The Libyan Journal of Agriculture: volume 7, 1978

Significance of *Bacillus thuringiensis* Lytic Bacteriophages in Soil

S. M. SALEH[1] AND R. F. HARRIS[2]

ABSTRACT

Under conditions favouring spore germination and growth of *B. thuringiensis* in soils inhabited by TV-1 and TV-2 bacteriophages, propagation of the phages commences within three hours of incubation with the host bacterium. Phage TV-1 attains levels higher than 1 million pfu/g soil within 48 hr, about 5-fold higher level than that noticed for TV-2 bacteriophage. Consequently, *B. thuringiensis* spores added to soil may considerably diminish in numbers with time, contrary to other results obtained for phage-free soils.

INTRODUCTION

Agricultural applications of *Bacillus thuringiensis* preparations as insecticides result in the addition of substantial amounts of viable spores of the organism to crops and associated soils. The possibility of *B. thuringiensis* establishing itself in soil and affecting soil biocoenosis has not been adequately investigated. Previous investigations indicated that spore viability is maintained in soils amended with high levels of *B. thuringiensis* (6). The microorganism was recovered at numbers ranging from 7.8×10^3 to 1.7×10^5 propagules, essentially spores, per gram from silty clay loam and silt loam soils to which either of the commercial products known as Thuricide and Biotrol had been applied for the control of insect pests on cabbage and lettuce crops (9). Under conditions favouring the growth of soil bacilli, such as neutral soil pH conditions and the presence of proteinaceous amendments, *B. thuringiensis* was found to germinate, compete vegetatively with soil microorganisms, and sporulate successfully to attain levels higher than 1 million spores/g soil (10). Irrespective of soil treatment performed, *B. thuringiensis* failed to propagate in just one out of 16 soil samples collected from the same location included in the previous study. The concerned soil sample was later found to inhabit two lytic bacteriophages to the organism.

Virulent phages for *B. thuringiensis* had been isolated from lysogenic bacteria (1), commercial preparations of the organism (3), fermentation broths and soil samples (4). No attempt, however, has been made to investigate the effect of phages on the persistence of *B. thuringiensis* in soils treated with billions of viable spores of the organism as an insecticide.

The purpose of this investigation was to evaluate the significance of indigenous bacteriophages in relation to the survival and competitive growth of the added microorganism to rather neutral soils amended with alfalfa.

[1] *Department of Soil and Water, Faculty of Agriculture, University of Alfateh, Tripoli, S.P.L.A.J.*

[2] *Department of Soils, University of Wisconsin, Madison, Wisconsin, 53706 U.S.A.*

MATERIALS AND METHODS

Bacterial strain

B. thuringiensis var. *thuringiensis* Berliner in the form of spore-crystal concentrate (Thuricide 90 T) containing about 3.2×10^{10} viable spores per milliliter (International Mineral and Chemical Corp. Libertyville, III.) was used to prepare a pure culture stock of the organism by serial dilutions in pH 7.1 tris buffer and successive streak-plating on nutrient agar. Freedom of the culture of related phage particles was ascertained by the conventional method (2) and ultra-violet induction technique (5). Appropriate dilutions of the stock culture were made with water or tris buffer immediately before use with soil or in phage assays respectively. The exact concentration of each final suspension was determined using nutrient agar.

Phages

The bacteriophages were isolated from Elliot soil sample (No. 12) as plaques on agar plates of host strain *B. thuringiensis* var. *thuringiensis*. A well-isolated circular or halo plaque was picked with a sterile wire and the phage was purified by serial single plaque isolations (2). The phages were given the designations TV-1 and TV-2 in accordance with Colasito and Rogoff system (4) for the circular and halo plaque forming particles respectively. High titre phage lysates were prepared by inoculating 50 ml nutrient broth made 0.002 M with respect to Ca^{2+} in 250 ml capacity flasks with *B. thuringiensis* spores and incubated at 25°C in a shaking waterbath for 16 hours. The phage was added to give a multiplicity of infection of about 0.1, and incubation continued for a further 8 hours. The lysate was freed of the whole cells and debris by centrifugation at 6000 g for 30 minutes and the supernatant fluid was filtered through a Millipore membrane filter with pore diameter of 0.22μm. The phages were assayed by the conventional soft agar overlay method (2).

Fate of *B. thuringiensis* in alfalfa-amended soils known to contain indigenous bacteriophages, and in sterile soil system.

Ten-gram samples of air-dried Elliot silt laom (No. 12) pH 6.4 were mixed thoroughly with sterile 0.4 g air-dried ground alfalfa, placed in petri plates, moistened to about field moisture capacity by inoculation with 2 ml *B. thuringiensis* spore suspension and incubated at 25°C in a humidor. Duplicate plates were combined for determination of numbers of cells and spores of *B. thuringiensis* and soil microorganisms, using nutrient agar amended to contain 5 ppm polymixin B and 4 ppm penicillin G (NPP selective agar) and the modified plate dilution frequency technique described previously (8). Spore enumeration involved pasteurizing 1:10 soil-water suspension at 80°C for 10 minutes (9).

Another set of ten-gram samples was prepared in the same manner but the samples were sterilized by intermittent autoclaving of 1 hour at 121°C on three successive days prior to inoculation with the test organism.

Growth of *B. thuringiensis* in sterile soils inoculated with soil microorganisms and bacteriophage lysate.

Three sets each of 10.0 g samples of sterile Elliot soil (No. 12) were prepared as above. Every 10-g portion of the appropriate set of samples was moistened uniformly

with 2 ml of a mixture of *B. thuringiensis* spore suspension combined with a 1:10 aqueous soil suspension of air-dried Elliot silt loam (No. 12), Elliot silt loam (No. 13), or Elliot silt loam (No. 13) mixed with 0.1 ml phage TV-1 lysate. The soil suspension was shaken mechanically for 30 min, allowed to stand for 1 min, and decanted of the residual soil before being used. Each inoculum contained about 3.0×10^5 *B. thuringiensis* spores/ml, equivalent to about 30×10^4 spores/g soil. Bacteriophage content of the last inoculum was about 1.6×10^6 pfu/ml, equivalent to about 1.6×10^4 pfu/g soil. Incubation conditions and microbiol determinations were as described in the first experiment.

RESULTS AND DISCUSSION

Fate of *B. thuringiensis* in alfalfa-amended soil known to contain indigenous bacteriophages, and in sterile soil system

Despite the prevailing favourable conditions for the growth of *B. thuringiensis* in this soil such as the soil reaction (pH 6.4) and the presence of an organic amendment, no significant increase in total numbers of the organism was observed throughout the incubation period (Table 1). A gradual decline in *B. thuringiensis* spore counts was noticed after 6 hours of incubation so that a 10-fold reduction over the control treatment was shown at 96 hours. Addition of alfalfa caused an increase in soil microbial numbers, and from the relationship between total cells and spores it is apparent that germination, growth, and subsequent sporulation of soil bacilli occurred within the first day of incubation. While a count of 1.8×10^8 cells/g was obtained for total soil microbes at 48 hours, no significant increase in numbers of spores of soil bacilli was observed but at 96 hours. At this time the total soil cells to spores reached a considerable high ratio of about 31:1 (Table 1).

In marked contrast to the performance of *B. thuringiensis* in natural soil samples, counts of the organism continued to increase in presterilized soils after a period of 12 hours only. Thus values of 7.96×10^7 total cells and 5.90×10^7 spores/g were attained at 96 hours (Table 2). The results in Tables 1 and 2 clearly emphasize the

Table 1 Numbers of *B. thuringiensis* and NPP-resistant soil microorganisms in incubated alfalfa-amended Elliot soils (No. 12) inoculated with *B. thuringiensis* spores.

Incubation time, hours	Microorganisms, thousands/g[a]			
	B. thuringiensis		*Soil microorganisms*	
	Total cells	Spores	Total cells	Spores
0	173	173	1,800	1,330
3	306	229	1,730	780
6	228	228	3,060	306
12	173	78	13,300	133
24	422	42.2	22,900	581
48	133	22.8	180,000	1,730
96	133	17.3	180,000	5,810

[a]95% confidence interval was d/2.47 to 2.47 d where d is the number of microorganisms.

Table 2 Numbers of *B. thuringiensis* in incubated alfalfa-amended presterilized Elliot soils (No. 12) inoculated with *B. thuringiensis* spores.

Incubation time, hours	*B. thuringiensis*, thousands/g[a]	
	Total cells	Spores
0	173	133
6	422	228
12	422	133
24	2,280	581
48	5,900	1,020
96	79,600	59,000

[a]95% confidence interval was d/2.47 to 2.47 d where d is the number of microorganisms.

magnitude of the deleterious effect exerted on *B. thuringiensis* by indigenous soil microflora. In this respect, it may be worthwhile to mention that the antimicrobial substances inhibitory to *B. thuringiensis* growth (7,11) were proved to be undetectable in this particular soil sample even when the diluted soil extract was brought to its natural concentration by vacuum evaporation at 35°C.

Indigenous bacteriophages, whose characteristics are described in a proceeding report, were found to increase in numbers significantly in Elliot soil samples amended with alfalfa and inoculated with *B. thuringiensis* spores. Maximum counts of 3.55×10^6 and 2.65×10^5 pfu/g were recorded for TV-1 and TV-2 phages at 24 and 48 hours respectively (Table 3). The results mentioned above evidently suggest a direct relationship between phage propagation and *B. thuringiensis* spore eradication reported in Table 1 for the soil under investigation.

Fate of *B. thuringiensis* in sterile soils inoculated with soil microorganisms and TV-1 phage lysate.

Presterilized Elliot soil (No. 12) was used throughout these experiments to ensure control of soil microbial population variable under evaluation. Elliot soil microorganisms extracted from samples numbers 12 and 13 were used as microbial sources to

Table 3 Numbers of *B. thuringiensis* bacteriophages in incubated alfalfa-amended Elliot soils (No. 12) inoculated with *B. thuringiensis* spores.

Incubation time, hours	PFU, thousands/g[a]	
	TV-1	TV-2
0	< 1.0	< 1.0
3	13.5	2.1
6	1,040	30.0
12	1,230	75.0
24	3,550	200
48	1,460	265
96	2,640	95.0

[a]95% confidence interval was f/3.46 to 3.46 f where f is the number of infective centers.

ascertain whether the observed elimination of *B. thuringiensis* spores from Elliot soil (No. 12) shown in Table 1 was related to the indigenous bacteriophages of this soil or to the other groups of soil microorganisms.

Elliot soil (No. 12) microorganisms added to alfalfa-amended presterilized soil and inoculated with *B. thuringiensis* spores increased in numbers from 2.28×10^4 to 1.05×10^8 propagule/g at 48 hours of incubation. However, *B. thuringiensis* added at a level of about 3.0×10^4 spores/g remained relatively constant, probably largely in the spore state, throughout the incubation period (Table 4a). Indigenous bacteriophages

Table 4 Effect of incubation on the numbers of *B. thuringiensis*, corresponding bacteriophages, and NPP-resistant soil microorganisms in alfalfa-amended presterilized Elliot soils (No. 12) inoculated with a mixture of *B. thuringiensis* spores and soil microorganisms with or without TV-1 bacteriophage.

Incubation time, hours,	Microorganisms, thousands/g		
	B. thuringiensis[a]	PFU[b]	Soil microorganisms[a]
	(a) Elliot soil (No. 12) microorganisms		
0	22.8	< 0.1	22.8
24	30.6	4.1	4,280
48	48.2	3,040	105,000
	(b) Elliot soil (No. 13) microorganisms		
0	30.6	Nd	30.6
24	422	Nd	3,090
48	4,220	Nd	309,000
	(c) Elliot soil (No. 13) microorganisms and TV-1 lysate		
0	17.3	10.2	17.3
24	78.0	42.0	18,000
48	22.8	1,860	428,000

[a]95% confidence interval was d/2.47 to 2.47 d where d is the number of microorganisms.
[b]95% confidence interval was n/3.61 to 3.61 n where n is the number of infective centers.
Nd: Undetectable.

in this case were shown to increase to a level of 3.04×10^6 pfu/g at 48 hours. Growth of *B. thuringiensis* in soil amended with Elliot soil (No. 13) microorganisms indicated that this organism competed very well with a considerably higher number (3.09×10^8 cells/g) of soil microbes to reach a count of 4.22×10^6 cells/g at 2 days in soils devoid of the specific lytic bacteriophages (Table 4b). At 24 hours of incubation, a mere 4.5-fold increase in counts of *B. thuringiensis* was observed in soil amended with Elliot soil (No. 13) microorganisms and TV-phage (Table 4c). *B. thuringiensis* numbers declined thereafter to about the initial level as TV-1 phage content of the soil increased to 1.86×10^6 pfu/g.

LITERATURE CITED

1. Ackermann, H. W., W. A. Smirnoff and A. Z. Bilsky. 1974. Structure of two phages of *Bacillus thuringiensis* and *B. cereus*. Can. J. Microbiol. 20:29–33.
2. Adams, M. H. 1959. Bacteriophages. Interscience Publishers Inc., New York.
3. Chapman, H. M. and J. R. Norris. 1966. Four new bacteriophages of *Bacillus thuringiensis*. J. Appl. Bacteriol. 29:529–535.

4. Colasito, D. J. and M. H. Rogoff. 1969. Characterization of lytic bacteriophages of *Bacillus thuringiensis*. J. Gen. Virol. 5:267–274.
5. Colasito, D. J. and M. H. Rogoff. 1969. Characterization of temperate bacteriophages of B. thuringiensis. J. Gen. Virol. 5:275–281.
6. Ignoffo, C. M. and H. M. Graham. 1967. Laboratory and field cage tests with *Bacillus thuringiensis* against pink-bollworm larvae. J. Invert. Pathol. 9:390–394.
7. Morris, O. N. 1969. Susceptibility of several forest insects of British Columbia to commercially produced *Bacillus thuringiensis*. I. Studies on the physiological properties of some commercial products. J. Invert. Pathol. 13:134–146.
8. Saleh, S. M., R. F. Harris and O. N. Allen. 1969. Method for determining *Bacillus thuringiensis* var. *thuringiensis* Berliner in soil. Can. J. Microbiol. 15:1101–1104.
9. Saleh, S. M., R. H. Harris and O. N. Allen. 1970. Recovery of *Bacillus thuringiensis* var. *thuringiensis* from field soils. J. Invert. Pathol. 15:55–59.
10. Saleh, S. M., R. F. Harris and O. N. Allen. 1970. Fate of *Bacillus thuringiensis* in soil: effect of soil pH and organic amendment. Can. J. Microbiol. 16:677–680.
11. Smirnoff, W. A. and P. M. Hutchinson, 1965. Bacteriostatic and bactericidal effects of extracts of foliage from various plant species on *Bacillus thuringiensis* var. *thuringiensis* Berliner. J. Invert. Pathol. 7:273–280.

اهمية فيروسات بسلس ثرنجيينسس
فى التربة

صالح محسن صالح ، روبن هاريس

المستخلص

ظهر بان هذه الفيروسات تبدأ بالتكاثر خلال ثلاث ساعات من توفر الظروف الملائمة لانبات سبورات البكترية باسلس ثرنجيينسس ٠ وكانت هذه الفيروسات على نوعين سمى احدهما ت٠ف ـ ١ والاخر ت٠ف ـ ٢ ٠ وقد بلغ عدد النوع الاول اكثر من مليون/ وحدة / جرام بينما بلغ عدد النوع الثانى ما يقارب ٢٠٠ر٠٠٠ وحدة / جرام بعد مرور ٨٨ ساعة على معاملة التربة بالبكترية ٤ وعليه فقد انخفض عدد البكترية انخفاضا كبيرا نتيجة لنشاط تلك الفيروسات ٠