopesticide, the virus was adapted to the original host twice to produce adaptive 2nd generation on the natural environment with high pathogenicity. The pathogenicity can be improved by repeated infection to its original host, the first infection showed 14% motility and the second was 63%.[11]

3.2. Bio-Insecticide Compatibility in Photo-Protectant

Treatment using a mixture *Splt*MNPV, *B. bassiana* spores, and NSE with 15% kaolin and EPMS provided the ultimate mortality of 93.3%. While the mixture with EPMS 15% or kaolin own mortality respectively 80% and 75%. Mortality was obtained in the treatment when compared to controls that biopesticide mix without being given photo-protectant mortality was only reached 68.3%. This contrasts with previous research resulted in combination of NSP (neem seed powder) + SlNPV without photo-protectant combination NSP + SlNPV caused the death of *S. litura* as much as 65% in the laboratory. This showed that

the addition of two kinds of photo-protectant could provide protection against pathogenicity of biopesticide mixtures containing *Splt*MNPV, *B. bassiana* spores and NSE.

The high mortality resulting in treatment was due biopesticide contains virus *Splt*MNPV that killed the host by taking over the genetic expression system of the host cell to produce new viruses. Thus the infected cells lysis and broken.[6,8] *B. bassiana* spores were also present in the formula after successfully entering the host cell. Contact between the skin epithelial cells with spores and the spores would germinate the spores and chitinase enzymes that can damage the cell wall of the host then entered the cell cytoplasm. In the cells of hyphae of *B. bassiana* will produce a toxin called beauverin, these toxins would enter hemolymph and destroy the cells that passed by the toxin.

Neem seed (NSE) contains Azadirachtin compound that directly absorbed by the host midgut and circulated throughout the body via the hemolymph. NSE also contains other active compounds from secondary metabolites such as salanin, meliantriol, nimbin and nimbidin azadirachtin. Addition to act as insecticides, NSE also has properties as fungicidal, virucidal, nematicides, bactericides, and acaricides. Azadirachtin acts as a blocker ecdyson a compound that inhibits hormone action in the insect metamorphosis process. A failure in this process resulted in death. Azadirachtin also affects the activity of eating. This is in accordance with previous research that mentioned that the active compound of neem effect on feeding, growth, a reproduction power, the process of molting, mating and sexual communication, lowered hatchability of eggs and inhibits the formation of chitin.[11]

The addition of kaolin and EPMS 15% were able to protect active ingredients biopesticide. Kaolin is known as a pest controller, sprayed kaolin will be some sort of film (white) which protect plants or fruit from pests an insect, it can even kill insects that eat them. In addition, it also binds oxygen free radicals with the help of silicon. Kaolin contains tiny particles that scatter and reflect UV light so that UV rays do not reach the active ingredients of biopesticide. Kaolin was a protective biopesticide physically active ingredient in the formula while EPMS acted as a protective chemical in biopesticide formulations protects the active compound by resonating during exposure to UV.[12] Resonance occurred when sunlight shined on the surface of EPMS, *Splt*MNPV, and *B. bassiana* spores, sunlight would break the double bond at one chain. This reaction was repeated until all the double bonds in EPMS were tired out. By repeating the reaction of solar energy would be depleted to such reactions. As a result of this reaction was the energy of sunlight could not reach the DNA of virus and fungus so that the effectiveness of *B. bassiana* and *Splt*MNPV can be stabilized.

This study also obtained larvae and pupae that were abnormal in the treatment of the biopesticide mixture with double or single photo-protectant. The highest percentage of abnormalities was obtained in biopesticide added with photo-protectant kaolin (23.3%) and then with no photo-protectant (20%), EPMS 15% (15%) and combination of EPMS 15% and Kaolin (5%). Addition of EPMS could protect the larvae from abnormalities because it could reduce the effects of sunlight on the growth of *S. litura* larvae. Thus increasing bio-insecticide microbial pathogenicity and inhibit abnormal larvae of *S. litura*.

Table I. Mortality, abnormality and normality percentage of *S. litura* by *Splt*MNPV *in vitro* (observed on day 17).

Treatment	Imago	Average (%)
A	Mortality	44
	Abnormality	14
	Normality	42
B	Mortality	40
	Abnormality	12
	Normality	48
C	Mortality	38
	Abnormality	12
	Normality	50
D	Mortality	18
	Abnormality	8
	Normality	74

Notes: *A* = *Splt*MNPV + *B. bassiana* + NSE + Kaolin (1:1:1:4), *B* = *Splt*MNPV + *B. bassiana* + NSE + EPMS 15% (1:1:1:15%), *C* = *Splt*MNPV + *B. bassiana* + NSE + EPMS + Kaolin 15% (1:1:1:4: 15%), *D* = *Splt*MNPV + *B. bassiana* + NSE-photo-protectant (positive control).

Table II. Biopesticide compatibility in a photo-protectant formulation in controlling *S. litura* according to mortality and abnormality (day 19).

Treatment	Stadium	Total	Average (%)
A	Dead larvae and pupa	450	75
	Abnormal imago	140	23,3
	Normal imago	10	1,7
B	Dead larvae and pupa	480	80
	Abnormal imago	90	15
	Normal imago	30	5
C	Dead larvae and pupa	560	93,3
	Abnormal imago	30	5
	Normal imago	10	1,7
D	Dead larvae and pupa	410	68,3
	Abnormal imago	120	20
	Normal imago	70	11,7

Notes: *A* = *Splt*MNPV + *B. bassiana* + NSE + Kaolin (1:1:1:4), *B* = *Splt*MNPV + *B. bassiana* + NSE + EPMS 15% (1:1:1:15%), *C* = *Splt*MNPV + *B. bassiana* + NSE + EPMS + Kaolin 15% (1:1:1:4: 15%), *D* = *Splt*MNPV + *B. bassiana* + NSE-photo-protectant (positive control).

3.3. Bio-Insecticide Potential in Greenhouse

The mortality of targeted pest treated by the combination of *Splt*MNPV, *B. bassiana* and NSE at the greenhouse with soybean as host plant were shown in Table III. It was shown that the mortality of of larvae of *S. litura* reached the goal in biopesticide added with EPMS and added with combination of EPMS and kaolin with 87.1% mortality.

Whereas by biopesticide mix with photo-protectant kaolin resulted in lower mortality of 70%, and control to reach 15.7%. One of the criteria for the effectiveness of an insecticide to kill defenseless when 80% or more. Thus, in this study were

Table III. Mortality of *S. litura* larvae treated with varied biopesticide combinations on soybean crop in the greenhouse (day 5).

Treatment	Total	Average (%)
A	490	70
B	610	87,1
C	610	87,1
D	510	72,9
E	110	15,7

Notes: *A* = *B. bassiana* spores + *Splt*MNPV + NSE + kaolin (1:1:1:4), *B* = *B. bassiana* + *Splt*MNPV + NSE + EPMS (1:1:1:15%), *C* = *Splt*MNPV + *B. bassiana* + NSE + kaolin + EPMS (1:1:1:4:15%), *D* = *B. bassiana* + *Splt*MNPV + NSE-photo-protectant, *E* = Distilled water.

said to be effective biopesticide is biopesticide *B. bassiana* + *Splt*MNPV + NSE + EPMS 15% (1:1:1:15%) treatment *B* and *C*, had 87.1% mortality.[13]

The high mortality in both treatment due to NSE added in the biopesticide particularly azadirachtin compound contained in NSE which could influence the change in histophysiology such as degeneration of midgut epithelial layer and matrix peritrophic larvae such as those found in the *S. frugiperda* larvae[2] with regeneration of the epithelial cells lining the midgut and the damage on peritrophic membrane decrease the larvae immune defense. Peritrophic membrane guarded midgut epithelial cells against microbes' invasion by food and other hazardous chemical materials.[14] In addition, azadirachtin also degenerated the cell epithelial midgut and membrane peritrophic so that the cell epithelial could not absorb food in midgut well and membrane peritrophic could not protect epithelial midgut cells.[2] Midgut also contained caliciform cells that produce various digestive enzymes.

Previous study stated that the caliciform cells are responsible for the digestive enzymes production in the insects.[15] With the degeneration of these cells, the production of digestive enzymes to be reduced. So the food was not digested properly lead to

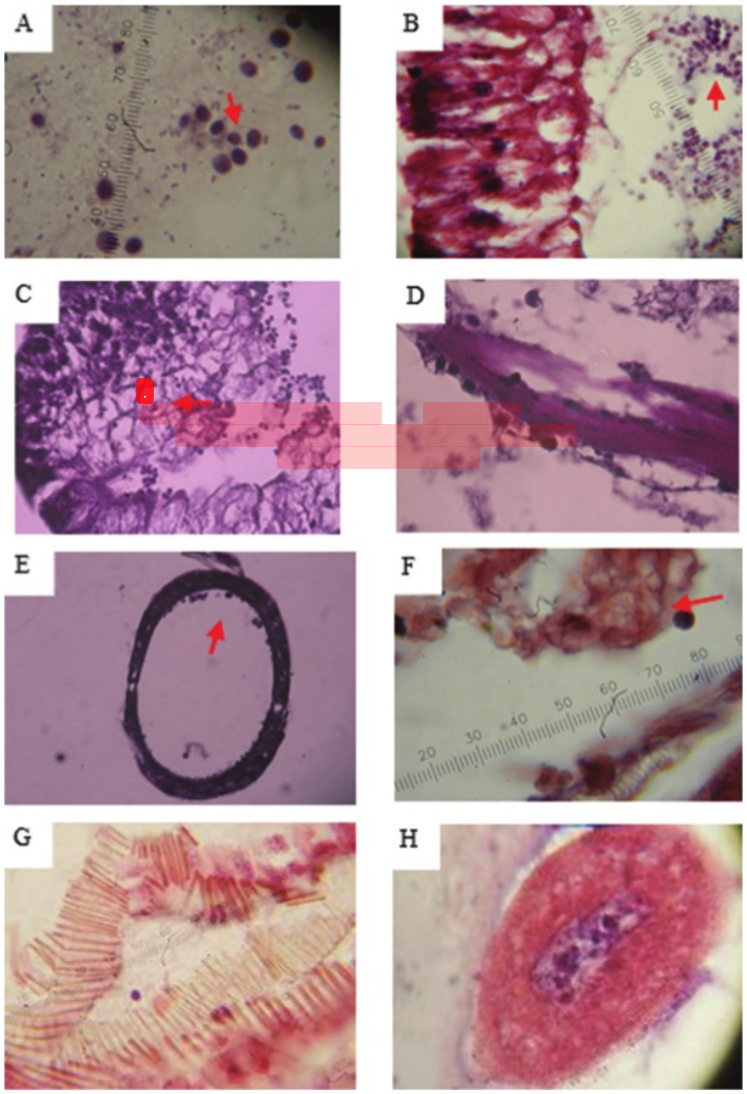

Fig. 1. PIBs *Splt*MNPV in: (A) Lumen midgut (1000×), (B) peritrophic membrane (400×), (C) midgut epithelium (400×), (D) muscle (1000×), (E) Malpighi tubules (400×), (F) fatty tissues (1000×), (G) trachea (1000×) and (H) blood vessels (100×).

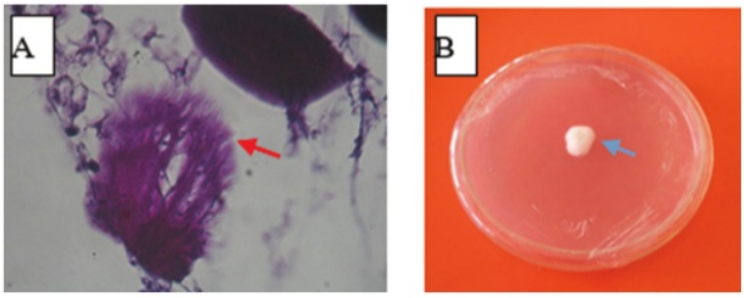

Fig. 2. (A) Histological image from *B. bassiana* grew under the cuticle on larvae of *S. litura* muscle tissue on a 5-day incubation. (B) *B. bassiana* grown on PDA after 5 days incubation.

improper development and decreased body immunity levels so that when other microbes that exist in the biopesticide, those were *Splt*MNPV and fungus *B. bassiana* entering larvae body, then the larvae could not fight it. In damaged peritrophic membrane, *Splt*MNPV would easily invade epithelial cells and spread to other cells. In previous study, the tissue of caterpillars treated with 0.4% neem oil showed clear necrosis on midgut epithelial cells and degradation on columnar cells. Peritrophic matrix was thickened, degraded and folded.[2] While the fungus *B. bassiana* were able to get into the larval body, it would produce a toxin called beauverin that will rapidly spread throughout the larval body and poison the cells in its path.

3.4. Histopathologic *S. litura* Larval Midgut Infected by the Fungus *B. bassiana* and *Splt*MNPV

In the histological picture of the infected larvae, presence of PIBs (polyhedral inclusion bodies) was visible in various organs such as midgut lumen, peritrophic membrane, epithelial midgut, fat tissue, muscle, malpighi tubules, and trachea (Fig. 1).

In the treatments using biopesticide, PIBs from *Splt*MNPV infected larvae could be found in almost every organ on 24–72-hours incubation time. PIBs has been seen in the lumen, epithelium of midgut, the peritrophic membrane, malpighi tubules, fat tissue, trachea muscles and blood vessels. This was in accordance with the result of previous study that *Sl*NPV attacked all organs (including the midgut, fat, blood vessels, and the basal membrane) at 48 hours after infection.[14] Meanwhile, according to another previous study, all organs (midgut, trachea, blood vessels, muscle cells, and epidermis cuticle) have been infected by *Ac*MPV 70 hours after incubation.[14]

Histological picture of *B. bassiana* fungus that attacked the larvae of *S. litura* seen in 5 days of incubation begun to form a set of hyphae on the epidermis under the cuticle as shown in Figure 2. This indicated that the growth of these fungi required longer incubation time than *Splt*MNPV. These results were consistent with the growth of *B. bassiana* performed *in vitro* on potato dextrose agar medium. On day 5 colony formed was still small at less than 1 cm.

4. CONCLUSIONS

The formulation of photo-protectant in this biopesticide with highest mortality against target pest was *Splt*MNPV + *B. bassiana* + NSE + kaolin + EPMS 15% (1:1:1:4:15%) with mortality obtained 93.5%. It was a compatible biopesticide formula to control target pest indicated by morphological and histological features by the presence of three symptoms in reaction with active ingredients, those were dead with aqueous body, followed by fungal hyphae growth, hardened body, the presence of PIBs virus and fungal hyphae as seen from histological images, as well as reduced epithelia in some cells.

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