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Influence of some environmental conditions on stability and activity of *Bacillus thuringiensis* formulations against the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

Moataz A. M. Moustafa[1*], Mohammed A. Saleh[2], Izat R. Ateya[2] and Mohamed A. Kandil[1]

Abstract

The entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) has been used in crop protection for the last 70 years; however, many environmental conditions affect its activity. The present study was directed to evaluate the influence of certain environmental conditions on stability and activity of *Bt* samples of the two commercial formulations (Dipel 2 × 6. 4% WP and Protecto 9.4% WP), when stored under accelerated hot storage, shelf, and outdoor storage. Photo degradation of the two formulations was studied in aqueous solution. The results revealed that the loss percentage of Protecto formulation was above the permissible limits of WHO specifications after 2 years of storage at ambient conditions and the thermal stability of the *Bt* was affected negatively depending on the storage periods. In accordance with this trend, the bioassay tests versus neonate and second instar larvae of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), showed a decrease in toxicity of the formulations to (60%) after storage at 35 ± 2 °C for 12 weeks and about (70%) after storage under sunlight for 2 days. Photolysis of aqueous solutions reduced the half-life of formulations by about 1–2 days. The stability of *Bt* should be evaluated prior to submission for registration as these products have showed less stability under storage at ambient conditions. The stakeholders and decision-makers can utilize these results and examine such products case by case.

Keywords: *Bacillus thuringiensis*, Shelf life, Photodegradation, Thermal stability, *Spodoptera littoralis*

Background

Bacillus thuringiensis (*Bt*) is one of the earliest developed entomopathogens and widely used as biopesticide. It produces insecticidal proteins (δ-endotoxins) which exhibit toxicity to many insect species belong to order Lepidoptera, Diptera, and Coleoptera. Recently, several gene coding for the insect toxins of *Bt* has been genetically incorporated into crop plants. These are referred to as *Bt*-crops representing (19%) of all GMO (Genetically manipulated organism) crops worldwide (Raddadi et al. 2009; Leng et al. 2011).

Shelf life of entomopathogens is often low, and there is a difficulty to achieve a viable product after 1 or 2 years under ambient conditions. As it is known that products based on natural molecules tend to be less stable than synthetic compounds, hence their residual effects are biodegradable (Gupta and Dikshit 2010; Villaverde et al. 2014). In addition, these products are not stable under natural environmental stresses such as temperature, ultra violet (UV) radiation, and sunlight. Radiation from sunlight or UV light is the main limitation that obviously reduced the potency of *Bt* crystals against different insect pests (Dunkle and Shasha 1988; Ignoffo 1992; Khorramvatan et al. 2014).

The present study was conducted to evaluate the stability and insecticidal activity for two formulations of *Bt*, exposed to temperature, light, and aqueous solutions.

* Correspondence: moataz.moustafa79@gmail.com
[1]Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, 6 ElGamma street, Giza 12613, Egypt
Full list of author information is available at the end of the article

Material and methods

Bio-insecticides

Two formulations of *Bacillus thuringiensis*, *subsp. kurstaki*, were used, Dipel 2× formulation (6.4% WP contain 23,000 international unit/mg) produced by Valent Corporation USA and Protecto formulation (9.4% WP contain 23,000 international unit/mg) produced by Biocide Production Unit, Plant Protection Res. Institute, Agriculture Research Center, Giza, Egypt.

Insect rearing and bioassays

Insect source

Spodoptera littoralis larvae were obtained from the laboratory of pesticide, Cairo University, Egypt, that reared under a complete absence of insecticides (Eldefrawi et al. 1964).

Bioassay

Bioassay was carried out using the leaf dipping technique (Ahmed 2009). Five serial concentrations, calculated as an active ingredient, ranging from 500 to 8000 mg/L, of the two tested formulations against the neonates and 2nd instar larvae of *S. littoralis*. The castor leaves were immersed in the aqueous solution of each concentration for 20 s, with gentle agitation, and then allowed to dry under an airflow. After drying, two leaves were placed in each glass jars. Twenty-five larvae were released into each glass jar. Four replicates were used per concentration; each replicate contained 25 larvae. Leaves dipped in water served as control. All glass jars were kept under 25 ± 2 °C and RH 65%. After 24 h of exposure, castor leaves treated with the bio-insecticides concentrations were removed and fresh non-treated leaves were added successively for 3 days. Mortality rates, 24 and 96 h post-treatments, as well the natural mortality were recorded.

Statistical analysis

Abbott (1925) corrected mortality data for control response. The median lethal concentration (LC_{50} value) was calculated according to Finney (1971), using the software 321,958 package Ldp lines analysis version 1.0. Toxicity index was calculated according to Sun (1950).

$$\text{Toxicity index} = \left(LC_{50} \text{ of the most effective sample} / LC_{50} \text{ of the sample}\right) \times 100$$

Physical studies

Storage tests

The formulated samples were stored according to (CIPAC, handbook J 2000, MT 46.3) as follows:

1- Accelerated hot storage: At 72 ± 2 °C for 3 days, 54 ± 2 °C for 14 days, and 35 ± 2 °C for 12 weeks. Storage procedure for solid formulation (wettable powder (WP)) were completed as follows: 20 g were placed in the beaker and spread, without applying pressure, in a smooth even layer of constant thickness. A disk was placed on the surface of the sample in a beaker; which placed in oven at the specified temperature and for the defined period. At the end of the time, the beaker was removed from the oven, the disk taken off, then placed in a desiccator and allowed to cool at room temperature.
2- Shelf storage: Samples were kept in original packaging, away from direct sunlight in stores for 2 years.
3- Storage in outdoor: Packaged samples were exposed to direct sunlight for 2, 7, and 15 days, at 35 ± 2 °C as the average of temperature.

Aqueous photolysis

Three different sources of water (Nile, ground, and drain water) were used in this test. These water samples were collected from Al-Bureejat Village (Beheira Governorate); physical and chemical properties for each source of water were determined and presented in Table 1 according to Lico et al. (1982) and Rice et al. (2012). Suspensions from the two *Bt* formulations were prepared by different concentrations from the three different sources of water according to Behle et al. (1997) and Naghavi et al. (2016) and placed in a clear bottle, which then exposed to direct sunlight for 1, 2, 4, 24, 72, and 120 h. Dominating atmospheric temperature ranged between 32 and 38 °C. At the end of time, samples were collected and analyzed.

Photo degradation by UV light

To study the effect of UV light on samples, UV Japan lamp (technical specification: G13T8 tube, 30 W, 254 nm) was inserted in a tightly locked wooden box and connected to an electrical source. The suspensions from the two *Bt* formulation samples were prepared in a clear bottle. These samples were exposed directly to UV light inside the box for 1, 2, and 4 h according to Talkhan et al. (2013) and Khorramvatan et al. (2014). At the end of time, samples were collected and analyzed.

Preparation of samples for analyses

Strain standard preparation of Bt

The standard strain of (*Bacillus thuringiensis kurstaki*) was collected from Microbiological Resources Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt, in a solidified medium (DSM Medium 1), consisting of peptone 5 g, meat extract 3 g, agar 15 g, and distilled water 1000 ml. Different concentrations

Table 1 Physiochemical properties of water

Parameter (s)	Nile water	Ground water	Drain water
pH*	7.17	7.33	7.41
Conductivity Ms*	463	775	787
Salinity %*	0.2	0.4	0.4
Total dissolved solids (TDS) mg/l*	219	370	378
Elements (ppb)**			
Cr	N.D	N.D	N.D
Co	N.D	25	37
Cu	N.D	N.D	N.D
Fe	N.D	N.D	N.D
Mn	41	49	48
Ni	33	40	45
Zn	N.D	35	N.D
Sn	29	33	42
Cd	N.D	N.D	N.D
Pb	N.D	N.D	N.D
Sb	0.9	0.88	0.97
As	N.D	N.D	N.D

*Determination was conducting according to Lico et al. (1982)
**Elements determination were carried out by using method of Rice et al. (2012)

were prepared to determine the LC_{50} according to Dulmage et al. (1971), Dulmage (1973), Navon et al. (1990), and Asan et al. (1993) in Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt.

Preparation of *Bt* formulations samples

Suspensions from the two *Bt* formulations were prepared by different concentrations (ranging from 0.98 to 2000 mg/l). The bioassay test was used to evaluate the active ingredient, and the samples exposed to UV and sunlight.

Determination the active ingredient of samples

The determination method was based on the number of international units per milligram (IU/mg), using bioassay test and the calculation formula according to McLaughlin et al. (1984) as follows:

$$\text{IU/mg test sample} = (LC_{50}\ \text{standard}/LC_{50}\ \text{sample}) \times \text{potency of the standard in IU/mg.}$$

The bioassay method described by Dulmage et al. (1971) was conducted by preparing artificial diet composed of dry kidney beans, dry yeast, dry agar, ascorbic acid, Nipagin, formalin, and water. After preparing the different concentrations of samples as mentioned above, they decanted in cups and kept at room temperature in a cap for 1 h to allow water to evaporate and condense. Ten neonate larvae of *S. littoralis* were placed into each cup. Three replicates were used for each concentration; each replicate contained ten larvae. A cup dipped in water served as control. All cups were kept at 25 ± 2 °C and RH of 65%. Mortality was recorded 24, 48, 72, and 96 h post-treatment and corrected for natural mortality by Abbott formula (Abbott 1925). LC_{50} values were calculated the according to Finney (1971).

Kinetic study

To study the rate of degradation of the tested bio-insecticide, the half-life time (T1/2) was calculated. The following equation according to Moye et al. (1987) was used:

$$\text{T1/2} = 0.693/\text{K, where } K = \text{rate of degradation, } K = 1/\text{Tx. ln a/bx}$$

where Tx = time in days or hours, a = initial residue, and bx = Residue at time.

Results and discussion

Effect of storage under accelerated hot storage and shelf life

The data presented in Table 2 indicated that the percentage of loss at 72 ± 2 °C for 3 days was higher than storage at 54 ± 2 °C for 14 days and 35 ± 2 °C for 12 weeks. The loss % was 13.42, 3.41, and 5.82% after storage at 72 ± 2 °C for 3 days, 54 ± 2 °C for 14 days, and 35 ± 2 °C for 12 weeks, respectively, for Dipel 2× formulation. However, the formulation of Protecto was less stable, when stored at different temperatures, where the loss percentage was (13.93, 8.03, and 12.52%) after storage at 72 ± 2 °C for 3 days, 54 ± 2 °C for 14 days, and 35 ± 2 °C for 12 weeks,

Table 2 Effect of storage under different temperatures on stability of *Bacillus thurigiensis* formulations

Temperature/time	*Bacillus thuringiensis*			
	Dipel 2×		Protecto	
	Content a.i IU/mg	Loss %	Content a.i IU/mg	Loss %
Zero time	31,289	0	30,155	0
72 ± 2 °C—3 days	27,089	13.42	25,955	13.93
54 ± 2 °C—14 days	30,222	3.41	27,734	8.03
35 ± 2 °C—12 weeks	29,156	5.82	26,379	12.52

Table 3 Effect of storage at ambient conditions for 2 years on stability of *Bacillus thurigiensis* formulations

Time	*Bacillus thuringiensis*			
	Dipel 2×		Protecto	
	Content a.i IU/mg	Loss %	Content a.i IU/mg	Loss %
Zero time	31,289	0	30,155	0
3 months	30,578	2.27	29,155	3.32
6 months	29,155	6.82	27,379	9.21
24 months (2 years)	26,665	14.79	23,372	22.49

respectively. On the other hand, when the two formulations were stored for 2 years at room temperature, the losses were (14.79 and 22.49%) for Dipel 2× and Protecto formulations, respectively (Table 3).

The permissible limit for loss at the stored bio-insecticide must be less than 16% of the active ingredient (WHO 2012). Although, the loss percentage for the two formulations was less than the permissible limit when stored under different temperatures. While for Protecto formulation stored for 2 years at room temperature, the loss percentage was higher than the permissible limit, while it was less than the permissible limit for Dipel 2× formulation. Therefore, the shelf life for Protecto formulation was less than 2 years. It was clear that these formulations had poor storage stability, where shelf life was often low and the viability of products did not exceed 2 years under ambient conditions. In addition, as known, the products based on natural molecules or contained natural active ingredients tend to be less stable than synthetic compounds (Gupta and Dikshit 2010; Villaverde et al. 2014). The results presented in Table 2 showed that temperature was one of the most important factors affecting degradation of the active ingredient during shelf or storage life. At storage temperatures of 35, 54, and 72 °C, the high temperatures negatively affected the stability of *Bt*, depending on storage periods. On the other hand, the low temperatures exhibited lightly effect. These results are in concinnity with Brar et al. (2005) and Sorokulova et al. (2008) who found that the temperature above 50 °C decreased the viability of spores and crystal protein. Ignoffo (1992) reported that the half-life of the toxin (Cry and Cyt) was less than 10 days at above 50 °C and the half-life of dry spores exposed at 50 °C was greater than 100 days. While wet spores had a half-life less than 60 days but those exposed at 60 °C were inactivated after an exposure of only 15 min, in addition, the temperatures between 10 to 20 °C were considered optimal for spores activity and toxin effect.

Effect of storage under sunlight

Results in Table 4 show that the loss percentage of stored formulations Dipel 2× and Protecto, after exposure to direct sunlight for 15 days, were 34.90 and 45.99%, respectively. It is observable from the data presented in Tables 2, 3 and 4 that temperature and exposure to sunlight also affected negatively on their stability. The results showed that the loss percentage of the active ingredients proportionally increased after exposure to direct sunlight. Protecto was less stable to different storage conditions than Dipel 2×. These results are in line with Ignoffo (1992) who found that spore viability of *Bt* was reduced by 50% after 30 min exposure to simulated sunlight. However, both formulations significantly been affected when stored under direct sunlight. In contrast, only the Dipel 2× formulation was more stable when stored away from sunlight and their shelf life may increase to 2 years.

Bioassays and determination of lethal concentrations

The lethal activities of Dipel 2× and Protecto formulations at different storage conditions including storage at 35 ± 2 °C for 12 weeks and storage under direct sunlight for 2 days against the neonate and 2nd instar larvae of *S. littoralis* are given in Table 5. Based on these results, the LC_{50} of the Dipel 2× formulation showed clear differences between LC_{50} values before and after storage under temperature of 35 ± 2 °C and under sunlight to 3.3 and 3.6 fold, respectively. In addition, the LC_{50} values were 2.2 and 4.3 fold at 35 ± 2 °C for 12 weeks and 2 days under sunlight, respectively for second instar larvae. Similarly, Protecto formulation, LC_{50} values for neonate larvae at 35 ± 2 °C for 12 weeks and 2 days under sunlight were 2.6 and 2.8 fold, respectively, and for 2nd Instar larvae were 2.6 and 3.2 fold at 35 ± 2 °C for 12 weeks and 2 days under sunlight, respectively.

Aqueous stability

Most bio-pesticide formulations are sold in concentrated form and have to be diluted in water before they can be applied. Stability of aqueous solution of bio-insecticides

Table 4 Effect of storage under direct sunlight on stability of *Bacillus thurigiensis* formulations

Time	*Bacillus thuringiensis*			
	Dipel 2×		Protecto	
	Content a.i IU/mg	Loss %	Content a.i IU/mg	Loss %
Zero time	29,155	0	28,311	0
2 days	24,889	14.63	23,610	16.60
7 days	21,689	25.61	19,789	30.10
15 days	18,979	34.90	15,289	45.99

Table 5 Activity of *Bacillus thuringiensis* formulations against neonate and 2nd larvae of *Spodoptera littoralis* at different storage conditions

Bioinsecticides	Conditions	LC_{50} (mg/L)	LC_{90} (mg/L)	x^2	Slope ± SE	Toxicity index
Neonate larvae						
Dipel 2×	0	948.18	9067.25	3.37	1.307 ± 0.12	100
	1	3156.95	24,948.50	4.53	1.428 ± 0.16	30.03
	2	3460.22	28,855.13	2.86	1.391 ± 0.22	27.40
Protecto	0	1577.92	33,715.81	1.16	0.935 ± 0.11	100
	1	4233.44	37,038.06	1.07	1.422 ± 0.19	37.27
	2	4504.29	38,769.82	2.88	1.371 ± 0.19	35.03
2nd larvae						
Dipel 2×	0	931.99	17,905.98	6.97	0.998 ± 0.11	100
	1	2069.06	10,281.37	1.26	1.841 ± 0.12	45.04
	2	4067.42	24,013.61	8.34	1.662 ± 0.19	22.91
Protecto	0	1530.31	59,547.43	0.56	0.806 ± 0.11	100
	1	4051.99	25,819.24	6.67	1.593 ± 0.19	37.77
	2	5000.50	71,826.85	1.02	1.107 ± 0.22	30.60

0—zero time where each mg contain 31,289 IU for Dipel 2× and 30,155 IU for Protecto
1—storage at 35 ± 2 °C for 12 weeks where each mg contain 29,156 IU for Dipel 2× and 26,379 IU for Protecto
2—storage under direct sunlight for 2 days where each mg contain 24,889 IU for Dipel 2× and 23,610 IU for Protecto

Table 6 Effect of photolysis in water for *Bacillus thuringiensis* formulations

Bioinsecticides	Time/hours	Water source					
		Nile water		Ground water		Drain water	
		IU/mg	Loss %	IU/mg	Loss %	IU/mg	Loss %
Dipel 2×	0	28,834	0	28,376	0	28,376	0
	1	27,965	3.01	27,644	2.58	27,920	1.61
	2	26,920	6.64	26,834	5.43	26,844	5.40
	4	25,400	11.91	25,465	10.26	25,320	10.77
	24	20,200	29.94	20,320	28.39	18,844	33.59
	72	13,712	52.44	13,200	53.48	13,010	54.15
	120	11,100	61.50	11,134	60.76	10,844	61.78
Regression equation (y =)		$-0.0185x + 10.19$		$-0.0162x + 10.19$		$-0.0153x + 10.18$	
Degradation rate (K)		0.01853		0.01621		0.01527	
$t_{½}$ h		37.40		42.76		45.40	
Determination coefficient (R^2)		0.97		0.96		0.94	
Protecto	0	27,560	0	27,560	0	27,560	0
	1	26,245	4.77	26,263	4.71	26,400	4.21
	2	24,710	10.34	24,534	10.98	24,821	9.94
	4	23,400	15.09	23,279	15.53	23,144	16.02
	24	18,555	32.67	18,979	31.14	17,910	35.01
	72	12,310	55.33	12,266	55.49	12,200	55.73
	120	9117	66.92	9355	66.06	8934	67.58
Regression equation (y =)		$-0.0259x + 10.13$		$-0.0263x + 10.13$		$-0.0254x + 10.13$	
Degradation rate (K)		0.02589		0.02632		0.02537	
$t_{½}$ h		26.77		26.33		27.32	
Determination coefficient (R^2)		0.98		0.98		0.97	

depends upon the source of water and the exposure period to sunlight. Naturally, the various sources of water are different in their physiochemical properties and this may be reflected on stability of the tested bio-insecticides. Data presented in Table 6 subjected to first-order kinetics, using regression analysis, where the regression equations and half-life values $t_{½}$ along with determination coefficient and degradation rate K values, for Dipel 2× and Protecto formulations, were calculated. The results showed that samples exposed to sunlight for after 120 h in different sources of water, loss more than 60 and 65% of its concentrations for Dipel 2× and Protecto, respectively. Tested bio-insecticides dissipated faster after exposure to sunlight in different sources of water, where the half-life of *Bt* was 1–2 days. Previous studies reported that aqueous photolysis of *Bt* was rapid and observed half-lives of endotoxin was from 1 to 4 days (Ignoffo 1992). As the spore viability was reduced by (80%) after exposure to natural sunlight for 1 day after application, while it reduced (50%) after 30 min exposure to simulated sunlight in filter and glass tube. In addition, endotoxin activity was reduced, but it required about eight times more sunlight exposure (3.8 h) to obtain a 50% loss in insecticidal activity. Behle et al. (1997) found that the activity of *Bt* formulations decreased about 20% after exposure to sunlight for 1 day, about 35% after 2 days and more than 80% after 7 days. Recently, Naghavi et al. (2016) noticed that the activity of *Bt* formulations decreased about 40% after exposure to sunlight for 3 days, and more than 50% after 7 days and no activity after 10 days. Data observed that the photolysis in water played a vital role in the degradation of the tested bio-insecticides. However, amount of samples kept away from sunlight for 5 days had not changed and remained stable as it was without any loss of its concentration.

Photo degradation under UV light

The data presented in Table 7 showed that after 8 h of exposing, more than 55% of concentrations of the two formulations were dissipated, where the half-life was 7.97 and 6.31 h of Dipel 2× and Protecto, respectively. Several studies investigated the role of UV light on degradation of *Bt* formulations. Cohen et al. (1991) tested the effect of ultraviolet irradiation at 300–350 nm of *Bt* where its activity decreased to 70 and 95%, after 6 and 12 h from irradiation, respectively. Khorramvatan et al. (2014) found that the activity of *Bt* decreased to 50 and 40%, respectively after 1 h from exposing to ultraviolet irradiation at 245 and 385 nm. Moreover, the same authors found that the addition of UV protectants could reduce photo degradation and reported that addition of sodium alginate to *Bt* formulations decreased of UV effect, as also the degradation rate is changed by changing the concentration of solutions that exposed to UV light.

Table 7 Effect of exposed to UV light of the *Bacillus thuringiensis* formulations

Time/hour	*Bacillus thuringiensis*			
	Dipel 2×		Protecto	
	IU/mg	Loss %	IU/mg	Loss %
0	28,800	0	27,733	0
1	26,667	7.41	24,533	11.54
2	22,400	22.22	20,267	26.92
4	17,066	40.74	16,000	42.31
8	12,800	55.56	9600	65.38
Regression equation y =	− 0.0870x + 10.25		− 0.1098x + 10.22	
Degradation rate (*K*)	0.08697		0.10985	
$t_{½}$ h	7.97		6.31	
Determination coefficient (R^2)	0.96		0.99	

Conclusions

The UV light and sunlight are a primary degradation pathway for this bio-insecticide and needs to be controlled. Likewise, the photolysis in water plays a vital role in the degradation of the tested bio-insecticides. The present study pointed out that the shelf life of the tested bio-insecticides is relatively short and does not exceed 2 years. Moreover, the tested bio-insecticides need to be stored in proper conditions in order to conserve their bioactivity. Finally, the growers and farmers could get benefit from data obtained regarding performance of the tested bio-pesticides under field conditions.

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Availability of data and materials

All data of the study have been presented in the manuscript, and the materials, which are used in this study, are of high quality and grade.

Authors' contributions

The authors carried out all the experiments including the bioassay tests, analytical part, analysis of data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

This study does not contain any individual person's data.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, 6 ElGamma street, Giza 12613, Egypt. [2]Department of Pesticides Analysis Researches, Central Agricultural Pesticides Laboratory, Agricultural Research Center, Dokki, Giza, Egypt.

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