COCONUT SHELL AND STRAW CHARCOALS TO PROTECT *Bacillus thuringiensis* AGAINST ULTRAVIOLET B AND SUNLIGHT TO CONTROL TOBACCO ARMYWORM, *Spodoptera litura* (FABRICIUS, 1775) (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

Crops, for examples tobacco, onion, chili, and *Brassica*, in Indonesia are vulnerable to the attack of the tobacco armyworm, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). The use of synthetic insecticides become the major component of tobacco armyworm control. *Bacillus thuringiensis* (*Bt*) is an effective biological control agent; however, it is easily deteriorated by the ultraviolet lights from sunlight. The aim of this research was to investigate the coconut shell and straw charcoals as ultraviolet B (UVB) and sunlight protectants for *Bt* as a biological agent for controlling tobacco armyworm. Charcoal extract solutions (2% w/v) were made and used for *Bt* formulations, then exposed under UVB lights in the laboratory for 0, 72, and 144 h as well as exposed under sunlight for 0, 1, 3, 7, 14, 21, and 28 d. The formulations were then tested against one-day-old 1st larval instar of tobacco armyworm. The results showed that *Bt* added with coconut shell and straw charcoals at two days after treatment of UVB had higher pathogenicity compared to *Bt* alone. The larval mortality in each treatment was >82, >85, and <60%, respectively. The mortality of tobacco armyworms between the coconut shells (100%) and straw (93.6%) charcoals after 28 days of sunlight treatment was not significantly different. This study suggested that coconut shell and straw charcoals gave good protection to *Bt* from UVB and sunlight.

Keywords: Bio-insecticides, carbon, ultraviolet, protectants, additives

ABSTRAK

Tanaman dagangan contohnya tembakau, bawang, cili dan *Brassica*, di Indonesia terdedah kepada serangan ulat ratus, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). Penggunaan

racun serangga sintetik telah menjadi komponen utama kawalan ulat ratus ini. *Bacillus thuringiensis* (*Bt*) ialah agen kawalan biologi yang berkesan, walau bagaimanapun, ia mudah rosak disebabkan oleh cahaya ultraungu yang terhasil daripada cahaya matahari. Tujuan penyelidikan ini adalah untuk mengkaji tempurung kelapa dan arang jerami sebagai pelindung dari ultraungu B (UVB) dan cahaya matahari untuk *Bt* bertindak sebagai agen biologi untuk mengawal ulat ratus. Larutan ekstrak arang (2% b/v) telah disediakan dan digunakan untuk *Bt* formulasi, kemudian didedahkan di bawah lampu UVB di makmal selama 0, 72 dan 144 jam serta didedahkan di bawah cahaya matahari selama 0, 1, 3, 7, 14, 21 dan 28 hari. Formulasi kemudiannya diuji terhadap larva pertama ulat ratus yang berumur satu hari. Keputusan menunjukkan bahawa *Bt* ditambah dengan tempurung kelapa dan arang jerami pada dua hari selepas rawatan UVB mempunyai sifat patogenik yang lebih tinggi berbanding *Bt* sendirinya. Kematian larva dalam setiap rawatan ialah >82, >85 dan <60%, masing-masing. Kematian ulat ratus antara tempurung kelapa (100%) dan arang jerami (93.6%) selepas 28 hari rawatan cahaya matahari tidak begitu ketara. Kajian ini membuat kesimpulan dan mencadangkan bahawa tempurung kelapa dan arang jerami mempunyai potensi perlindungan yang baik untuk *Bt* untuk melindungi daripada UVB dan cahaya matahari.

Kata kunci: Bioinsektisid, karbon, ultraungu, pelindung, bahan tambahan

INTRODUCTION

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) is a phytophagous insect affecting at least 120 plants. It is presented in 59 countries, including Indonesia (CABI 2019). In Indonesia, the loss of soy bean caused by this insect might reach up to 85%. It damages many important crops such as soybean, ground nut, tobacco, chillies, onion, spinach, and tomatoes (BALITKABI 2015). Farmers depend on the use of chemical insecticides for controlling this pest. Most of the farmers usually applied the insecticides regularly based on the schedule or age of the crops without considering the pest population. The excessive use of insecticides will be deleterious to the natural enemies as well as causing environmental pollution and lead to the resistance and resurgence of the pests. In long term, the application of insecticides will cause residual effects.

The residues of insecticides might pollute the environment and be poisonous to other organisms. In Indonesia, the impacts of insecticide residues on the environment have been reported. For example, soil contaminations in South Sumatra rice fields (Wartono et al. 2018), in several landmarks (Utami & Widijanto 2015), highland and low land areas (Sjoeib 1994). The insecticide residues also possibly moved to mangrove ecosystems (Bhattacharya et al. 2003). The insecticide residues also affect non-target organisms, for example in catfish (Rahmawati et al. 2013) and crops in Indonesia (Ardiwinata et al. 2018). The consumption of contaminated crops and fish also could be dangerous to the human being. The residues could be accumulated in human milk (Burke et al. 2003; Sudaryanto et al. 2006), thus harmful to the infant.

The increasing society's awareness of the need for insecticide-free crops stimulates the growth of organic farming. There are many benefits of organic farming compared to conventional farming. Organic farming potentially produces higher productions, richer organic matter and nitrogen in the soil, lower fossil energy needs, and provides more soil moisture and water resources (Pimentel et al. 2005) compared to that insecticide-dependent farming. One challenge in organic farming is the alternation of chemical insecticides (Trewavas 2001) by using biological insecticides. The study on the comparison of insecticide residues in carrots and potatoes produced by organic and conventional farming showed that organic farming produces

lower residues than those conventional (Rahmawati et al. 2017). Studies on the use of botanical insecticides have been used for reducing residues such as the use of *Momordica charantia* L. (Atiqah et al. 2015), *Ocimum canum* Sims., *Rhinacanthus nasutus* (L.) Kurz., and *Ocimum sanctum* L. (Kamaraj et al. 2008), *Vitex negundo* L., *Strychnos nux-vomica* L., *Murraya koeingii* (L.) Sprengel, *Abrus precatorius* L., and *Zanthoxylum limonella* (Dennst.) Alston. (Arivoli & Tennyson 2013) extract as anti-feedant for controlling *S. litura*.

Organic farming relies on the use of biological agents for insect pest management. One biological agent that has been widely used for tobacco armyworm is *Bacillus thuringiensis* var. *kurstaki* (*Bt*) (Duman et al. 2018; Linh et al 2018; Maqsood et al. 2019; Murali et al. 2018; Palma et al. 2014; Patel et al. 2018; Singh et al. 2019). This bacterium is effective against several insect pests and safe for humans and ecologically safer. During sporulation, *Bt* produced insecticidal crystal protein (ICP) or endotoxins (Cry proteins) (Sansinenea 2012). Unfortunately, when applied in the field *Bt* is facing environmental limiting factors such as ultraviolet (UV) from the sunlight (Zogo et al. 2019) which made toxin protein degraded or inactivated rapidly (Liu et al. 1993; Pozsgay et al. 1987).

Some additives have been used for the protection of biological agents such as spinosyn, and baculovirus to give protection from UV irradiation (Sukirno et al. 2017; Sukirno et al. 2018; Sutanto et al. 2017). A previous report indicated that carbon could enhance the persistence of *Bt* when applied in freshwater habitats for mosquito larvae (Watts et al. 2019). As an agricultural country, in Indonesia, there are a lot of carbon sources which are available as non-expensive UV protectant materials, for example, coconut shells and straw charcoals. Our work was focused on the use of coconut shells and straw charcoals as *Bt* protectants against UVB and sunlight for controlling *S. litura*. This research is important to support the Integrated Pest Management (IPM) program for tobacco armyworms in Indonesia which has a sunny and warm climate all the year, which made the application of *Bt* for controlling insect pests vulnerable to environmental conditions, especially against UVs from sunlight. The objective of this research was to explore the potential of coconut shell and straw charcoals as additives for protecting *Bt* against UVB and sunlight for supporting IPM of *S. litura*.

MATERIALS AND METHODS

Mass Rearing of *Spodoptera litura*

The *S. litura* at larval stages were collected from cabbage plantations in Mount Andong, Kopeng, Central Java, Indonesia. The larvae were brought to the Entomology Laboratory Faculty of Biology Universitas Gadjah Mada, Indonesia and reared on an artificial diet (Sutanto et al. 2017), which was modified from Shorey & Hale (1965). The larvae were maintained in 90 ml plastic cups (d= 60 mm, h= 40 mm) provided with as much as ¼ of cup volume until pupae. The five days old pupae were then collected, and surface sterilized using 1% chlorox (Bayclin® Reguler, SC Johnson & Sons, Inc. IN) for a minute then rinsed in tap water for 5 minutes then air-dried at room temperature prior to pupae incubation. The pupae were incubated in glass jars (d= 150 mm, h= 150 mm) provided with opaque paper for egg deposition substrate. When the adults emerge, honey-soaked cotton balls (2% v/v) were added to the jar for adult feeding. One-day-old first larval instar of the 4th generation were used for the experiment. We used the first larval instar as a sensitive indicator for evaluating the *Bt* formulations effectivity.

Bioassay of *Bt* and Additive Formulations Exposed Under UVB

A total of 25 grams for each coconut shell and straw charcoals from a farmer in Gantiwarno Klaten Central Java, Indonesia was crushed with mortar and pestle and then blended in a

commercial blender by adding with up to 250 ml final volume of autoclaved dH_2O to produce a 10% (w/v) solution. The mixture was then filtered using four layers of muslin cloth. Four ml of the solution was taken and added with autoclaved dH_2O to reach 20 ml final volume (2% w/v) concentration. This solution then was used for making *Bt* formulations at $2x10^8$ spores/ml in 2% charcoal suspension. The suspension then was homogenized using a vortex, and then 1 ml was poured homogenously onto a disposable petri dish (d = 60 mm x 10 mm). Autoclaved dH_2O was used as a control treatment. After that, it was exposed under 2x10 watt ultraviolet B lights (Philips, IN) for 0, 72, and 144 hours. Each treatment was using three replicates. After respective exposure, the treated formulations were kept at -20ºC.

Prior to the bioassay, each UV-treated formulation was recollected by adding 10 ml of autoclaved dH_2O, then homogenized and transferred into a 15 ml conical tube. One ml of this *Bt* solution (LC_{95}) was poured evenly onto the surface of a 20 ml artificial diet prepared in a disposable petri dish (d = 90 mm x 10 mm). After that, it was left air-dried at room temperature (25ºC) for 2 hours. After that, 25 individuals of one day old 1^{st} larval instar of tobacco armyworms were introduced onto the contaminated diet. Each of the treatments was tested using three replicates. The mortality of the larvae was observed at 48 h after the treatment.

Bioassay of *Bt* and Additive Formulations Exposed Under Sunlight

Each additive at 2% (w/v) concentration was used to suspend *Bt* up to 50 ml final volume ($2x10^8$ spores/ml). After that, the suspension was homogenized and one ml of the suspension was taken and put homogenously onto a sterile disposable petri dish (60mm x 10mm). After that, it was exposed to sunlight in a greenhouse at Sawit Sari Research Station, Faculty of Biology Universitas Gadjah Mada for 0, 1, 3, 7, 14, 21, and 28 days. The experiment was using three replications for each treatment. *Bt* alone (*Bt* suspended in autoclaved dH_2O) and autoclaved dH_2O treatments were used as controls. After respective sunlight exposures, the formulations were harvested by adding 10 ml of autoclaved dH_2O and put into a 15 ml conical tube. The procedure of pathogenicity test of sunlight exposed- *Bt* was following the bioassay procedure for the previous work on *Bt* and additive formulations exposed under UVB. At this stage, the experiment was done using 30 individuals of one-day-old 1^{st} larval instar of tobacco army-worms.

Effect of Sunlight Exposures to *Bt* and Additives Formulations Persistency

The persistency evaluation of *Bt* and additives formulations after exposure to sunlight was done using total plate colony count methods. Each of 10 µl of 10^{-3} *Bt* suspension of each treatment after being exposed to different sunlight exposures was taken and inoculated onto disposable petri dishes (90mm x 10 mm) containing 20 ml nutrient agar medium. The inoculum then was homogenised by smooth agitation. After homogenized, the petri dish was wrapped and then incubated at room temperature (±27ºC). Bacterial colonies were counted after of 24 and 48 h after incubation.

Experimental Design and Statistical Analysis

The experiment was done using a complete randomized design. The percentage of mortality of each treatment was corrected using Abbot's formula (Finney 1971). The dependent variables of the treatments were analysed statistically using analysis of variance (one-way ANOVA). The separation of the mean was using Tukey's HSD at α: 0.05. All the statistical analysis was done in SPSS 16.0 (SPSS Inc. 2008).

RESULTS AND DISCUSSION

Table 1 showed the mortality of 1st larval instar of tobacco armyworm after treated with *Bt* formulations without UVB exposure. The results showed that coconut shells and straw charcoals have a higher mortality rate compared to *Bt* alone. On the 1st day after the treatment, the mortality of tobacco armyworm in *Bt* straw charcoal was the highest. While in the 2nd, 3rd, and 4th day after the treatment, the mortality in *Bt* coconut shell and straw charcoals added were significantly higher than that alone formula. These indicated that the addition of charcoal might have a synergistic effect with *Bt* thus elevating the mortality significantly. The previous study on *Bt* pathogenicity showed inclination when mixed with sericin extract of *Samia ricini* Drury (Sukirno et al. 2022). The work on the use of starch wastewater also showed the enhancement of *Bt* pathogenicity as well as increased toxin productivity (Ndao et al. 2019; Kumar et al. 2019). The study on the use of biochar as an additive of *Bt*, baculovirus, and *Beauvaria bassiana* also showed that carbon is capable to increase the bio-pesticides pathogenicity (Sayed et al. 2018).

Table 1. Percent mortality (mean±SE) of 1st larval instar of tobacco armyworm after treated with *Bt* formulations at 0-hour UVB exposure

Formulations	**Mortality(%)**			
	Day 1	**Day 2**	**Day 3**	**Day 4**
Bt – coconut shells charcoal	16±4[a]	90.7±5.8[b]	100±0[b]	100±0[a]
Bt – straw charcoals	34.7±4.8[b]	93.3±3.5[b]	100±0[b]	100±0a
Bt alone	8±4.6[b]	60±10.1[b]	66.7±13.5[b]	66.7±13.5[b]

Numbers in the same column followed by the same letter is not significant different at α: 0.05.

The effects of UVB lights for 72 hours exposure on the pathogenicity of *Bt* formulation is depicted in Table 2. The mortality of tobacco armyworm on the 1st day after treatment showed that there were no significant differences between the formulations. In contrast, after the 2nd, 3rd, and 4th day, the mortality in *Bt*-charcoals was significantly higher than *Bt*- alone. The addition of coconut shell charcoal was the highest. The mortality comparison in the *Bt* alone after exposure for 72 hours and *Bt*-alone unexposed indicated that the pathogenicity at the fourth day after treatments fall from above 94% to 33.3%. At this exposure period, the UVB might have deactivated or degraded the *Bt* toxin. Whereas, the addition of charcoals indicated that the pathogenicity of *Bt* after being exposed to UVB for 72 hours remains high. Studies on *Spodoptera exigua* NPV showed that the addition of charcoal might protect the baculovirus against UV (Samsudin et al. 2011). It has been proved that the addition of charcoal mixture produced dissolved organic carbon which consisted of benzenoid containing unsaturated aliphatic bonds which can absorb UV lights (Bai et al. 1993).

Table 2. Percent mortality (mean±SE) of 1st larval instar of tobacco armyworm after treated with *Bt* formulations at 72-hours UVB exposure

Formulations	**Mortality (%)**			
	Day 1	**Day 2**	**Day 3**	**Day 4**
Bt – coconut shell charcoal	18.7±9.3[a]	92±2.3[b]	98.7±1.3[b]	98.7±1.3[b]
Bt – straw charcoal	29.3±8.1[a]	88±6.1[b]	94.7±5.3[b]	94.7±5.3[b]
Bt alone	13.3±9.6[a]	28±22.3[a]	33.3±27.6[a]	33.3±27.6[a]

Numbers in the same column followed by the same letter is not significant different at α: 0.05

Table 3 showed the percentage mortality of tobacco armyworms after the treatment of UVB exposed *Bt* formulations for 144 hours. The data showed that the mortalities in both charcoals were still higher than in *Bt* alone. Table 4 showed that the larval mortality caused by *Bt* with the addition of coconut shell and straw charcoals, at 28 d of sunlight exposure was 100% and 93.62%, respectively. These were significantly higher than that in exposed (74.71%) *Bt* alone treatment. This means that the *Bt* added with coconut shell charcoal can enhance the pathogenicity although has been exposed under sunlight. The porous physical properties of charcoal have a role as an absorbent capable of high-capacity absorption of many materials both liquids and gasses (Lempang 2014). Its capacity may reach up to 672 mg/g (Harsanti et al. 2013). Straw charcoal is rich in potassium carbonate, potassium bicarbonate, silica, calcium, iron and aluminium (Maulinda & Jalaluddin 2012) by which silica has protectant activities against insect pests (Aldryhim 1993; Barbosa et al. 1994; El-Samahy et al. 2015; Yu et al. 2020). Charcoal is also very hygroscopic which can absorb a lot of water as well as increasing carbon and sodium uptake in plants. When charcoal is mixed with water, the organic carbon dissolved, for example, benzenoid and unsaturated aliphatic bonds, that can absorb UV rays (Brandstetter et al. 1996).

Table 3. Percent mortality (mean±SE) of 1st larval instar of tobacco armyworm after treated with *Bt* formulations at 144 hours UVB exposure

Formulation	**Mortality(%)**			
	Day 1	**Day 2**	**Day 3**	**Day 4**
Bt – coconut shell charcoal	12±12[a]	82.7±1.3[b]	98.7±1.3[b]	100±0[b]
Bt – straw charcoal	16±9.2[a]	85.3±3.5[b]	93.3±6.7[b]	93.3±6.7[b]
Bt alone	13.3±11.4[a]	29.3±11.6[a]	33.3±13.9[a]	40±10.6[a]

Numbers in the same column followed by the same letter is not significant different at the 95% confidence level.

Table 4. Percent mortality of 1st instar of tobacco armyworms after treatment of *Bt* formulation which has been exposed to sun light for 28 days

Treatments	**Sunlight Exposures (day)**						
	0	**1**	**3**	**7**	**14**	**21**	**28**
Bt-coconut shell	98.9±1.1 aA	94.5±4.0 aA	100±0.0 aA	97.1±1.7 aA	100±0.0 aA	96.1±1.1 aA	100±0.0 aB
Bt-straw	98.1±1.9 aA	99.1±0.9 aA	94.6±5.4 aA	100±0.0 aA	97.9±2.1 aA	99.4±0.6 aA	93.6±6.4 aAB
Bt alone exposed	96.5±2.4 bA	98.0±2.0 bA	100±0.0 bA	100±0.0 bA	96.7±1.9 bA	98.2±0.9 bA	74.7±10.1 aA
Bt alone unexposed	96.3±0.4 bA	95.1±4.9 abA	97.7±1.2 bA	98.9±1.1 bA	93.3±3.9 abA	100±0.0 bA	82.9±2.8 aAB

Numbers in the same row (comparison between different sunlight exposures) followed by the same lowercase letter show no significant different at α:0.05, while numbers in the same column (data comparison between different *Bt* formulations) followed by the same uppercase letter show no significant different at α:0.05

The addition of charcoal also increases the pH level to alkaline by which enhancing *Bt* infection capability in the insect midgut. When infects the midgut, *Bt* toxins cause swelling, sloughing, and damage to the epithelial cells of the tobacco armyworms. The nucleus of infected cells will enlarge and the endoplasmic reticulum will to resembles vacuoles (Mafazah & Zulaika 2017). *Bt* toxins are not soluble in water or organic solvents and are denatured by heat, gastric acid, and gastric protease.

The persistence of *Bt* is shown in Figure 1. The data showed that on the one-day sunlight exposure, the number of recovered *Bt* in coconut shells and straw charcoals was 173 x 10^5 cells/ml 143 x 10^5 cells/ml, respectively. These were higher than those on exposed (3 x 10^5 cells/ml) and unexposed (8.33 x 10^5 cells/ml) *Bt* alone. However, along with the longer sunlight exposures, the *Bt* persistence in all treatments was decreasing. At 28 days of exposure, the recovered *Bt* in coconut shells and straw charcoals was 7.33 x 10^5 cells/ml and 1 x 10^5 cells/ml. Whereas, Figure 2 showed that after one day of sunlight exposure, the number recovered *Bt* in coconut shell charcoal (228.33 x 10^5 cells/ml) was higher compared to straw charcoal treatment 120 x 10^5 cells/ml). This persistence was significantly higher than those on exposed (2.33 x 10^5 cells/ml) and unexposed (1.33 x 10^5 cells/ml) *Bt* alone. These results are in accordance with Widayani et al. (2018), the longer exposure to bio-insecticide, the more degradation.

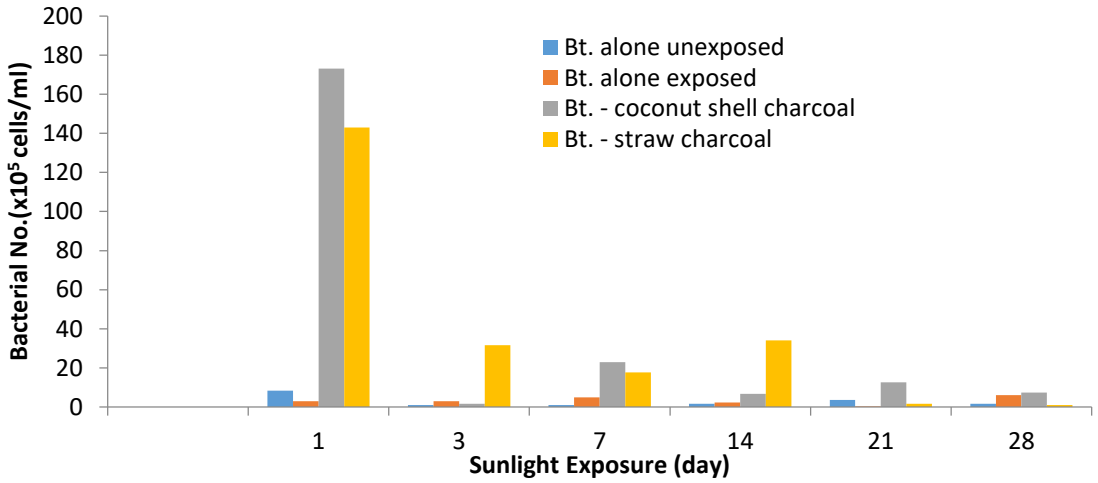

Figure 1. The persistence of *Bt* alone and *Bt* added with coconut and straw charcoals (x10^5 cells/ml) after exposed under sunlight for 1 – 28 days based on 24 h incubation in NA medium

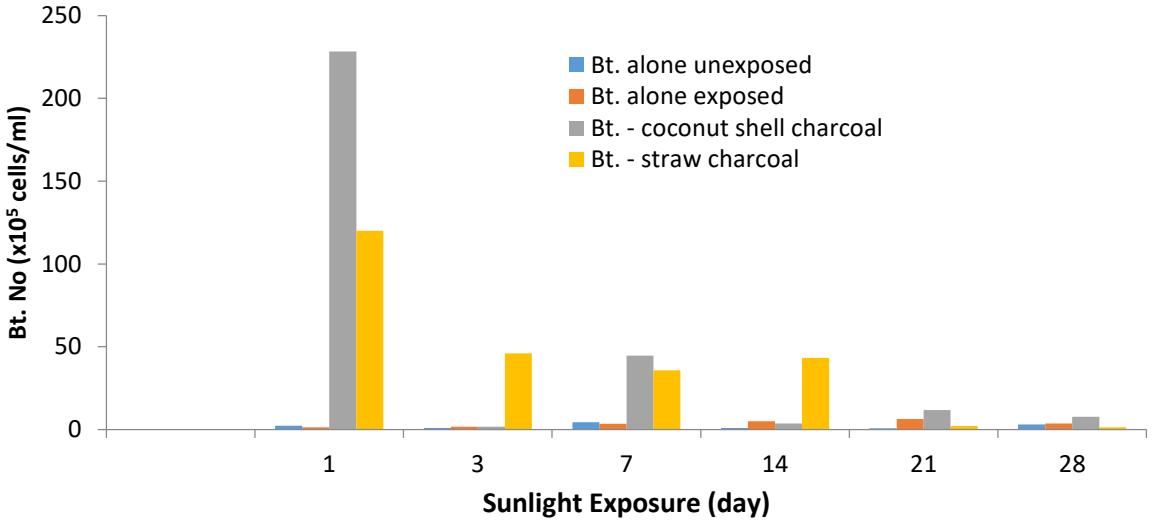

Figure 2. The persistence of *Bt* alone and *Bt* added with coconut shell and straw charcoals (10^5 cells/ml) after exposed under sunlight for 1 – 28 days based on 48 h incubation in NA medium

On the 28 days of sunlight exposure, based on 48 h incubation in nutrient agar medium, the *Bt* persistence in both coconut shell and straw charcoals was decreasing as the same pattern as of 24 h incubation time. When compared with the larval mortality of tobacco armyworms from day 0 to day 14, *Bt* added with charcoals showing higher mortality compared to *Bt* alone. Whereas, in the 21 and 28 d of sunlight exposures, charcoal additions had higher larval mortality as well as higher in *Bt* persistence when compared to that of in *Bt* without additive. The addition of charcoal might also provide alkaline conditions which increase the ability of *Bt* toxins to penetrate the peritrophic membrane of the midgut, thus resulting in higher pathogenicity. From these, it was concluded that when *Bt* applied without protectant has lower pathogenicity than those using charcoals as UV protectants.

CONCLUSION

The results of the study conclusively showed that coconut shells and straw charcoals have the potential to protect *Bt* against ultraviolet light B and sunlight. Charcoal formulations significantly increased the pathogenicity of *Bt* compared to *Bt* alone and might be applied to enhance the persistence of *Bt* as one of the biological agent of *S. litura* in Indonesia.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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