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Study on using gamma radiation to inactivate *Bacillus thuringiensis* spores in biopesticide

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Abstract: *Bacillus thuringiensis* (Bt) produces different types of toxin that have potent and specific insecticidal activity. In recent years, Bt toxins have been used as the safe biological control agents to protect crops replacing for chemical insecticides. Bt-based biopesticides that have been commercialized as the alternative products to control pests and insects for sustainable agriculture, contain toxicity crystals and a significant number of spores that affects to the soil microflora. These uncontrollable changes may contaminate the cultivation soil, and eventually cause adverse effects to human and animal health. Therefore, the living cells and spores existing in the Bt-biopesticides should be controlled. This study evaluates the effects of gamma radiation on spore viability, germination and growth of the existing spores after spraying on the soil and the insecticidal effectiveness of a Bt-based biopesticide (VBT) against lepidoptera larvae. We attempted to identify the optimal dose that could inactivate Bt spores but the toxicity of Bt still retain highly. The results revealed that the dose of 20 kGy is enough to control all living cells and spores in the product that consists of approximately 5.2×10^7 spores in the initial VBT. Though the growth of existing spores after spraying on the soil reduced by 85% or more by irradiation, their insecticidal activity against *Heliothis armigera* larvae reduced by 20-30% only as compared to that of the initial VBT. It suggested that gamma irradiation can be applied as useful way to control the living cells and spores existing in the commercial Bt-based bio-pesticides, and the radiation dose of 20 kGy is enough to kill all spores in VBT, but still kept its insecticidal effect for *Heliothis armigera* larvae.

Keywords: *Bacillus thuringiensis, gamma irradiation, spore, insecticidal activity, Heliothis armigera larvae.*

I. INTRODUCTION

Bio-pesticides are types of pesticides which are derived from such natural materials as animals, plants, bacteria, and certain minerals. They are developing, commercializing and gradually replacing for traditional chemical pesticides because of their strong environmental and human health safety records. *Bacillus thuringiensis* (Bt) is a soil bacterium that produces spores during its growth. The spores always link with toxic crystals, called as δ-endotoxins that have potent and specific insecticidal activity. Therefore, Bt has been extensively studied and Bt toxins have been used as safe topical pesticides since 1981 in over the world [1]. Nowadays, 90% of bio-pesticide products based on Bt, are used to protect crops against lepidoptera insects and other pests in agriculture and forest [2]. The total value of Bt-based bio-pesticides has been increased each year. The global market for pesticides should reach $60.2 billion in 2016 and $78.7 billion in 2021. The global bio-pesticides market should grow from nearly $4.0 billion in 2016 to $7.7 billion in 2021, at a five-year CAGR of 14.1% [1].

Though Bt bio-pesticides have been considered as the safe and effective products for controlling insects and pests in agriculture [3]. However, most of Bt products still contain living cells and spores, which may germinate and grow after spraying on the soil.

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This may cause the risks to microflora as well as the changes in crops. Bacterial cells and spores may become "foreign" and cause allergic reactions if they are inhaled or rubbed into the skin. Therefore, the number of Bt living spores in bio-pesticides must be controlled. In Germany, only the Bt products that do not contain living bacilli or spores can be applied [5]. Since spores are highly resistant to heat, radiation and chemical, the inactivation of bacterial spores has been considered as a challenge for human health, environmental quality and food safety [6]. There are actually three methods of destroying bacterial spores [7]. First is heat treatment (120-130 °C), which can sterilize but the toxin protein may be denatured at high temperature. As a result, the insecticidal activity of the treated Bt was significantly reduced or completely lost [8]. Furthermore, the package may be destroyed during heating. Second is chemical treatment, a simple and inexpensive, but high toxicity method, which may cause undesired effects to human's health and environment [7]. Gamma Co^{60} radiation effectively inactivates living cells and spores. This method is clean, high efficiency, low re-infection and widely applied to food irradiation and sterilization of medical products [8].

In Vietnam, though Bt bio-pesticides that have been studied and developed for long time, but were still only produced in small scale and did not met the requirements [9]. According to the Ministry of Agriculture and Rural Development, the number of commercialized bio-pesticides was rapidly increased from 2 in 2009 to 221 in 2015 and to 334 in 2017 [10]. However, the spores existing in the Bt products and their impacts on the soil microflora after spraying were not adequately concerned. In this study, VBT, a commercial biopesticide was irradiated by γ-rays at different doses, and the effects of gamma radiation on viability of *bacilli* and spores, their growth after spraying on the soil and toxicity for 4^{th}-stage larvae of *Heliothis armigera* sworm were investigated in laboratory scale.

II. EXPERIMENTS

A. Sampling and Irradiation

VBT biopesticide (16000 IU) with an activity of 16000 IU/mg was purchased from Vietnam Green Garden Company Ltd. The product was packed in the carton boxes and irradiated by γ-rays at the absorbed doses of 5, 10, 15, 20 and 25 kGy (with the average dose rate 1 kGy/h) measured by the ECB dosimeters.

B. Radiation effect on spore viability

The plates were prepared with agar and NB broth to culture Bt cells and spores. Briefly, 10 g VBT samples were transferred into 250 ml flask contains 90 ml sterilized peptone water under magnetic stirring. After homogenization, serial dilutions were prepared in order to make sure that the number of spores in countable limits. 100 µl of dilute sample was smeared onto nutrient agar plate, then incubated at 30 °C for 24 and 48 h, according to the protocol described by Becker [4]. At least 3 plates were applied for each dilution, and the average number of Bt spores existing in each sample was calculated to determine the effect of gamma radiation on Bt spores viability.

C. Insecticidal activities of VBT irradiated at various doses against fourth stage *Heliothis armigera* larvae

In this experiment, *Heliothis armigera* adults were collected from tomato field and reared in laboratory to collect the larvae at various larval stages. 1440 of *Heliothis armigera* larvaes at fourth stage (4^{th}-stage) were used. Baby corn was used as testing material to feed the larvae. VBT solutions of 0.01, 0.05, 0.10, 1.125, 10, 50, 100 g/L were prepared from initial and irradiated VBT samples. Insecticidal activities of various VBT to 4^{th}-stage *Heliothis armigera* larvaes were determined as follow: First, baby corns were immerged into the VBT solution for 5 mins, then air dried for 20 mins. Second, the corns treated with each VBT solution were divided into 3 test cups, ten 4^{th}-stage larvaes of *Heliothis armigera* were added to each cup. All larvaes were reared in the laboratory of Plant Protection Institute at 26 ±1 °C and relative humidity of 70-85%. Mortality data of the larvae was recorded after 24 and 48 h corresponding to the VBT solution.

Insecticidal activity or toxicity of the VBT was evaluated by median lethal concentration (LC_{50}) of VBT bio-pesticide of killing 50% of the tested larvae population. The data were analyzed by using probit method [11].

D. Growth of Bt spores on the soil sprayed with VBT

The soil was divided into styrofoam box of 600 mm × 450 mm × 375 mm (length × width × height) size for cultivating of borecole. VBT solution of optimal concentration was sprayed on the plants and soil following the guide of manufacturer (18 g per 16-20 liters of water, 400-600 liters/ha). The borecole styrofoam boxes were separately placed in order to avoid spreading of the pesticide solutions to each other. Every week, same VBT solution was sprayed on the same styrofoam boxes, and 10 g of surface layer soil was collected after 3 times spraying. The soil was diluted with 90 ml of sterilized peptone water for sampling. The sample was well shaken and then placed in a hot water bath at 80 °C for 10 mins to kill all *bacilli* and spores that were not heat resistant as reported by Berker [4]. After that, the flasks were placed in a refrigerator at 5 °C, to prevent any new growth of heat resistant spores. The sample was further diluted and cultured in LB plates. Survival and growth of Bt spores on the soil were determined with irradiated VBT samples as mentioned above.

III. RESULTS AND DISCUSSION

A. Radiation effect on spore viability

As presented in Fig. 1, 1 gram of initial VBT contains 5.2×10^7 spores. This Bt spore number significantly decreased by gamma irradiation, and there are no spore that can be grown by radiation at 20 kGy. It is obviously that the number of spores in VBT was linear reduced with radiation dose. The decimal radiation dose (D_{10}) that can be estimated as about 3.2 kGy, mean that the effective dose of $[3.2 \times \log(5.2 \times 10^7)] \approx 24.7$ kGy must be applied to kill all spore in VBT pesticide.

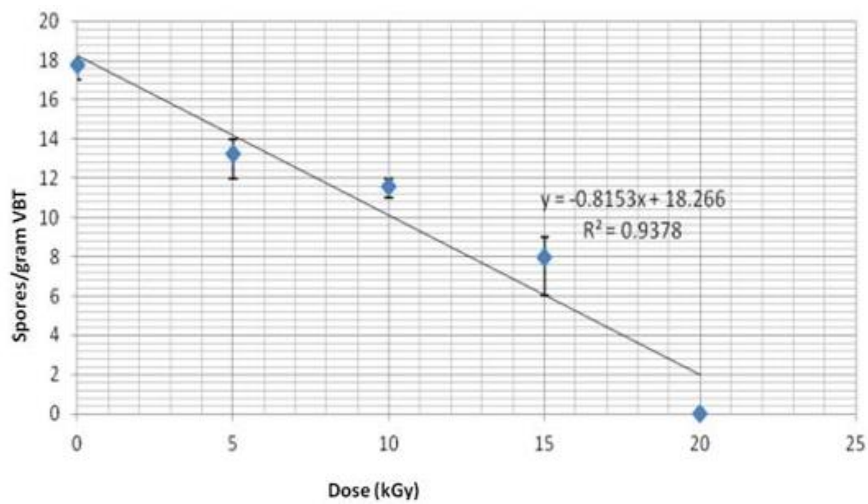

Fig. 1. Number of viable *Bacillus thuringiensis* var. *kurstaki* spores after gamma irradiation with various doses.

According to manufacturer, VBT pesticide can be preserved within 2 years so that the density of *bacillus* spores was also determined after irradiation from 3 to 12 months. The results in Table I revealed that the viability of the spores in VBT after 12 months storage increased 40 times. However, it was slightly increased for the irradiated VBT. There are no viable spores could be observed in the VBT irradiated at 20 kGy or higher dose even after 12 months storage in laboratory.

Table I. Effect of gamma irradiation on spore viability of VBT after 0, 3 and 12 months from irradiation.

Dose (kGy)	Viable spores/mg	Viable spores/mg after 3 months from irradiation	Viable spores/mg after 12 months from irradiation
0	0.052×10^9	0.42×10^9	2.1×10^9
5	0.055×10^6	1.02×10^6	1.2×10^6
10	0.100×10^4	0.45×10^4	10×10^4
15	2.700×10^2	ND	5.5×10^3
20	ND	ND	ND
25	ND	ND	ND

ND: not detected

From these results, the minimum dose required to sterilize the Bt spores in VBT was estimated as about 20 kGy, similar to the other results [4, 12]. In studies on the radiation sensitivities of some *Bacillus thuringiensis* strains, Sun et al. reported that 9, 12, 15 and 19 kGy were effective dose for killing 4 types of Bt spores particularly WP, HD-1, TnY and TnX spores, respectively [5]. The radiation sensitivities among the Bt strains depend on their physiological characteristics and their abilities to recover from radiation injury.

B. Estimation of radiation effect on the toxicity of VBT pesticide

As one can see from Table II that the toxicity of VBT against *Heliothis armigera* larvae was slightly decreased by gamma irradiation. Insecticidal activity of VBT samples irradiated with dose below 10 kGy were about 90% in comparison with initial VBT. It reduced by 20 and 29% for the pesticide irradiated at 20 and 25 kGy, respectively. These results are consistent with other research that irradiation at a dose of 20-25 kGy caused a 20-30% reduction in the effectiveness of Bt-based pesticide against mosquito larvae and the reduction of toxicity of Bt by radiation follows a rather linear model [5].

Table II. Effect of gamma irradiation on toxicity of VBT against *Heliothis armigera* larvae

Dose (kGy)	LC_{50} (range)	Toxicity (%)
0	8.987 (5.358 – 15.070)	100
5	11.150 (3.335 – 37.250)	88
10	21.28 (9.103 – 49.730)	95
15	30.59 (16.32 – 57.35)	76
20	19.25 (17.95 – 20.63)	80
25	27.02 (16.62 – 49.90)	71

According to Sun et al., the insecticidal effectiveness of Bt products against lepidoptera pests did not reduce, but their toxicities against mosquito were significantly reduced by gamma irradiation [5]. Differences in toxicities of the irradiated Bt-based bio-pesticides in this study and others are attributed to the differences of physiochemical structure and properties of toxin proteins by various Bt strains.

There are several methods for inactivation of the spores in Bt-based biopesticides such as heat, chemical agent, radiation. However, these methods do not completely kill all spores and significantly reduce the toxicity of products. The present study tried to determine the optimal dose of gamma irradiation for sterilizing VBT but still keeping its toxicity. The results suggest that the radiation dose of 20 kGy can be applied to kill all spores and keep an acceptable insecticidal activity of VBT. Becker also reported that 20 kGy is the maximum dose for routine sterilization of Bt products that would maintain the effectiveness of the product [4].

C. Growth of *Bacillus thuringiensis* in the soil treated with irradiated and unirradiated VBT

Growth of Bt spores were observed after incubating with nutrient agar. Their morphological characteristics of Bt colonies seem not to be changed during experiment. For the soil treated with VBT, about 0.6 - 3.0 $\times$ 10^6 spores geminated and grown into colonies from 1 g soil (CFU/g). However, only small amount of spores can be found in the soil treated with the irradiated pesticide. The density of spore in soil treated by irradiated VBT was about 15% as many as in soil treated by un-irradiated VBT. The amount of viable spores in VBT was reduced 99% by irradiation treatment at doses higher than 15 kGy so the difference among density of spores in soils which were treated with these VBT may be itself spore in initial soil.

Table III: Assessment of radiation effect on density in areas treated with irradiated and un-irradiated VBT

Dose (kGy)	The density of spores in product (%)	Toxicity (%)	The density of spore in soil (%)
Control	100 (5.2 × $10^{7)}$	100	100
5	0.102 (5.5 × $10^{4)}$	83.68	23.29 ± 4.90
10	0.021 (0.1 × 10^4)	81.18	22.60 ± 4.40
15	0.005 (2.7 × 10^3)	67.85	15.30 ± 4.30
20	ND	72.50	20.00 ± 2.48
25	ND	73.60	20.30 ± 1.68

ND: not detected

The results also revealed that Bt spores can accumulate in soil and overgrow when the environment conditions become suitable for *bacilli*. According to German Mosquito Control Association, the soil which was treated by non-sterilized Bt biopesticides twice a year may contains 0.7 × 10^6 to 44 × 10^6 spores per gram. But there are no or less than 10^5 Bt spores per gram of soil were found in the areas treated by irradiated biopesticide [4]. Therefore, gamma irradiation may be considered as one of the most useful methods to inactivate spore for improving the use not only Bt-based products, but also other biopesticides in agriculture.

IV. CONCLUSIONS

Spore viability was greatly reduced at 15 kGy and no spores survived radiation dose of 20 kGy and higher. Gamma irradiation can cause a reduction of insecticidal toxicity of VBT against *Heliothis armigera* larvae. About 90% of toxicity of VBT remained after irradiation with dose below 10 kGy, but the reductions were 20 and 29% for the irradiated pesticide with 20 and 25 kGy, respectively. The density of spore in soil treated irradiated VBT only get 15% as many as soil treated with unirradiated VBT. The spores after spraying irradiated VBT on the soil reduced by 85% or more as same as control. A radiation dose of 20 kGy fullfills the requirements of killing all spores in VBT and maintaining the effectiveness of products.

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