Turkish Journal of Biology

Volume 28 | Number 1 Article 3

1-1-2004

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Turk J Biol
28 (2004) 15-21

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Received: 15.12.2003

Abstract: The effects of 9 commercial pesticides used commonly in agriculture on spore germination and larvicidal activity of the mosquito pathogenic *B. thuringiensis* var. *israelensis* and *B. sphaericus* 2362 strain were examined. Six of the pesticides was had an inverse impact on spore germination and growth of the test strains. It was determined that the pesticides with the most adverse effect were copper sulfate, methiocarb, and dalapon on spore germination of the microorganisms. Higher concentrations or MIC values of the pesticides reduced the heat-resistant spore numbers of both bioinsecticides, suppressing spore germination. These results indicated that the spores of the bacteria are sensitive to chemical pesticides at the same level. Moreover, the study revealed that the spore germination and larvicidal activity of both biological control agents are affected by pesticides equally. The findings should be considered for assessing the effects of pesticides when both microbial agents are used in field applications.

Key Words: Pesticides, *B. thuringiensis* var. *israelensis*, *B. sphaericus* 2362, Biological control

Bazı Pestisitlerin *Bacillus thuringiensis* var. *israelensis* ve *Bacillus sphaericus* 2362 suşunun Spor Çimlenmesi ve Larvasidal Aktivitesi Üzerine Etkileri

Özet: Bu çalışmada, zirai amaçla yaygın olarak kullanılan dokuz ticari pestisitin sivrisinek patojenleri *B. thuringiensis* var. *israelensis* ve *B. sphaericus* 2362 ırklarının spor çimlenmesi ve larvasidal aktivitesi üzerine etkileri incelenmiştir. Üç tanesi hariç, bütün pestisitler test ırklarının büyümesi ve spor çimlenmesini olumsuz etkilenmiştir. Mikroorganizmaların spor çimlenmesi üzerine olumsuz etki gösteren pestisitlerin bakır sülfat, methiocarb ve dalapon olduğu belirlenmiştir. Pestisitlerin MİK veya daha yüksek konsantrasyonları spor çimlenmesini baskılayarak her iki biyoinsektisitin ısıya dayanıklı spor sayılarını düşürmüştür. Bu sonuçlar bakteri sporlarının kimyasal pestisitlere karşı aynı seviyede duyarlı olduğunu göstermiştir. Ayrıca, çalışmada pestisitlerle muamele edilmiş biyoinsektisitlerin larvasidal aktivitelerinin de benzer seviyede etkilendiği belirlenmiştir. Her iki mikrobiyal kontrol ajanı arazi uygulamalarında kullanıldığında pestisitlerin etkilerinin tespit edilmesine ait bulgular dikkate alınmalıdır.

Anahtar Sözcükler: Pestisitler, *B. thuringiensis* var. *israelensis*, *B. sphaericus* 2362, Biyolojik kontrol

Introduction

Recently, several pathogenic strains of the *B. thuringiensis* and *B. sphaericus* were reported that they have a high level of biological activity against mosquito larvae in laboratory and field studies (1,2). Preparations from sporulated cultures used as larvicides yield excellent control of many species belonging to the genera *Anopheles*, *Culex* and *Psorophora,* as well as of some species of the genus *Aedes* (3). Barjac and Sutherland (1) summarized the effect of biotic and abiotic factors on the viability, toxin stability and larvicidal activity of both biological control agents against many species of mosquito larvae. One of the most important environmental factors affecting the larvicidal activity of these bacteria is water pollution rate and deepness (4-7). Pathogenic strains of *B. thuringiensis* can lose their toxic activities in habitats polluted with organic materials, and also exhibit lower persistence (3,8,9). In contrast, other studies have reported that the larvicidal activity of *B. sphaericus* may persist against many species of mosquito larvae in organically enriched habitats (6,10-12).

It was reported that pathogenic strains of *B. thuringiensis* have a greater chance of survival in aquatic habitats owing to the presence of various carbohydrates, but *B. sphaericus* does not use carbohydrates for growth (13). In addition to persistence of toxins, residual larvicidal activity also may be facilitated by the ability of the bacterium to recycle within larval cadavers under certain conditions (14). Moreover, some researchers have suggested that aluminum carboxymethylcellulose, carrageenan and alginate encapsulated forms of *B. sphaericus* and *B. thuringiensis* var. *israelensis* might be used more efficiently than their free forms in the control of mosquito larvae when they are exposed to different pH levels, high temperature and UV radiation (15).

So far, no studies on the effects of chemical pesticides have been carried out despite the existence of several studies on different chemical compounds on the larvicidal activity and spore viability of *B. thuringiensis* var. *israelensis* and *B. sphaericus* (16-18). The aim of this study was to determine the effects of different commercial pesticides on the spore germination and larvicidal activities of *B. thuringiensis* var. *israelensis* and *B. sphaericus* 2362 strain.

Materials and Methods

Bacteria and Spore Suspensions: The *B. thuringiensis* var. *israelensis* and *B. sphaericus* 2362 used in this study were obtained from Prof. Dr. A.A. Yousten (VPI & State University, Blacksburg, VA, USA). Microorganisms were cultivated in nutrient yeast salt medium broth (19) by overnight incubation and then inoculated onto NYSM agar plates. The plates were incubated at 30 ºC for 5 days for complete sporulation, having been examined at regular intervals under a phase-contrast microscope (Nikon, Japan). Spores were then collected from the surface of the medium and washed 3 times with sterile distilled water. Afterwards spores were resuspended in sterile distilled water and adjusted to 2.2 x 10^{10} spores ml^{-1} by plate counting onto NYSM agar after heating at 80 ºC for 12 min.

Preparation of Pesticide Stock Solutions and MIC Assays: The commercial pesticides used in this research were obtained from the Agricultural Research and Control Institute of Turkey (Yenimahalle, Ankara). Four grams of each pesticides was dissolved in 10 ml of distilled water and then autoclaved at 121 ºC for 15 min and kept at 4 ºC. To determine the minimal inhibitor concentrations (MICs) of the pesticides, 2 ml of stock solution of each pesticide was added to sterile tubes containing 2 ml of NYSM broth (20). Serial dilutions of pesticides were prepared from 200 to 3.125 mg ml^{-1}. Pure NYSM broth medium was used as a control group. Subsequently, 100 µl of spore suspensions of test bacteria were inoculated into each tube containing pesticides and control NYSM broth medium. Inoculated serial tubes were then incubated on a rotary shaker at 30 ºC for 48 h at 150 rpm. The minimal inhibitor concentrations of pesticides were determined by checking the growth of bacterial culture against the control group. All experiments were carried out in triplicate and MIC values were calculated as mg ml^{-1}.

Preparation of Samples for Spore Counts: Two milliliters of bacterial samples from cultures incubated for 48 h were transferred into sterile Eppendorf tubes and kept at -70 °C until use. The heat-resistant spore counts of bacterial cultures treated with pesticides were performed by heating 1 ml samples at 80 °C for 12 min and then plating on NYSM agar using the pour-plate technique. The number of spores was determined from the mean of at least 3 platings.

Phase-Contrast Microscopy: Fresh preparations of test strains were examined after 48 h of incubation and used to investigate bacterial growth, the structure of spore-toxin complex and spore germination a under phase-contrast microscope (Nikon, Japan) using x40 and x100 magnification.

Bioassays: The larvicidal activities of the test bacteria grown at different pesticide concentrations were tested against 2nd and 3rd instars of *Culex quinquefasciatus* larvae. *C. quinquefasciatus* culture was provided by Elisabeth Davidson (Arizona State University, Department of Zoology, Az, USA) in egg form and was maintained in the laboratory at 25 ± 1 ºC with a photoperiod of 12 h of light and 12 h of darkness at 70% relative humidity. Larvae were fed 3 times daily with a 1:2 mixture of yeast (Nutrex 430, Amber Laboratories, and Juneau, WI, USA). Spore samples were added to plastic cups containing 50 mosquito larvae in 30 ml of sterile tap water in different concentrations in triplicate, and percentage mortality was determined after 48 h of incubation at 25 ºC in the dark.

Statistical Analysis

The results of the heat-resistant spore count and larvicidal activity of test bacteria treated with pesticides

were tested by analysis of variance (ANOVA). The significance of differences in the spore counts and larvicidal activities related to different pesticide concentrations was determined at the 95% confidence limit (p=0.05). Differences among treatments were examined for levels of significance by the least significant differences (LSD) test (SAS Inst., Inc., Cary, NC, USA).

Results

MIC values of 9 commercial pesticides against *B. thuringiensis* var. *israelensis* and *B. sphaericus* 2362 strain can be seen in Table 1. This study determined that copper sulfate, a fungicide, has the most adverse effect on bacterial growth. One of the fungicides, copper carbonate had a slight effect on the spore germination of test bacteria while the other 2 fungicides, carbendazim and benomyl, inhibited spore germination of the test strains significantly. The herbicide, with the largest inverse effect on the spore viability of both microbial control agents was dalapon.

Heat-resistant spore numbers were determined for bacteria grown on different pesticide concentrations (Figures 1,2). Spore numbers of the heat-resistant test strains were reduced 10^3-10^4 -fold compared to initial spore numbers and the control group. However, the number of spores of *B. sphaericus* 2362 strain was higher than that of *B. thuringiensis* var. *israelensis* after 48 h of incubation. The results of MIC, phase-microscopy observation and direct count study showed that spore germination of both strains was inhibited after 48 h of incubation in different pesticide concentrations but a loss of parasporal toxin crystals did not occur (data not shown). Vegetative growth of bacteria from spores requires the integration of both external and internal stimuli, including an abundance of nutrition and cell cycle signals the nature of which is unclear. Our results suggest that pesticides might inhibit internal factors that extensively control vegetative growth from sporulation in the test bacteria. This observation supports the conclusion that pesticides did not mutate the genome, or at least the DNA segment coding for insecticidal toxin proteins. Insufficiently developed genetics of *B. sphaericus* and *B. thuringiensis* var. *israelensis* hinders the progress of this investigation. Attempts are now underway to detect and identify DNA segments involved in information governing vegetative growth.

Table 1. Pesticides used in the study and their commercial names, type, active ingredient, and MIC values.

Pesticides			Bacteria	
			B. thuringiensis var. israelensis	*B. sphaericus* 2362
Commercial Name and Formulations	Type	Active Ingredient	Minimal Inhibitor Concentrations (mg ml^{-1})	
Pirimor (Powder)	Insecticide	Primicarb	*	*
Mesurol (Powder)		Methiocarb	200	200
Akkükürt (Powder)		Sulfur	*	*
Derosal 50 WP (Powder)	Fungicide	Carbendazim	200	200
Cupramaag (Powder)		Copper Carbonate	*	*
Koruma Göztaşı (Powder)		Copper Sulfate	12,5	12,5
Benor (Powder)		Benomyl	100	100
Dowpon (Powder)	Herbicide	Dalapon	12,5	25
Nata (Powder)		TCA	25	25

* No effect at all concentrations tested

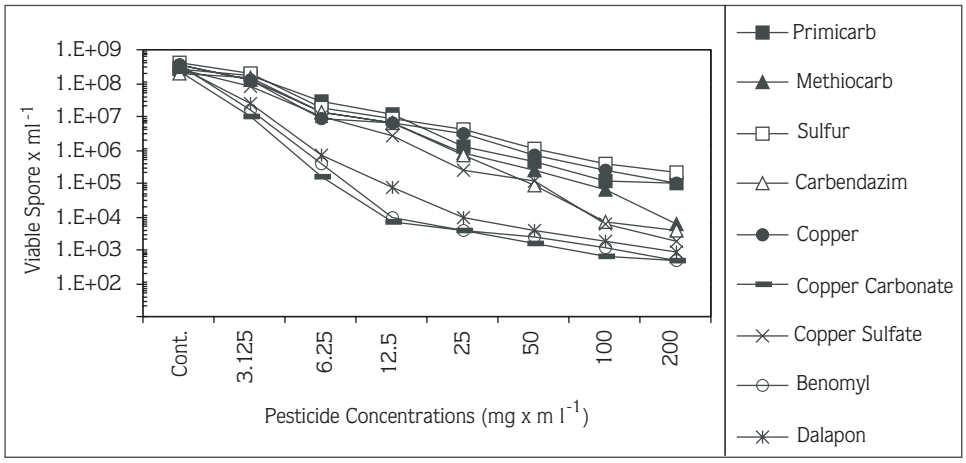

Figure 1. Heat-resistant spore numbers of *B. sphaericus* 2362 strain grown in media containing pesticides.

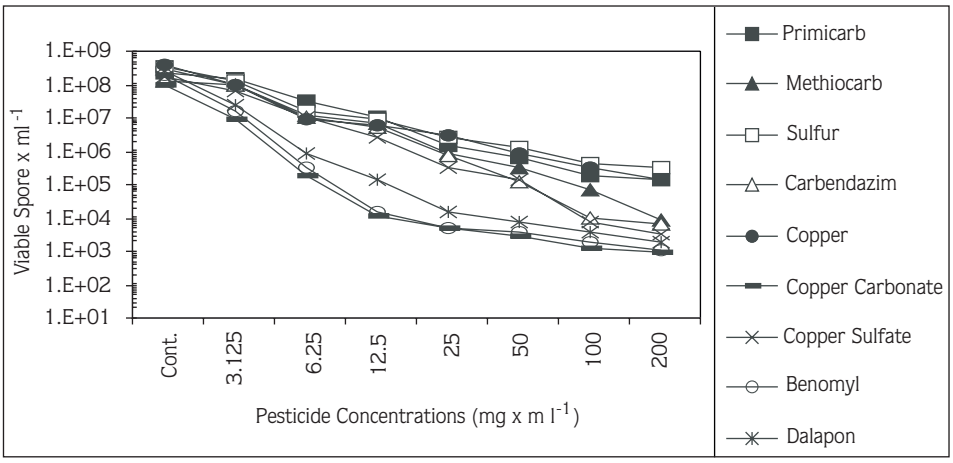

Figure 2. Heat-resistant spore numbers of *B. thuringiensis* var. *israelensis* grown in media containing pesticides

The larvicidal activities of the test bacteria grown in serial concentrations of 9 different pesticides against *C. quinquefasciatus* 2nd and 3rd instars after 48 h of incubation are given in Tables 2 and 3. No significant difference was observed between the larvicidal activities of the 2 test bacteria but they were lower compared to the control group. The tables show that 6 of the concentrations of commercial pesticides have larvicidal activity at or over MIC values. The 3 that did not were primicarb, sulphur and copper carbonate. They have no effect on the larvicidal activity at any concentration. Therefore, ANOVA showed that the concentration of pesticides had a significant impact ($p < 0.05$) on heat-resistant spore numbers and larvicidal activity.

Discussion

In an earlier study, it was shown that *B. sphaericus* 1593 lost its spore viability after 4 h of UV exposure, while its activity remained unchanged (21). Cokmus et al. (22) showed that larvicidal activity was lost after the treatment of test strains of a biological agent with UV for 12 h while parasporal crystals were present in pathogenic *B. sphaericus* strains. In the present study, larvicidal activity disappeared on media containing pesticides at and over MIC values. Parasporal inclusions were also found in the bacteria. The reason for the disappearance of larvicidal activity in bacteria might be unstable toxin proteins degradated by the formation of H^+ ions and the free radicals those are OH^- and peroxide in media. The

Table 2. Percentage of larvicidal activity of *B. thuringiensis* var. *israelensis* treated with serial concentrations of 9 different pesticides against *C. quinquefasciatus* 2nd and 3rd instars after 48 h of incubation.

Active Ingredient	(%) Larval Mortality							
	Pesticide Concentrations (mg ml^{-1})							
	C**	3.125	6.25	12.5	25	50	100	200
Primicarb	100	100	100	100	100	94	86	75
Methiocarb	100	100	100	100	88	73	54	*
Sulfur	100	100	100	100	92	88	73	70
Carbendazim	100	100	100	94	81	58	52	*
Copper carbonate	100	100	98	92	86	74	72	68
Copper sulfate	100	67	48	*	*	*	*	*
Benomyl	100	100	84	74	68	54	*	*
Dalapon	100	76	48	*	*	*	*	*
Trichloroacetic acid	100	66	52	44	*	*	*	*

* No larvicidal activity observed for these concentrations of pesticides tested.
** Control

Table 3. Larvicidal activity of *B. sphearicus* 2362 strain treated with serial concentrations of 9 different pesticides against *C. quinquefasciatus* 2nd and 3rd instars after 48 h of incubation.

Active Ingredient	(%) Larval Mortality							
	Pesticide Concentrations (mg ml^{-1})							
	C**	3.125	6.25	12.5	25	50	100	200
Primicarb	100	100	100	100	100	93	87	78
Methiocarb	100	100	100	100	86	74	55	*
Sulphur	100	100	100	100	95	90	73	70
Carbendazim	100	100	100	95	85	61	52	*
Copper carbonate	100	100	98	96	85	76	74	71
Copper sulfate	100	68	52	*	*	*	*	*
Benomyl	100	100	88	72	65	56	*	*
Dalapon	100	66	42	40	*	*	*	*
Trichloroacetic acid	100	73	58	48	*	*	*	*

* No larvicidal activity observed for these concentrations of pesticides tested.
** Control

findings of the spore numbers and the larvicidal activity were in parallel with each other. In a recent study related to the effects of chemical compounds on spore viability, larvicidal activity and toxin stability of *B. sphaericus* 2362 strain it was reported that the reason for the loss of larvicidal activity is the chemical degradation of toxin proteins by the generation of free radicals and pH differences (23). Our results are in good agreement with the findings of Cokmus et al. (22) and Berber (23).

It was reported that mosquito pathogenic strains of *B. thuringiensis* could lose their toxic activity in habitats polluted with organic and chemical materials, and also

exhibit lower persistence, whereas the insecticidal activity of *B. sphaericus* was prolonged in this kind of habitat (8,9-12). Nevertheless, the results of these experiments confirmed that there was no significant difference between spore germination and larvicidal activity in either biological control agent treated with pesticides. In addition, statistical analysis of the results of the heat-resistant spore counts and larvicidal activities of the bacteria treated with pesticides showed that concentrations of various commercial pesticides considerably impede the spore germination and larvicidal activities of test strains ($p < 0.05$). However, the insecticidal activity of *B. thuringiensis* var. *israelensis* and *B. sphaericus* 2362 spores was quite tolerant to inactivation by the pesticides applied.

There is still no general mechanism to describe how the accelerated degradation of pesticides occurs. Some scientists speculate that, as with microbial resistance to antibiotics and heavy metals, the genes for pesticide breakdown may be carried on plasmids that can be treated freely among various microbes to speed adaptation to the pesticides (24). It would be better to use genetically modified strains that contain genes resistant to pesticides in habitats polluted with chemicals.

It was shown that encapsulated spore-toxin complex provided prolonged protection compared to free spores when they were exposed to different pH, temperature, and UV light levels (15,25-27). In conclusion, the effect of encapsulation on spore viability and toxin stability for both microbial insecticidal agents in the presence of pesticides should be investigated. It is really important to take into consideration these findings under laboratory conditions when determining the larvicidal activity and toxin stability of these microbial agents applied for the control of mosquito larvae in field studies. These results indicated that pesticides prevented the effect of bioinsecticides, thus causing unreliability in biological control methods and resulting in the loss of millions of dollars spent on biological control.

Acknowledgments

We would like to thank Prof. Dr. A.A. Yousten (VPI & State University, Blacksburg, VA, USA) for the bacterial strains.

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