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(54) Title: INSECTICIDAL COMPOSITIONS CONTAINING A DELTA-ENDOTOXIN

(57) Abstract: This invention relates to two new varieties of Bacillus thuringiensis, viz Bacillus thuringiensis ATCC 55266 and Bacillus thuringiensis ATCC 55267, to delta-endotoxins obtained from these varieties, to insecticidal compositions containing the delta-endotoxins, optionally in combination with spores of the varieties, and to the control of insects, especially of the Order Lepidoptera, using the delta-endotoxins and the combination of delta-endotoxins with spores.

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(56) Documents cited: US - 5061489 WO - 91/07481 WO - 92/19106 WO - 92/13941

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BACKGROUND OF THE INVENTION

This invention relates to new varieties of Bacillus thuringiensis, to delta-endotoxins obtained from these varieties, to insecticidal compositions containing the delta-endotoxin, optionally in combination with spores of the varieties, and to the control of insects using the delta-endotoxins and the combination of delta-endotoxins with spores.

Bacillus thuringiensis (B.t.) is a well known rod-shaped Gram positive bacterium characterized by its ability to produce crystal protein inclusions known as protoxins or delta-endotoxins during its sporulation phase. Reviews, "Insecticidal Crystal Proteins of Bacillus thuringiensis", by Hofte et al., Microbiol. Rev. (1989) 53 : 242-252 and Rowe and Margaritis; CRC, Vol. 6, part 1 (1987) have been published on this topic. After the sporulation phase the cells lyse, releasing spores and crystals into the culture medium. The pathogenicity of B.t. to a variety of sensitive insects, such as those in the orders Lepidoptera, Coleoptera and Diptera, is essentially due to the delta-endotoxin. The crystals can comprise up to 20% of the cell dry weight. They are composed entirely of protein, they are heat labile, and they are insoluble in water or organic solvents but readily solubilized in alkaline reducing conditions. If the crystals (protoxin) are ingested by most insects or by vertebrates they either pass unchanged through the gut or are inactivated by acidic conditions in the stomach. However many Lepidoptera have a highly alkaline mid-gut (pH 10,2 to 10,5) and in these conditions the crystals dissolve and the toxin is released by proteolytic enzymes. Affected species show a rapid gut paralysis, causing the insect to cease feeding. Many insects are killed by the toxin crystal alone, but in other cases a combination of spores and crystals is necessary and this combination is generally present in the commercial formulations. The spores germinate when the pH of the gut is lowered by equilibration with the blood. The vegetative cells then invade the tissues and cause a lethal septicemia.

Bacillus thuringiensis is considered as an environmentally safe bioinsecticide, and it has been used for a number of years to control insect pests of agricultural crops, forest trees and ornamentals.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided Bacillus thuringiensis ATCC 55266 or a biologically pure culture thereof.

According to a second aspect of the invention there is provided Bacillus thuringiensis ATCC 55267 or a biologically pure culture thereof.

According to a third aspect of the invention there is provided a delta-endotoxin obtained from a culture of Bacillus thuringiensis ATCC 55266 or Bacillus thuringiensis ATCC 55267 after sporogenesis.

According to a fourth aspect of the invention there is provided an insecticidal composition containing as its active ingredient a delta-endotoxin as described above.

According to a fifth aspect of the invention there is provided an insecticidal composition containing as its active ingredient a combination of a delta-endotoxin as described above and the spores obtained from cultures of Bacillus thuringiensis ATCC 55266 or Bacillus thuringiensis ATCC 55267 after sporogenesis.

According to a sixth aspect of the invention there is provided a method for controlling insects, especially of the order Lepidoptera, at a locus, which includes the steps of treating the locus to be protected with an amount of a delta-endotoxin as described above or a combination of the delta-endotoxin and the spores as described above.

DESCRIPTION OF EMBODIMENTS

The strain of Bacillus thuringiensis which is most commonly used commercially is HD-1 (B.t. in Crop Protection by S Rigby, Asgrow, 1991) which is available from the collection of Bacillus thuringiensis strains maintained by the US Department of Agriculture. The present invention relates to novel strains of Bacillus thuringiensis having generally similar properties to HD-1, but distinguished therefrom by improved insecticidal activity against some lepidopteran pests.

The first and second aspects of the invention are novel strains of Bacillus thuringiensis, designated by the internal codes BM7 and I52, and deposited in the American Type Culture Collection (ATCC) under the accession numbers 55266 and 55267 respectively.

The strain I52 was isolated from a lepidopteran larva collected dead in Transvaal crop plantations in South Africa. The strain BM7 was isolated from contaminated artificial insect diet in which lepidopteran larvae had been maintained. In colony morphology and biochemical properties they are similar to HD-1. The biochemical properties of the strains are compared in Tables 1A and 1B.

Biochemical tests	HD-1 Thuricide	HD-1 Dipel	BM7	I52
Nitrate utilization i.e. Nitrate → Nitrite (NO_3)	+	+	+	+
Indole Production (TRP)	-	-	-	-
Acidification of glucose (Glu)	-	-	-	-
Arginine utilization (ADH) i.e. arginine dihydrolase	+	+	+	+
Urea utilization (URE) i.e. urease	+	+	+	+
Esculin hydrolysis (ESC) i.e. β-glucosidase	+	+	+	+
Gelatine hydrolysis (GEL) i.e. protease	+	+	+	+
p-Nitro-phenyl-β-D-galacto-pyranoside (PNPG) i.e. β-galactosidase	-	-	-	-
Glucose assimilation (GLU)	+	+	+	+
Arabinose assimilation (ARA)	-	-	-	-

TABLE 1A continued

Biochemical tests	HD-1 Thuricide	HD-1 DIPEL	BM7	I52
Mannose assimilation (MNE)	-	-	-	-
Mannitol assimilation (MAN)	-	-	-	-
N-acetyl glucosamine assim. (NAG)	+	+	+	+
Maltose assimilation (MAL)	+	+	+	+
Gluconate assimilation (GNT)	V	V	V	+
Caprate assimilation (CAP)	-	-	-	-
Adipate assimilation (ADI)	-	-	-	-
Malate assimilation (MLT)	+	+	+	+
Citrate assimilation (CIT)	-	-	-	-
Phenyl-acetate assimilation (PAC)	-	-	-	-
MRVP	+	+	+	+
Starch hydrolysis	+	+	+	+
Casein hydrolysis	-	-	-	-
Lactose assimilation	-	-	-	-
Lecithinase	+	+	+	+
Sucrose utilization	-	-	-	-
Salicin utilization	+	+	+	+

+ = positive; - = negative; V = variable.

TABLE 1B

Antibiotic Profiles Concent	HD-1	BM7	I52
1. Ampicillin 25	V	V	R
2. Chloramphenicol 50	S	S	S
3. Nitrofurantoin 200	S	S	S
4. Streptomycin 25	S	S	S
5. Colistin Sulphate 10	R	R	R
6. Nalidixic Acid 30	S	S	S
7. Rifampicin 2	S	S	S
8. Cephaloridin 25	S	S	S
9. Oxilinic Acid 2	S	S	S
10. Vancomycin 30	S	S	S
11. Lincomycin 15	S	S	S
12. Carbenocillin 100	V	V	V
13. Erythromycin 10	S	S	S
14. Sulphamethoxazole/ Trimethoprim 25	S	S	S
15. Sulphafurazole 500	S	S	S
16. Kanamycin 30	S	S	S
17. Gentamycin 10	S	S	S

S = sensitive; R = resistance; V = variable

Preliminary biochemical classification following Martin and Travers (Applied and Environ. Microbiol., 1989 : 2437-2442) based on the utilization of salicin and sucrose and the hydrolysis of esculin and lecithin, places the strains BM7 and I52 in the kurstaki subspecies.

These results were further confirmed by flagellar serotyping studies using antibodies from the Institute Pasteur. Strain HD-1 also belongs to the subspecies kurstaki.

Electrophoresis of plasmid preparations on agarose gels, showed that strains I52 and BM7 contain six to seven extrachromosomal DNA bands. Plasmid size ranged from approximately 6 Mda to 100 Mda.

Crystal preparations separated by sodium-dodecyl-sulphate (SDS) polyacylamide gel electrophoresis (PAGE) showed the protoxin band of approximately 133.4 KDa and a smaller protein of approximately 59.6 KDa.

Crystal protein genes were located in the plasmid screens using a gene-specific probe (a ^{32}P labelled PVU II fragment internal to the toxin gene from plasmid pES1, Schnepf H.E. and Whiteley H.R., Proc. Natl. Acad. Sci. 1981. 78 : 2893-2897), after transferring the DNA to nitrocellulose filters. It was determined that strains BM7 and I52 both contain the anti-lepidopteran genes CryIA(a) and CryIA(c). In I52 both genes are present in chromosomal and plasmid DNA. In BM7 both genes are present in chromosomal DNA, however plasmid DNA does not have the CryIA(a) gene.

Presence of the anti-coleopteran/anti-lepidopteran Cry V gene was detected on strain I52 in total DNA preparations (not determined in strain BM7)

The strains according to the invention may be cultured under suitable conditions in an appropriate medium. Such conditions and media are well known to the art. The media will, for example contain a nitrogen source (e.g. yeast extract, corn steep liquor), and a carbohydrate source such as glucose. Suitable conditions include temperatures in the range 25-30°C, and an approximately neutral pH (6,8 - 7,2). Typically fermentations are carried out for periods of 1-3 days.

The third aspect of the invention is a delta-endotoxin obtained from a culture of either of the two varieties, after the sporogenesis and the successive lysis of sporangium.

The fourth aspect of the invention is an insecticidal composition containing as its active ingredient this delta-endotoxin and the fifth aspect of the invention is an insecticidal composition containing the delta-endotoxin and the spores obtained after sporogenesis.

For example, insecticidal compositions according to the invention may be made by centrifuging or filtering the fermented media followed by spray drying the B.t. spores and crystals. Formulating agents can be incorporated to the B.t. mixture before or after spray drying. Useful formulating agents include for example wetting agents, stickers, dispersing agents, UV stabilizers, and carriers (e.g. silica, kaolin).

The sixth aspect of the invention is a method for controlling the insects, especially of the order Lepidoptera, at a locus, which includes the step of treating the locus to be protected either with an amount of a delta-endotoxin as described above or with an amount of a combination of a

delta-endotoxin and spores as described above. Preferably, the delta-endotoxin or the combination of delta-endotoxin and spores are applied as a composition. For example, the composition of the invention containing B.t. may be applied by spraying onto plants infested with, or liable to infestation, by lepidopteran insects.

Insects which are controlled by the process of the invention are of the Order Lepidoptera, for example those in Table 2 below.

TABLE 2

COMMON NAME	LATIN NAME
Cotton bollworm or American bollworm	Heliothis armigera
Sorghum stalk borer	Chilo partellus
Plusia looper	Chrysodeixis acuta
Potato tuber moth	Phthorimaea operculella
European corn borer	Ostrinia nubilalis
Fall army worm	Spodoptera frugiperda

Specific examples of commercially important plants to be protected by the invention are vegetables (such as tomatoes, beans, peas), citrus, deciduous fruits (such as apples, peaches, pears and plums) and cereals (mainly maize and sorghum).

The following examples illustrate the invention.

Example 1

Isolation of the B.t. Strains I52 and BM7

Strain I52 was isolated from a dead lepidopteran larva collected in the field. This larva was homogenized in 5ml of water using a pestle and mortar. Double serial dilutions of the homogenate were plated onto nutrient agar. The plates were incubated for 3-4 days at 26°C, after which time slides were prepared from B.t.-like colonies and viewed by phase contrast microscopy (at a magnification of 1000x using the oil immersion lens) for the presence of refractive bipyramidal shaped crystals. Crystal-positive colonies were streaked onto nutrient agar in order to ensure a pure culture, and incubated for 3 days at 26°C. Isolated colonies were re-examined for purity and for crystal formation. Purified colonies were transferred to nutrient agar slants and stored at 4°C. Purified cultures were also stored at -70°C. Strain BM7 was isolated from contaminated artificial insect diet, following the general procedure described above.

Example 2

Propagation of the B.t. strains according to the invention

a. Propagation in shake flasks

Inoculum of BM7 or I52 was transferred from a slant or from a culture stored at -70°C to a nutrient agar plate and incubated at 26-30°C for 18 to 24 hours. Six to ten Erlenmeyer flasks containing 50ml nutrient broth supplemented with 0,1M di-potassium hydrogen phosphate (K_2HPO_4) were inoculated with a loopful of bacterial culture from the plate and incubated at 26-30°C with agitation (150 rpm) for 3 to 5 days, until formation of spores and crystals occurred.

Cells, spores and crystals harvested by centrifugation (16300 x g, 4°C for 10 min) were washed once with 1M NaCℓ and three times with distilled water. Subsequently the preparations were freeze-dried.

b. Propagation in 14L fermentors

Fresh cultures on nutrient agar plates were prepared from stock cultures kept at -70°C. Nutrient broth (500ml) distributed into three 1L flasks was inoculated with cultures from the fresh plates, and incubated until logarithmic phase (12-16 h) at 26°C to 30°C with agitation (150 rpm). These cultures were pooled and used to inoculate a 10L working volume fermenter. Fermentations were carried out in fed batch mode using a protein and carbohydrate feed. A typical defined medium contained at the beginning of the fermentation for example: 5-10 g/l glucose; 10 g/l yeast extract; 10 g/l peptone; 4 g/l L-aspartate; 6,8 g/l KH_2PO_4; 8,7 g/l K_2HPO_4; 2 ml/l antifoam; and a salt mixture ($CaC1_2.2H_2O$ 0,13 g/l, $MgCl_2.6H_2O$ 0,1 g/l, $MnSO_4$ 0,.028 g/l, $ZnSO_4$ 0,005 g/l, $FeSO_4$ 0,001 g/l, $CuSO_4$ 0,001 g/l. The total feed consisted of approximately 47 g/l glucose and a peptone-yeast extract mixture containing 5 g/l of each.

An example of a typical medium using corn steep liquor as a raw source of protein contained: corn steep liquor at a protein concentration of 10 g/l (as determined using the Lowry et al method, (1951) J.Biol.Chem 193: 265-275); 5-10 g/l glucose; 6,8 g/l KH_2PO_4; 8,7 g/l K_2HPO_4; 4 g/l aspartate; 2 ml/l antifoam; and a further 47 g/l glucose added as a feed during the fermentation.

Fermentations were run at 30°C, pH 7,2, with an aeration of 1L/L/min and agitation of 600-800 rpm. p02 was kept at a minimum of 25% by sparging pure oxygen when necessary. Fermentations were generally

completed in between 18 to 24 hours.

Spores and crystals were harvested from the fermented medium by centrifugation or microfiltration to 20% of the original volume (cream) and lyophilized or spray dried.

Example 3

Toxicity Bioassays

Newly hatched larvae were individually placed in pill vials containing an artificial agar-based diet. After 3 days the larvae were transferred to new vials containing the diet supplemented with B.t. spores and crystals.

Bioassays were carried out mainly as described by Dulmage H.T., Boening O.P., Rehnborg C.S. and Hansen G.D. (J. Invertebr. Pathol. 18:240,1971). Freeze-dried spore/crystal preparations were suspended in bioassay buffer (0,85% NaCℓ; 0,6% K_2HPO_4 and 0.3% KH_2PO_4) at a 2mg/ml concentration. These suspensions were prepared with a freshly weighed batch of powder. The s/c suspensions were made homogeneous by placing them in a sonicating bath for 20 mins. Tween 20 (0,01%) was added to wet the B.t. suspensions when required.

The insect diet was then dispensed in 30ml aliquots into 50ml beakers, cooled and maintained at 55°C, until the s/c suspension was mixed in, using an Ultra Turrax homogenizer. The diet was supplemented with 5 to 7 different concentrations of toxin (ranging from 0 to 500μg of toxin per ml diet). The diet was mixed, poured into petri plates and allowed to solidify. The diet was then cut into 25 pieces with a scalpel blade, and each piece placed into a pill vial. One larva was added per vial. The vials were plugged with cotton wool and incubated for 7 days at 25°C, after which the

mortality in the test samples and untreated control was determined. The dose-mortality data was converted to probits and the LC_{50} values of the samples were compared with that of a standard preparation (HD-1-S-1980), assayed in parallel; to calculate potency values. Since the standard has an assigned potency in international units (IU) per mg (16000 IU/mg), the potency of strains BM7 and I52 can also be calculated (Beegle C.C. in the 198th National Meeting of the American Chemical Society, Hickle and Fitch (eds). 1990).

Potency values were calculated only for Heliothis armigera, percent mortality is indicated for the other insect species.

TABLE 3

Potency of B.t. isolates against Heliothis armigera

STRAIN	MEAN POTENCY IU/MG	CV
BM7	41892	0,1
I52	97896	0,04
HD-1-S-1980	16000	

Bioassays were carried out in triplicate at 5 to 7 different concentrations.

These results indicate that BM7 and I52 are 2,6 and 6 times more toxic than HD-1-S-1980, respectively.

TABLE 4

Mortality of Chilo partellus caused by BM7 and I52 spore crystal preparations

STRAIN	% Mortality at 120µg/ml	
	x	δ
BM7	75	2
I52	50,1	11,8
HD-1-S-1980	32	2,1

Both strains show a higher toxicity than the HD-1-S-1980 standard.

Mortality values were calculated from eight independent tests which were run with ten replicates each and three neonate larvae per replicate.

Table 5

Mortality of Ostrinia nubilalis (European corn borer) caused by BM7 and I52 spore/crystal preparations

STRAIN	% Mortality at 10µg/ml	
	x	δ
BM7	93	5,5
I52	83	17,9
HD-1	98,5	1,7

Mean values were calculated from eight independent tests which were run with ten replicates each and three neonate larvae per replicate.

Table 6

Mortality of Spodoptera frugiperda (Fall army worm) caused by BM7 and I52 spore/crystal preparations

STRAIN	% Mortality at 10µg/ml	
	x	δ
BM7	67,5	26
I52	80.0	28,5
HD-1	35,8	29

Mean values were calculated from eight independent tests which were run with ten replicates each and three neonate larvae per replicate.

Example 4

Heliothis Armigera Control on Tomatoes

A field trial using I52 freeze-dried spore/crystal preparations was conducted on tomatoes (Zest-table tomatoes) infested with bollworm and plusia looper in the Eastern Transvaal Lowveld. Treatments were arranged in a randomized block design with four replicates, each consisting of 5 x 2m rows. The crop was planted on 22 August 1992 and insecticide applications were done on 2, 9, 13, 19, 25 and 30 November and on 7, 14, 18 December 1992. All applications were done with a backpack sprayer with 2 x D4 hollow cone nozzles.

Comparisons were made with the commercially available product Dipel (Abbott Laboratories).

Bollworm and plusia looper damage was assessed by inspecting each fruit harvested at 6, 7 and 8 weeks after the first application.

TABLE 7

Bollworm Control on Tomatoes

TREATMENT	No of IU/ha	Mean % Fruit Harvested with Bollworm Damage		
		No of weeks after first application		
		6	7	8
Dipel (16000 IU/mg)	$0,8 \times 10^{10}$	12,3 AC	2,6 BC	6 CE
Dipel (16000 IU/mg)	$1,6 \times 10^{10}$	11,6 AC	5,7 B	7,7 BCD
I52 (16000 IU/mg)	$1,6 \times 10^{10}$	6,5 CDE	2,2 BC	5,4 CE
I52 (16000 IU/mg)	$3,2 \times 10^{10}$	2,4 E	2,4 BC	1,6 DEF
I52 (16000 IU/mg)	$6,3 \times 10^{10}$	2,4 E	2,4 BC	2,2 DEF
Untreated	-	18,4 A	15,7 A	15,6 AB
F-test Prob (%)		0,03	0,01	0
Std Error (Single plot)		5,8	5,4	6,3
Coeff of Var				
5% LSD		8,3	7,7	9,0

Means within column followed by the same letter are not significantly different.

All I52 treatments (at 6, 7 and 8 weeks after the first biopesticide application) resulted in significantly lower damage than the untreated control. Dipel treatments were not significantly different to the control at 6 weeks, but at 7 and 8 weeks control of bollworm was improved. I52 and Dipel gave similar control when compared at the same concentration ($1,6 \times 10^{10}$ IU/ha).

TABLE 8

Looper Control on Tomatoes

TREATMENT	No of IU/ha	Mean % Fruit Harvested with Looper Damage		
		No of weeks after first application		
		6	7	8
Dipel (16000 IU/mg)	$0,8 \times 10^{10}$	10,0 AB	6,4 AE	1,7 CD
Dipel (16000 IU/mg)	$1,6 \times 10^{10}$	0,6 CD	0,3 G	2,0 CD
I52 (16000 IU/mg)	$1,6 \times 10^{10}$	0,8 CD	3,6 CDEG	2,8 C
I52 (16000 IU/mg)	$3,2 \times 10^{10}$	0,1 D	0,6 FG	0 D
I52 (16000 IU/mg)	$6,3 \times 10^{10}$	1,5 CD	0,2 G	0,2 CD
Untreated	-	16,1 A	14,7 AB	12,2 AB
F-test Prob (%)		0	0	0
Std Error (Single plot)		6,9	6,9	6,6
Coeff of Var				
5% LSD		9,8	9,8	9,4

Means within column followed by the same letter are not significantly different.

All I52 treatments and Dipel at 1,6 x 10^{10} IU/ha resulted in significantly lower damage than the untreated control. I52 and Dipel gave similar control when compared at the same concentration (1,6 x 10^{10} IU.ha).

Example 5

Insectidical Composition

Upon completion of the fermentation cycle spores and crystals of Bacillus thuringiensis ATCC 55266 or ATCC 55267 were harvested from the fermented medium as described in Example 2. Wettable powder formulations were prepared by adding to the cream 0,5 to 5% wetting and dispersing agents (such as Goulac which is a mixture of lignosulphonate, and Empicol L2, which is sodium lauryl sulphate), and a carrier (aluminium silicate hydroxide), the amount of carrier depending on the potency of the technical concentrate and the desired potency of the wettable powder. The mixture was then dried. Oil suspension concentrate formulations were prepared by mixing at high speed in a Silverson homogenizer spray-dried Bt solids (Bt sprayed-dried together with hydrophobic silica) with an emulsifier (such as Emulsogen LP) and sunflower oil. This suspension concentrate was then milled with 2mm beads in a Dyno mill.

CLAIMS

1.
Bacillus thuringiensis ATCC 55266 or a biologically pure culture thereof.

2.
Bacillus thuringiensis ATCC 55267 or a biologically pure culture thereof.

3.
A delta-endotoxin obtained from a culture of Bacillus thuringiensis ATCC 55266 after sporogenesis.

4.
A delta-endotoxin obtained from a culture of Bacillus thuringiensis ATCC 55267 after sporogenesis.

5.
An insecticidal composition containing as an active ingredient a delta-endotoxin according to claim 3.

6.
An insecticidal composition containing as an active ingredient a delta-endotoxin according to claim 4.

7.
An insecticidal composition containing as an active ingredient a combination of a delta-endotoxin according to claim 3 and the spores obtained from a culture of Bacillus thuringiensis ATCC 55266 after sporogenesis.

8.

An insecticidal composition containing as an active ingredient a combination of a delta-endotoxin according to claim 4 and the spores obtained from a culture of Bacillus thuringiensis ATCC 55267 after sporogenesis.

9.

A method for controlling insects at a locus which includes the step of treating the locus to be protected with an amount of a delta-endotoxin according to claim 3 or claim 4 or a combination of a delta-endotoxin and the spores according to claim 7 or claim 8.

10.

A method according to claim 9 wherein the insects are of the order Lepidoptera.

DATED THIS 19th DAY OF April 1993

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ABSTRACT

This invention relates to two new varieties of Bacillus thuringiensis, viz Bacillus thuringiensis ATCC 55266 and Bacillus thuringiensis ATCC 55267, to delta-endotoxins obtained from these varieties, to insecticidal compositions containing the delta-endotoxins, optionally in combination with spores of the varieties, and to the control of insects, especially of the Order Lepidoptera, using the delta-endotoxins and the combination of delta-endotoxins with spores.