Volume 12 – No. 11 – 2003
REPRINT pp. 1338 - 1344

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EFFECT OF GRAMOXONE® HERBICIDE ON SPORE VIABILITY AND LARVICIDAL ACTIVITY OF *Bacillus sphaericus* 2362 AND 1593 STRAINS

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SUMMARY

Spore viability and larvacidal activity of *B. sphaericus* 2362 and 1593 strains were monitored in media containing Gramaxone® herbicide. Minimal Inhibitory Concentrations (MICs) of the herbicide were determined for each of the mosquito pathogenic strains. Addition of paraquat into the media suppressed the spore germination after 24 and 48 h, but no influence was observed on parasporal inclusions containing insecticidal toxin proteins. SDS-PAGE analysis confirmed the presence of the so-called binary toxin comprising two proteins that are deposited in parasporal crystals treated with the herbicide. Moreover, the number of heat-resistant spores and total cell numbers of test strains were reduced drastically when test strains were grown in media containing 50, 100 and 200 mg ml^{-1} of paraquat. In contrast, there were no differences between heat-resistant spore counts of both strains when grown under diet concentrations of less than 50 mg ml^{-1}. This study revealed that *B. sphaericus* 2362 strain has a higher mosquito larvacidal activity than 1593 strain in the presence of paraquat in the media. Thus, the use of Gramaxone® herbicide can have harmful effects on mosquitocidal biological control agents.

KEYWORDS: *Bacillus sphaericus*, herbicide, spore viability, larvicidal activity, paraquat.

INTRODUCTION

Gramoxone® (1,1'-dimethyl-4,4'-bipyridylium dichloride) is the commercial name of a herbicide consisting of 10% paraquat as the active ingredient, that is commonly used for weed control in agricultural fields [1]. Although extensive use of pesticides has improved agricultural production, it has also caused many problems for the natural environment and effects on human health [2, 3]. After application of some herbicides to soil, the active ingredients can reach aquatic habitats. Once an aquatic system has become contaminated with pesticides, the contamination can last for extended periods [4, 5].

There has been considerable research on the effects of the pesticides on soil microorganisms [6-11]. *Bacillus thuringiensis* and some pathogenic strains of *B. sphaericus* are used as microbial control agents to combat mosquitoes [12]. Mulla et al. [13] and Lacey [14] summarized the effect of biotic and abiotic factors on 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 [15-20]. Pathogenic strains of *B. thuringiensis* can lose their toxic activities in habitats polluted with organic materials and also exhibit lower persistence [21-24]. In contrast, other studies have reported that larvicidal activity of *B. sphaericus* may persist against many species of mosquito larvae in organically enriched habitats [19, 25-28]. To date, related studies with chemical pesticides have not been carried out despite several studies reporting the effects of different kinds of chemical compounds on the larvicidal activity and spore viability of *B. sphaericus* strains [29-32]. The purpose of our study was to investigate the effect of Gramoxone® herbicide on spore viability and mosquito larvicidal activity of *B. sphaericus* 2362 and 1593 strains that have been commonly used as biological agents in mosquito control.

MATERIALS AND METHODS

Bacteria and Spore Suspensions

B. sphaericus 2362 and 1593 strains were cultured overnight in Nutrient Yeast Salt Broth [33], inoculated onto NYSM agar plates (Difco, Detroit, MI), and then incubated at 30 °C for 5 days to obtain complete sporulation. The progress of sporulation was examined periodically by phase-contrast microscopy. Spores were then collected from the surface of the NYSM agar plates and washed three times with sterile distilled water. A stock solution of spore suspensions were prepared in sterile distilled water and adjusted to 2.2 x 10^{10} spores ml^{-1}. Spores were counted on the NYSM agar plates after incubation for 12 min in a water bath at 80 ºC.

Preparation of Gramoxone® Stock and MIC Assays

Gramoxone® herbicide was provided by the Agricultural Research and Control Institute of Turkey. The herbicide was sterilized by filtrating the product through cellulose acetate membranes (Sartorious, 0.25 µm diameters) and stored at +4 ºC. The minimal inhibitor concentrations (MICs) of the pesticide assays were carried out as described by Clause [34] with minor modifications. Serial dilutions 200, 100, 50, 25, 12.5, 6.25, and 3.125 mg ml^{-1} of the pesticide were prepared and 2 ml of pesticide solution was added into sterile tubes containing 2 ml NYSM broth. NYSM broth medium alone was used as control medium. Then 100 µl of the two stock spore suspensions were inoculated into each tube containing the pesticide and NYSM broth control medium. The inoculated tubes were incubated on a rotary shaker at 30 ºC for 48 h at 150 rpm. After incubation the MIC values of pesticides were determined by checking the growth of the bacterial culture against that of the control group [34]. MIC values were determined as mg ml^{-1} active ingredient in media.

Total Cell and Spore Count

Aliquate samples (500 µl) from each bacterial culture incubated for 24 and 48 h were transferred into sterile Eppendorf tubes to determine total cell and heat-resistant spore numbers per ml. The samples were washed with sterile distilled water 3-4 times to remove the pesticide residues. Total cell and heat-resistant spore numbers per ml were determined on NYSM agar plates after incubation at 30 ºC for 24 h. Bacterial samples were kept at 80 ºC for 12 min to determine heat-resistant spore numbers per ml, but the heating was omitted for the determination of total cell counts. Experiments were carried out separately for *B. sphaericus* 2362 and 1593 strains. All of the tests were carried out in triplicate to find out reproducibility of the assays.

Phase-Contrast Microscopy

Fresh inoculations of test strains after 24 and 48 h of incubation were used to investigate the bacterial growth, the structure of spore-toxin complex, the bacterial sporulation, and, finally, were photographed under phase-contrast microscopy.

Bioassays

Larvacidal activities of samples were tested against 2nd and 3rd instars of *Culex quinquefasciatus* larvae reared in our laboratory. Samples were added to plastic cups containing 35 mosquito larvae in 30 ml sterile tap water and percentage of mortality was determined after incubation for 48 h at 25 ºC in the dark (light : dark = 0 : 24) and all experiments were carried out in triplicate.

SDS-PAGE

Samples were centrifuged at 15,000 rpm for 10 min and proteins were extracted from the spores by suspending them directly in electrophoresis sample buffer (0.06 M Tris, 2.5 % glycerol, 0.5% SDS, 1.25% β-mercaptoethanol; pH 6.8) and boiling for 10 min. Solubilized proteins were subjected to SDS-PAGE in gel slabs of 0.75 mm thickness (1.5 cm, 4% stacking and 13.5 cm, 10% resolving gels) as described by Laemmli [35] and the gels stained with Coomassie Brillant Blue R-250.

RESULTS AND DISCUSSION

MIC values demonstrated that *B. sphaericus* 1593 did not germinate on media containing 200 mg ml^{-1} active ingredient, but 2362 test strain was grown in all serial concentrations of paraquat, evidencing a much better growth after incubation for 48 h. Thus, *B. sphaericus* 1593 is more sensitive to paraquat than the 2362 strain. In addition, the phase-contrast microscopy confirmed the presence of spore-toxin inclusions produced by both strains grown in the different paraquat concentrations (Figs. 1 and 2).

The number of heat-resistant spores and total cells were determined for both bacterial strains grown at each of the pesticide concentrations. The initial spore numbers (2.2 x 10^7 spores ml^{-1}) of the 1593 strain decreased to 0.1 x 10^3 spores ml^{-1}, 0.1 x 10^4 spores ml^{-1}, and 0.5 x 10^4 spores ml^{-1}, and the spore count of the 2362 strain was reduced to 1.4 x 10^4 spores ml^{-1}, 2 x 10^4 spores ml^{-1}, and 9.1 x 10^4 spores ml^{-1} when grown on media containing the three highest paraquat concentrations after 24 h of incubation (Fig. 3). In addition, the heat-resistant spore numbers at the three highest paraquat concentrations did not change in cultures incubated for 48 h (Fig. 4). Thus, the heat-resistant spore numbers for the two test strains were reduced 10^3-10^4-fold compared to the initial counts. The total cell numbers of the 2362 strain grown on media containing paraquat after 24 h of incubation ranged between 0.2 x 10^5 and 0.4 x 10^8 CFU ml^{-1}, while the total cell numbers of the 1593 strain were found to be between 0.1 x 10^5 and 0.2 x 10^8 CFU ml^{-1}. The numbers of heat-resistant spores and total cells of both strains were reduced when paraquat was added to the media. The 2362 strain showed a higher reduction rate after either 24 or 48 h of incubation (Figs. 3 and 4).

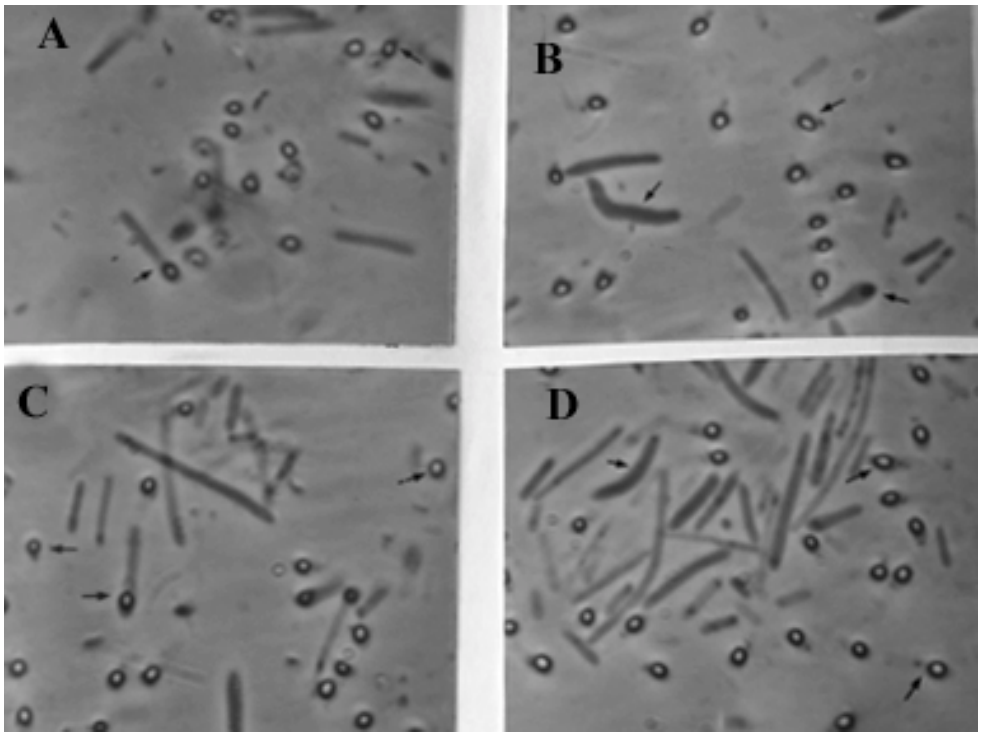

FIGURE 1 - Phase-contrast microscopy of *B. sphaericus* 2362 strain grown in different paraquat concentrations after 48-h incubation. (A): 200 mg ml^{-1}, (B): 100 mg ml^{-1}, (C): 50 mg ml^{-1} and (D): Control.

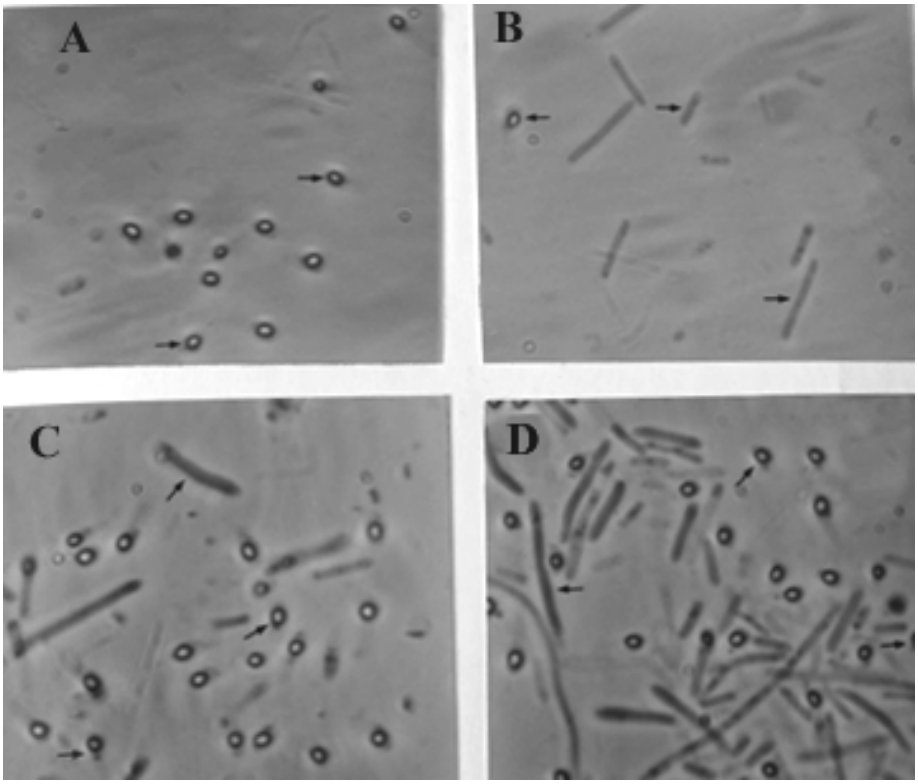

FIGURE 2 - Phase-contrast microscopy of *B. sphaericus* 1593 strain grown in different paraquat concentrations after 48-h incubation. (A): 200 mg ml^{-1}, (B): 100 mg ml^{-1}, (C): 50 mg ml^{-1} and (D): Control.

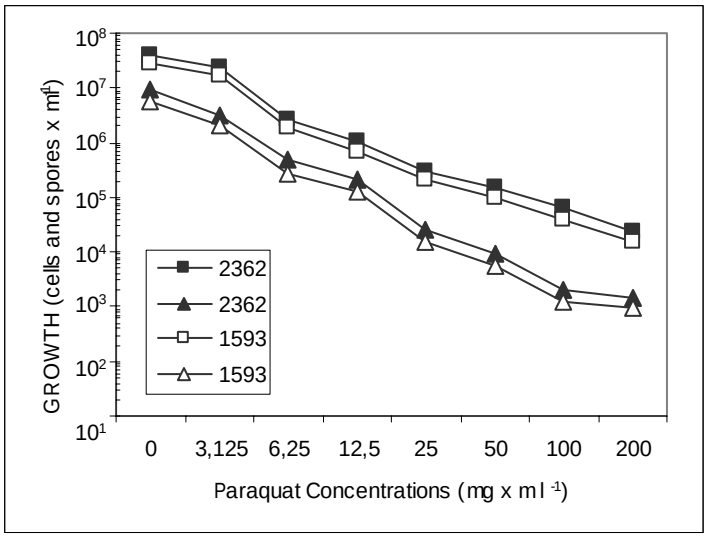

FIGURE 3
Total numbers of cell and spore of *B. sphaericus* 2362 and 1593 strains grown on media containing different paraquat concentrations after 24-h incubation. (■ and □: total cell counts; ▲ and Δ : heat-resistant spore counts).

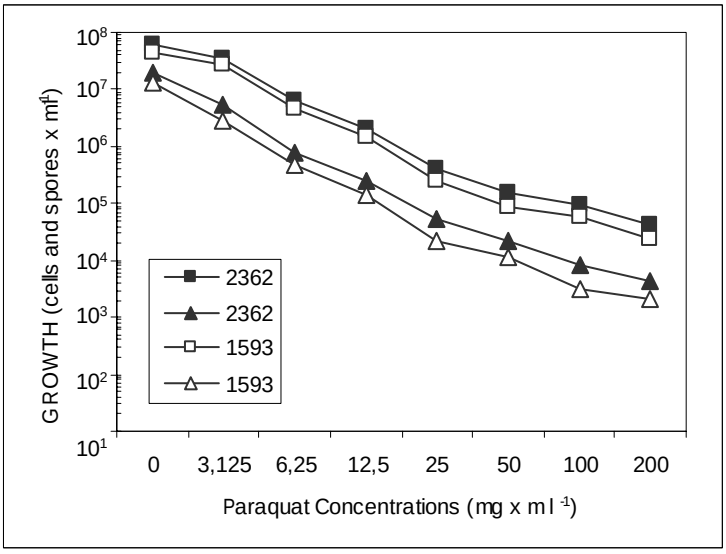

FIGURE 4
Total numbers of cell and spore of *B. sphaericus* 2362 and 1593 strains grown on media containing different paraquat concentrations after 48-h incubation (■ and □: total cell counts; ▲ and Δ : heat-resistant spore counts).

The results of the MIC tests, the phase-microscopy and the direct counts showed that spore germination of both strains was inhibited after 24 and 48 h of incubation in 200 mg ml^{-1} paraquat. However, none of the strains lost its parasporal toxin crystals. SDS-PAGE assays confirmed that the so-called binary toxin, comprising two proteins at 41.9 kDa and 51.4 kDa for both test strains grown on media containing various paraquat concentrations, did not disappear (Fig. 5). Vegetative growth of bacteria from the spores requires the integration of both external and internal stimuli, including an abundance of nutrition and cell cycle signals. Our results suggest that paraquat might have inhibited internal signals that control the vegetative growth from sporulation in *B. sphaericus* strains. The transition from the spore to the vegetative form was reduced even when paraquat-treated spores were washed and transferred to new media. This suggests that paraquat did not mutate the genome, at least for the

binary toxin-coding sequence of the DNA segment. Insufficiently developed genomic data for *B. sphaericus* is a limiting factor, and attempts are now made to detect and identify DNA segments governing the vegetative growth.

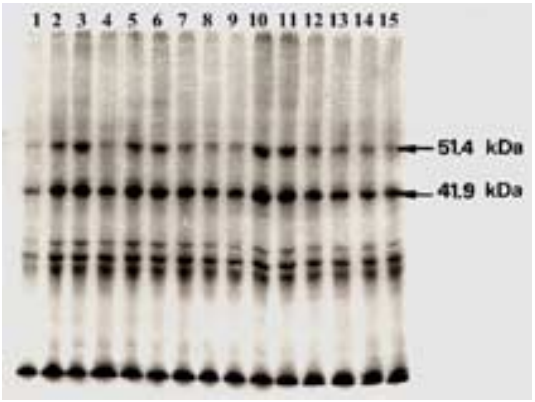

FIGURE 5 - SDS-PAGE analysis of *B. sphaericus* 2362 and 1593 strains after 48-h incubation on media containing different paraquat concentrations. Lanes 1-7 belong to 1593 strain, lanes 8-14 to 2362 strain and line 15 to control. Arrows show the binary toxin bands.

The larvicidal activities of *B. sphaericus* 2362 and 1593 strains grown in different paraquat concentrations against 2nd and 3rd instar *C. quinquefasciatus* are presented in Table 1. The differences in larvicidal activity of the two test strains was minimal, and 100% larvicidal activity was observed for control treatments and both strains grown on media containing 1.562 mg ml^{-1} of paraquat. In an earlier study, it was demonstrated that *B. sphaericus* 1593 lost its spore viability after 4 hours of UV exposure, although mosquitocidal activity remained [36]. The results of this study show that larvacidal activities of both *B. sphaericus* strains were maintained despite the damage caused by paraquat that inhibited spore germination.

Some researchers have reported that factors such as UV-light, pH, organic pollution and temperature have inverse effect on larvicidal activity and spore viability of different pathogenic strains of *B. sphaericus* [27, 37-41]. Cokmus et al. [16] showed that parasporal crystals of UV-irradiated spores of pathogenic strains of *B. sphaericus* might not contain these specific toxin proteins even though they appeared to be undamaged. Other papers reported the effects of some chemical compounds on spore viability of *B. sphaericus* [29, 31, 32, 42]. Berber [43] studied the effects of chemical compounds on spore viability, larvicidal activity and toxin stability of the *B. sphaericus* 2362 strain. He reported that the reason for loss of larvicidal activity was the chemical degradation of the toxin proteins by free radicals and changes of pH values. The results of our study showed that increasing concentrations of paraquat have no degradative effects on the toxin proteins of both the 1593 and 2362 strains. There is still no general mechanism to describe how accelerated degradation of the pesticides occurs. Some scientists have speculated that, similarly as with microbial resistance to antibiotics and heavy metals, the genes for pesticide degradation may be carried on plasmids that can be transferred freely to various microbes in order to speed up adaptation to the pesticides [11, 44].

It would be better to use genetically modified strains that contain resistant genes against pesticides in polluted habitats with chemical pesticides. The habitats where pathogenic strains of *B. sphaericus* have been used to combat mosquitoes tend to be aquatic areas, which are often exposed to chemical and organic pollution [15], possibly affecting the larvicidal activity of pathogenic bacteria [12]. Therefore, it is important to determine how the spore viability and insecticidal activity of pathogenic *B. sphaericus* strains are affected in these above-mentioned habitats. There the applied control strategies for mosquitos will be impacted not only in terms of activity, but also by altered persistence [43]. Both laboratory and field studies have demonstrated that persistence of pathogenic effects can be extended under certain conditions by larval cadaver recycling [19, 25, 28].

The residual larvicidal activity of both tested strains depends on the continued presence of the spore-toxin complex, which should include the toxic binary proteins. Myers et al. [45] showed that *B. sphaericus* 1593 vegetative cells are many thousand-fold less insecticidal than are the spores. Our study shows that the bacterial recycling rate in the presence of paraquat decreases the viable spore count, and, thus, the residual larvicidal activity decreases. Encapsulated spore-toxin complex have provided prolonged protection when tested at different pH values, temperatures, and UV-light exposures [46-49]. Therefore, it may be useful to use encapsulated spore-toxin complex to prolong larvicidal activity in organically and chemically polluted habitats. A breakdown in the effectiveness of the biological controls against mosquito larvae would not only result in an annual loss of millions of dollars, but also undermine the consumers` confidence in the introduction of biological methods for the control of mosquito species in general.

TABLE 1 - Percentage of larvicidal activities of *B. sphaericus* 2362 and 1593 strains grown on media containing different paraquat concentrations.

Strains	% larval mortality for 48-h treated pesticide samples									
	paraquat concentrations (mg x ml^{-1})									
	200	100	50	25	12.5	6.25	3.125	1.562	Control	MICs
2362	70	71	78	84	88	91	98	100	100	None
1593	67	68	76	80	85	90	97	100	100	200

ACKNOWLEDGEMENTS

The *B. sphaericus* 2362 and 1593 strains were generously provided by Prof. A. A. Yousten (VPI & State University, Blacksburg, VA).

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Received for publication: January 15, 2003
Accepted for publication: June 23, 2003

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