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Research Article

# The Effectiveness of Red Spinach (*Amaranthus tricolor* L.) and Green Spinach (*Amaranthus hybridus* L.) Extracts for *Bacillus thuringiensis* var. *kurstaki* Protectant against UVB Radiation for the Control of Armyworm (*Spodoptera litura* Fab.)

**Siti Sumarmi[1], Mifta Arlinda[2], Sukirno Sukirno[1]***

1) Entomology Laboratory, Faculty of Biology, Gadjah Mada University, Jalan Teknika Selatan, Sekip Utara, Sleman, Yogyakarta, Indonesia 55281
2) Undergraduate student, Faculty of Biology, Gadjah Mada University, Jalan Teknika Selatan, Sekip Utara, Sleman, Yogyakarta, Indonesia 55281

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## ABSTRACT

*Spodoptera litura* Fab. is an insect that damage cultivated plants in Indonesia. Efforts to control it can be done by using biological agents for example by using *Bacillus thuringiensis kurstaki (Btk.)*. Unfortunately, the *Btk.* is easily degraded by UV radiation. This research aimed to study the effectiveness of red and green spinach as UVB protection for *Btk.* and to observe the pathogenicity of *Btk.* formulations against armyworm. Furthermore, the sublethal effect of *Btk.* against *S. litura* was investigated. The morphology of the endospore, protein crystal, and bacterium were observed under a contrast phase microscope. The extracts at 2% (w/v) were mixed with *Btk.* suspensions at 5 x $10^4$, 5x$10^5$, and 5x$10^6$ (spores/ml), respectively. The formulations then exposed under Ultraviolet B (UVB) lights for 3, 6, and 9 hours then tested against the 3rd larval instar of armyworm. The larval mortality was observed daily and the analysis of variance was analyzed by one way anova. The sublethal effects of the treatment to the pupal and adult stages were observed when the moths emerge. The results showed that the larval mortality caused by *Btk.* mixed with red spinach ranged from 11.7 to 26.7%. The sublethal effects of *Btk.* resulted in smaller sizes of pupae and imago, darker pupae, and wings abnormality of the adult stage, compared to any control treatment. The morphological observation of the bacteria showed that extracts gave UV protection against UVB. These results suggested that red and green spinach potentially can be used as a protectant for *Btk.* against UVB.

**Keywords:** *Bacillus thuringiensis* var. *kurstaki (Btk.)*, Pathogenicity, Spinach, *Spodoptera litura*, UVB

## INTRODUCTION

*Spodoptera litura* Fab. is an insect that causes damage and losses on a cultivated plant in Indonesia. The high losses caused by these insects on cabbage plantations caused efforts to control these pests become a priority. Many control measures were made to control this pest including the use of chemical, herbal, and biological agents. The chemical controls such as using chemical insecticides resulting in resistance and resurgence of the pests (Marwoto & Neering, 1992). Whereas, the use of herbal controls, for example, using frangipani (*Plumeria* sp.) and chickweed (*Ageratum conyzoides* L.). One of the biological control agent commonly used is *Bacillus thuringiensis kurstaki* (*Btk.*) (Feitelson *et al.,* 1992).

*Btk.* easily degrades when exposed to ultraviolet (UV) lights. It causes the spores to be inactive (Griego & Spence, 1978). Several antioxidants can be used as additives for anti ultraviolets (El-Sharkawey *et al.,* 2009). The use of red and white dragon fruits (Sukirno *et al.*, 2017), aloe vera (Tarigan, 2019), and tea leaves (Ningrum, 2019) have been used for the study of *Btk.* protectants. Spinach contains compounds of vitamin A, vitamin C, vitamin E, flavonoids, and phenol

***Corresponding author**
Email: sukirnobiougm@ugm.ac.id

which can be used as antioxidants (Amin *et al.*, 2006). Therefore, in this research red spinach and green spinach extracts were used as protectants of *Btk*. against UV B to control armyworm. It is expected that the addition of protectants can increase the pathogenicity of *Btk*. against the pest.

## MATERIALS AND METHODS

### Materials

The materials used in this study include the 3rd instar of *S. litura* larvae, *Btk*. (Dipel WP ®, Abbot Co., IN) as a biological agent, red spinach, and green spinach extract as protectants of *Btk*., brain heart infusion (BHI) (Oxoid, ThermoScientific, UK), and bacteriological agar (BA) (Oxoid, ThermoScientific, UK) for culture media of *Btk*.

### Methods

#### Insect Collection

Armyworm at the larval stage for the initial stock was collected from cabbage farming in Cangkringan, Sleman. It was collected directly by taking the infested cabbage leaves. As many as 60 larvae were used as parental. The collected larvae were maintained in an artificial diet at Entomology Laboratory, Faculty of Biology, Gadjah Mada University.

#### Artificial Diet

White bean-based artificial diet (Sutanto *et al.*, 2016, Sukirno *et al.*, 2017; 2018) was used for the armyworm mass rearing. As much as 125 g of white bean was soaked overnight in tap water. Then, the bean was boiled until soft. After that, it was grinded in a commercial blender by adding 500 ml of distilled water. After that, the mixture was added with 50 g agar powder, 80 g fermipan, 10 g sodium benzoic, and 750 distilled water, and then boiled. After boiled, the mixture was left at room temperature for 10 minutes until the temperature drops to ± 50°C. After that 5 g of ascorbic acid was then added to the mixture and homogenously. 25 ml of the artificial diet was poured into a 90 ml plastic cup. The cup then kept at room temperature for 45 minutes then stored in a refrigerator at 4 °C until used.

#### Mass Rearing of Armyworm

Larvae collected from the field were transferred on to an artificial diet (Shorey & Hale, 1965) with some modifications until pupation. The seven days old pupae then collected and surface sterilized in 5% (v/v) chlorox (Bayclin ®, SC Johnson, IN), then air-dried for 30 minutes and transferred into a glass jar (d: 20 cm, h: 40 cm) for the adult emergence. After emerges, the adults were provided with 10% honey solutions for adult feeding and opac paper for egg-laying substrate. The laid eggs were collected daily and transferred into an artificial diet for larval feeding. The $F_2$ of armyworm were used for the bioassay.

#### *Btk*. Culture on BHIA

*Btk*. from the commercial product (Dipel WP®, Abbot Co., IN) were cultured BHIA medium in a 15 ml test tube. *Btk*. isolate then incubated for five days at room temperature (28°C) until the protein crystals and spores were formed. The formation of these was observed under a contrast phase microscope (Nikon BX -1, JP).

#### Red and Green Spinach Extraction

As much as 20 g of each of stems and leaves of red and green spinach were weighed and washed in running tap water. Then blended with a commercial blender by adding 180 ml of distilled water. The suspension then filtered using two layers of muslin cloth then stored in a refrigerator (4°C) as a 10% (w/v) stock.

#### *Btk*. Formulations Exposure under UVB

Additive extracts at 2% (w/v) were used for making *Btk*. serial concentrations at $5x10^5$, $5x10^6$, and $5x10^7$ (spores/ml). One ml of each of the formula was taken and homogenously dropped into a disposable plastic petri dish (d: 6cm, h: 1.5 cm), then exposed under UVB lights (2x 10 W Phillips tube, IN) for 3, 6, and 9 hours. After exposed, the treated suspensions then collected by adding 9 ml of autoclaved distilled water to get $5x10^4$, $5x10^5$, and $5x10^6$ (spores/ml) final concentration for the bioassay. The effects of UVB to the morphology of vegetative cell, spore, and crystal protein were observed under a phase-contrast microscope.

#### *Btk*. Bioassay against the 3rd instar of Armyworm

Each of the above final concentrations of *Btk*. suspension was taken and homogenously added into the surface of an artificial diet provided in a disposable petri dish (d: 6cm, h: 1.5 cm), then air-dried at room temperature for one hour. After that, 20 larvae of the 3rd instar of armyworm were added carefully using a soft brush. The bioassay was carried out using 3 replicates for each treatment. The addition of autoclaved distilled water was used as a control. Mortality parameters were measured at 24, 48, and 72 hours after treatment. Sublethal effects were observed until the 10th day after treatment.

### Experimental Design and Statistical Analysis

This study was done using a completely randomized design. The effects of the treatments to the variance of means of larval mortality were done using one-way anova at 95% significance, then followed by LSD if the anova was significant. The pathogenicity prediction of the formula was predicted using $LC_{50}$ and $LC_{90}$ calculation based on probit analysis (Finney, 1949). All the analysis procedures were done by using SPSS ver. 21.

## RESULTS AND DISCUSSION

### The effects of UV B to *Btk.* Morphology

The observation on the effects of UVB to *Btk.* morphology was done (Figure 1, 2, and 3). On the UVB radiation for 3 hours, spores and protein crystal were clearly can be observed. Whereas, on 6 and 9 hours UVB radiation the protein crystal and spores were degraded, and only they were remained in fewer numbers compared to controls. UV rays resulting in the loss of toxicity and cell pathogenicity. The longer exposure time to UV made the protein crystal and spores degraded (Griego & Spence, 1978) and caused the loss of pathogenicity (Khasdan *et al.*, 2001). Spinach leaves contain vitamin A, vitamin C, vitamin E, flavonoids, and phenols as antioxidants (USDA, 2020). Carotenoids, vitamins, and anthocyanins have important roles to fight free radicals (Anbhudasan, 2014). The number of spores was more visible in the red spinach protectant. This could be caused by the red spinach by which containing higher antioxidant compounds compared to green spinach. By this, it can give more protection to the *Btk.* compared to green spinach.

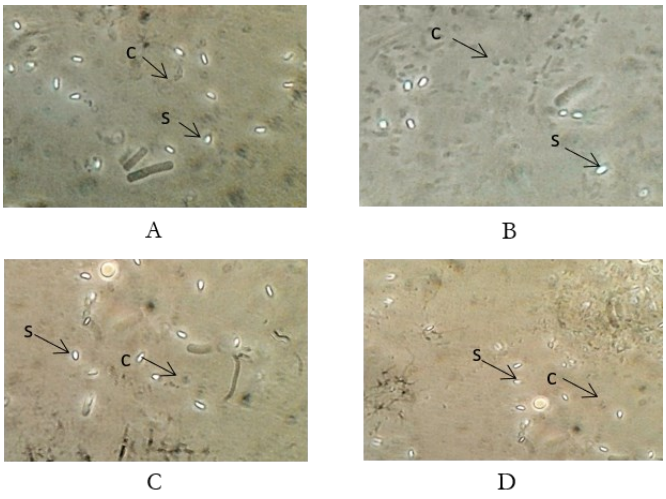



**Figure 1.** The effects of UVB exposure for 3 hours to *Btk.* spores and protein crystals (1,200 magnification under a contrast phase microscope) under concentrations of $5x10^6$ spores/ml in red and green spinach extracts (A: *Btk.* formulated with red spinach exposed to UVB; B: *Btk.* formulated with green spinach exposed to UVB; C: *Btk.* formulated with red spinach un-exposed to UVB; D: *Btk.* formulated with green spinach un-exposed to UVB; c: protein crystals); s: spore).

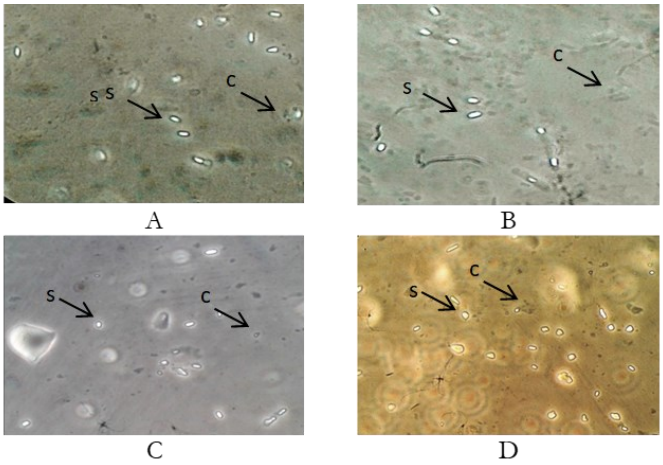



**Figure 2.** The effects of UVB exposure for 6 hours to *Btk.* spores and protein crystals (1,200 magnification under a contrast phase microscope) under concentrations of $5x10^6$ spores/ml in red and green spinach extracts (A: *Btk.* formulated with red spinach exposed to UVB; B: *Btk.* formulated with green spinach exposed to UVB; C: *Btk.* formulated with red spinach un-exposed to UVB; D: *Btk.* formulated with green spinach un-exposed to UVB; c: protein crystals); s: spore).

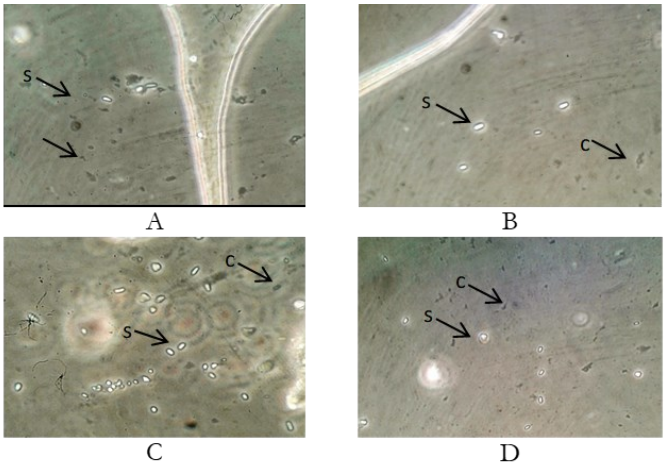



**Figure 3.** The effects of UVB exposure for 9 hours to *Btk.* spores and protein crystals (1,200 magnification under a contrast phase microscope) under concentrations of $5x10^6$ spores/ml in red and green spinach extracts (A: *Btk.* formulated with red spinach exposed to UVB; B: *Btk.* formulated with green spinach exposed to UVB; C: *Btk.* formulated with red spinach un-exposed to UVB; D: *Btk.* formulated with green spinach un-exposed to UVB; c: protein crystals); s: spore).

### The Pathogenicity of *Btk.* Formulations against Armyworm Larvae

Based on the observations of armyworm larval mortality treated with *Btk.* formulations which exposed to different periods of UVB were shown in Figure 4.

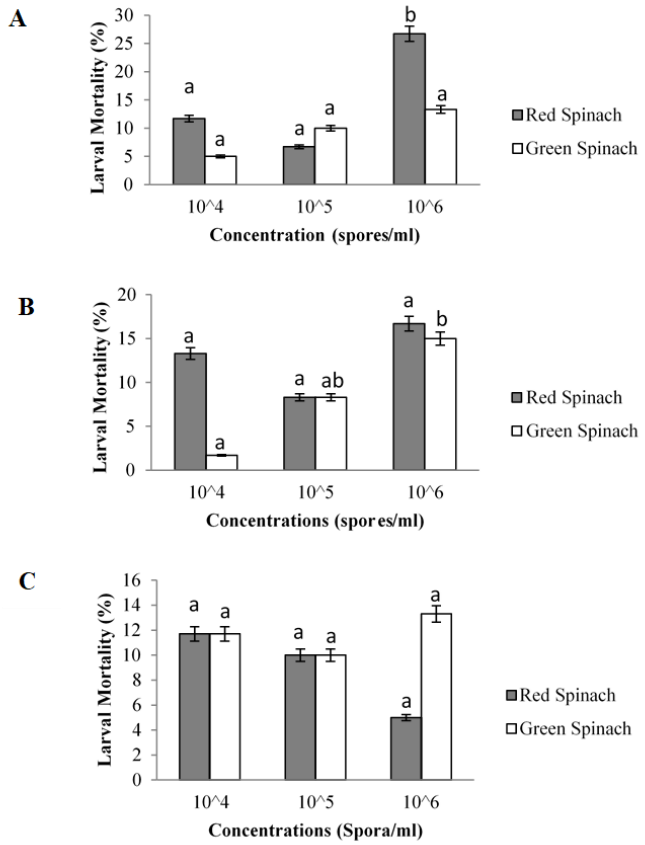



**Figure 4**. The mortality percentage of 3rd instar larvae of armyworm during after by treated by *Btk*. formulated with red spinach and green spinach extracts which was exposed at UVB for 3 hours (A); UVB for 6 hours (B); UV B for 9 hours (C).

In the UVB treatments at 3 and 6 hours of exposures, *Btk*. with protective red spinach in the concentration of $5x10^6$ spores/ml had a mortality of 26.7 % and 16.7%, respectively. This is because the levels of antioxidants that function to protect *Btk*. from UV light on red spinach are higher than green spinach. The number of spores and protein crystals in red spinach is higher than green spinach, so it is better at killing *S. litura*. Whereas, at 9 hours of UVB exposures, *Btk*. with green spinach extract at the concentration of $10^6$ spores/ml had the highest mortality (13.3%), while for concentrations of $10^5$ and $10^4$ spores have mortality <7% in third instar larvae.

Mortality increases in parallel with periods of the treatments (Bouda *et al*., 2001). The larval mortality in the treatment of green spinach extract at a concentration of $10^6$ spores/ml at 3 and 9 hours of UVB exposures was higher compared to the mortality at 6 hours exposure. The percentage of larval mortality in *Btk*. with red spinach protectant was higher than in green spinach. This can be due to the amount of protein crystals and spores in *Btk*. with red spinach were higher than *Btk*. with green spinach, thus it had higher pathogenicity. The protein crystal that enters the body of the insects passed through the insect's digestive tract and was activated by the alkaline conditions in the digestive tract to become δ-endotoxin or protoxin proteins. Protoxin will become a toxin if activated by the insect protease enzyme and is bound specifically to receptors in the digestive tract (Schunemann *et al*., 2014). The symptoms of sub-lethal effects to the larvae caused by *Btk*. are hardened of the body, stiff, blackish in color/ melanization and their size is shorter than the size before treatment (Khetan, 2001). The bacterial toxin infection may damage the digestive system of the larvae and may cause mortality (Schnepf *et al*., 1998).

**Table 1**. The lethal concentrations (spores/ml) of *Btk*. formulated with red and green spinach treated under UVB lights for 3, 6, and 9 hours against armyworm 3rd larval instar (Arlinda, 2019).

| UVB Exposures (h) | Protectants | Lethal Concentrations (spores/ml) | |
|---|---|---|---|
| | | $LC_{50}$ | $LC_{90}$ |
| 3 | Red spinach | $1.5 \times 10^4$ | $1.9 \times 10^5$ |
| | Green spinach | $2.8 \times 10^4$ | $4.1 \times 10^5$ |
| 6 | Red spinach | $5.0 \times 10^8$ | $6.8 \times 10^{14}$ |
| | Green spinach | $8.5 \times 10^3$ | $3.1 \times 10^4$ |
| 9 | Red spinach | $3.8 \times 10^2$ | $1.0 \times 10^3$ |
| | Green spinach | $3.8 \times 10^{13}$ | $4.1 \times 10^{19}$ |

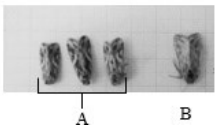
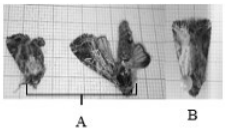
In this study, $LC_{50}$ and $LC_{90}$ values (Table 1) were used as the prediction of the pathogenicity for *Btk*. formulation. At 3 and 6 hours UVB exposures, the red spinach treatment was more pathogenic than the green spinach, with $LC_{50}$ $1.5 \times 10^4$ and $LC_{90}$ $1.9 \times 10^5$ (spores/ml), respectively. Red spinach gave a better UVB protection compared to green. This is possible because of antioxidant contents in red spinach is higher than green spinach.

### Sub- lethal Effects of *Btk*. formulations exposed to UVB against armyworm larvae.

Some of the treated armyworms were survived and developed into pupa and adult stages but posed abnormal morphologies (Table 2). In this study, the survived larvae were able to become pupae but are in smaller sizes, darker in color, and shorter than those control. Adults in *Btk*, treated also had relatively smaller sizes compared to control. Additionally, the pupae that succeeded emerge had an abnormal wing with the characteristics of curly and short wings. A similar result was observed on the studies on the effects of genetically modified maize and *Bt*. genes

on corn borer (*Ostrinia nubilalis*) (Cagan and Barta, 2008). It showed that the growth and development of the larval stage were slower compared to control.

**Table 2**. The sub- lethal effects of *Btk*. formulated with spinach extracts to the pupal and imago stages of armyworm (Arlinda, 2019).

| Parameters | Description |
|---|---|
| 1. Pupae   | The size of pupae in *Btk*. treated were smaller and shorter and the color were darker compared to control |
| 2. Adults size   | The size of adults in *Btk*. treated were smaller than the control |
| 3. Adult's wings   | The wings of the adults in *Btk*. treated were abnormal and had curly wings |

Description: A and B respectively correspond to the morphological performance of pupae as the result of *Btk* and control treatment during the larval stage (A: *Btk*. treated; B: control).

## CONCLUSION

Based on the research that has been done, it can be concluded that *Btk*. with red spinach extract gave more protection to *Btk*. when exposed under UVB compared to green spinach. The sub-lethal effects of *Btk*, formulations include the lesser of the growth and development of larvae, pupae, and adults, as well as wings abnormality.

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