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Nematicidal activity of *Bacillus thuringiensis* isolates

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Summary – The nematicidal activity of the spore-crystal mixtures of three *Bacillus thuringiensis* isolates against hatched juveniles and adults of *Caenorhabditis elegans* was investigated. Toxicity was determined by adding 50-µl aliquots of the spore-crystal mixtures to microtitre plate wells containing 50-µl aqueous suspensions of 200-400 hatched juveniles and adults of *C. elegans*. Nematode mortality was observed from 8 hours incubation onwards; after 24 hours incubation no more significant increases in nematode mortality occurred. Nematode mortality varied from about 50 to 60 % when the nematicidal activity was tested in distilled water and was usually somewhat higher (but less than 10 %) when tested in axenic medium. Toxicity varied between the three isolates. Concentrations of at least 10^8 particles/ml were necessary to cause a nematode mortality higher than 30 %. Nematicidal activity was only observed when spore-crystal mixtures from at least 2-day-old cultures, consisting of about 50 % of vegetative cells, often containing a spore, and for about 50 % of a mixture of spores and crystals, were used. Heating to 75 °C and higher for 24 hours and autoclaving at 120 °C for 20 min destroyed the nematicidal activity of all three isolates. Differences in stability of the nematicidal activity were observed between the three isolates. In two isolates the nematicidal activity did not decline after storage at 28 °C for 15 days; in the third isolate the nematicidal activity declined after storage at 28 °C for 7 days. Multiple freezing at – 20 °C or – 70 °C and thawing had no effect on the nematicidal activity of two isolates but decreased the nematicidal activity of the third isolate. pH changes resulted in differences in stability of the nematicidal activity between the three isolates. These results may indicate the presence of different toxins.

Résumé – *Activité nématicide de trois isolats de* Bacillus thuringiensis – L'activité nématicide de mélanges de spores et d'inclusions cristallines de trois isolats de *Bacillus thuringiensis* envers les juvéniles et les adultes de *Caenorhabditis elegans* est étudiée. La toxicité est déterminée en ajoutant 50 µl d'un mélange de spores et d'inclusions cristallines à 50 µl d'une suspension de 200 à 400 juvéniles et adultes de *C. elegans*. La mortalité des nématodes est observée après 8 heures d'incubation; aucune augmentation significative du pourcentage de mortalité n'est observée après 24 heures d'incubation. Ce pourcentage varie d'environ 50 à 60 % lorsque les tests sont réalisés avec de l'eau distillée et il augmente légèrement (moins de 10 %) si les tests sont effectués en milieu axénique. La toxicité varie selon les isolats. Un minimum de 10^8 particules/ml est nécessaire pour provoquer un effet létal supérieur à 30 %. L'activité nématicide n'est observée qu'avec des mélanges de spores et d'inclusions cristallines provenant de cultures âgées d'au moins 2 jours et constituées d'environ 50 % de cellules végétatives – contenant souvent une spore – et d'environ 50 % d'une mixture de spores et d'inclusions cristallines. Le chauffage à 75 °C ou plus pendant 24 heures ou un autoclavage à 120 °C pendant 20 minutes détruit l'activité nématicide des trois isolats. Des différences de stabilité de l'activité nématicide sont observées entre les trois isolats. Celle-ci ne diminue pas pour deux isolats maintenus à 28 °C durant quinze jours, mais elle diminue pour le troisième s'il est stocké sept jours à 28 °C. De même, l'activité nématicide de deux des isolats n'est pas modifiée après plusieurs congélations à – 20 °C ou – 70 °C suivies de décongélations, mais elle diminue pour le troisième isolat. Les variations du pH des mélanges de particules entraînent des variations dans la stabilité des activités nématicides des trois isolats. Ces résultats pourraient indiquer la présence de toxines différentes.

Key-words : *Caenorhabditis elegans, Bacillus thuringiensis,* toxicity.

Bacillus thuringiensis Berliner is a bacterium which produces several insecticidal metabolites (Lüthy *et al.*, 1985). The α-exotoxin is a protein which is toxic upon injection into insects and mice (Krieg, 1971). The β-exotoxin, also called thuringiensin, is a nucleotide secreted by the vegetative cells of several isolates. It is active not only against invertebrates but also against vertebrates (Sebesta *et al.*, 1981). However, the δ-endotoxins or insecticidal crystal proteins have made *B. thuringiensis* known worldwide as a biological control agent (Lambert & Peferoen, 1992). These proteins are produced within the cytoplasm during sporulation. Today, a wide range of biopesticides based on the δ-endotoxins are available for the control of agricultural insect pests. Also, insect-resistant transgenic tobacco, tomato, potato, cotton and maize plants have been developed by transfer of the genes coding for the insecticidal crystal proteins from *B. thuringiensis* into the plant genomes. So

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far, the nucleotide sequences of 24 different genes have been described each encoding for proteins with a very specific insecticidal activity. Based on their spectrum of insecticidal activity the crystal proteins are classified in four classes : *Cry* I (active against Lepidoptera), *Cry* II (Lepidoptera and Diptera), *Cry* III (Coleoptera) and *Cry* IV (Diptera). Recently, two new classes of nematode-active crystal proteins, *Cry* V and *Cry* VI, were added (Feitelson *et al.,* 1992).

Nematostatic or nematicidal effects of the β-exotoxin on free-living (*Panagrellus redivivus* and *Aphelenchus avenae*), plant-parasitic (*Meloidogyne incognita* and *Heterodera glycines*) and zoo-parasitic (*Trichostrongylus colubriformis* and *Nippostrongylus brasiliensis*) nematodes have been reported (Gevrey & Euzeby, 1966; Prasad *et al.,* 1972; Ignoffo & Dropkin, 1977; Bone, 1989; Noel, 1990).

Nematicidal effects of several commercial *B. thuringiensis* preparations on plant-parasitic (*Meloidogyne javanica* and *Tylenchulus semipenetrans*) and zoo-parasitic (*T. colubriformis* and *N. brasiliensis*) nematodes have also been reported (Osman *et al.,* 1988; Bone, 1989). These preparations only contain δ-endotoxins. Recently, in a series of patent applications by Mycogen Corporation, San Diego, U.S.A., several δ-endotoxins with nematicidal activity against juveniles and adults of *Caenorhabditis elegans, P. redivivus* and *Pratylenchus* spp. were claimed (see e.g. Edwards *et al.,* 1989; Narva *et al.,* 1991).

Nematicidal activity of natural strains of *B. thuringiensis* was mainly observed against the eggs and, exceptionally, the first three juvenile stages of zoo-parasitic nematodes, including *T. colubriformis, N. brasiliensis, Ancylostoma caninum, Haemonchus contortus, Cooperia punctata, Cooperia oncophora* and *Ostertagia ostertagi* (Ciordia & Bizzell, 1961; Bottjer *et al.*, 1985; Meadows *et al.,* 1989 *a, b*) and two free-living nematodes, *Caenorhabditis briggsae* and *Turbatrix aceti* (Bottjer *et al.,* 1985; Meadows *et al.,* 1990).

The susceptibility of nematode eggs and juveniles to the *B. thuringiensis* toxins (Bottjer *et al.,* 1985; Meadows *et al.,* 1990), the effect of the toxins on the morphology of the nematode egg-shell and juvenile (Bone *et al.,* 1985, 1987; Bottjer & Bone, 1987; Wharton & Bone, 1989) and several factors influencing the activity of the toxins (Bottjer *et al.,* 1985; Bone *et al.,* 1985, 1987; Bone & Coles, 1987; Bone *et al.,* 1988; Meadows *et al.,* 1989 *a, b,* 1990) were studied. A fraction with ovicidal activity against eggs of *T. colubriformis* was isolated from crystals of *B. thuringiensis israelensis* (Bone *et al.,* 1986).

Screening of *B. thuringiensis* isolates from the PGS collection for nematicidal activity on juveniles and adults of *C. elegans,* a bacteriophagous nematode, resulted in the identification of two nematicidal isolates. In the present study, the effects of incubation time, particle concentration, bacterial culture age, temperature, pH and multiple freezing and thawing on the nematicidal activity of the spore-crystal mixtures of the nematicidal *B. thuringiensis* isolates were investigated.

Materials and methods

Preparation of spore-crystal mixtures

Bacillus thuringiensis isolates, obtained from the PGS collection stored in 25 % glycerol at – 70 °C, were grown on 400 ml CBI (Culturing Bacillus Isolates) medium in 2-1 Erlenmeyer flasks on a rotary shaker (100 rpm) at 28 °C for 5 to 7 days. Composition of the CBI medium : bacto-peptone 7.5 g; glucose 1 g; KH_2PO_4 3.4 g; K_2HPO_4 4.35 g; distilled water to 1 l. After adjustment to pH 7.2 and sterilization at 120 °C for 20 min, two salts solutions were added : $MgSO_4 \cdot 7H_2O$ 2.46 g; $MnSO_4 \cdot H_2O$ 0.04 g; $ZnSO_4 \cdot 7H_2O$ 0.28 g; $FeSO_4 \cdot 7H_2O$ 0.4 g; distilled water to 100 ml and $CaCl_2 \cdot 2H_2O$ 3.66 g; distilled water to 100 ml. The two salts solutions were first filter-sterilized (0.2 μm). Upon lysis, vegetative cells, spores and crystals were harvested by centrifugation (3000 rpm) for 15 min. The pelleted particles, mainly consisting of spores and crystals, were resuspended in PBS (NaCl 8 g; KCl 0.2 g; Na_2HPO_4 1.15 g; KH_2HPO_4 0.2 g; distilled water to 1 l; pH 7-8) and stored in concentrations of $2\ 10^9$ particles/ml at – 20 °C until use.

Nematicidal bio-assay

Nematicidal activity of the spore-crystal mixtures was determined by adding 50-μl aliquots of the mixtures to microtitre plate wells containing 50-μl aqueous suspensions (distilled water) of 200-400 juveniles and adults of *Caenorhabditis elegans.* Control wells consisted of 50-μl aqueous nematode suspensions supplemented with 50 μl PBS. Nematodes were obtained from axenic cultures on soy-peptone and yeast extract supplemented with haemoglobin. The axenic cultures were kept at 20 °C and subcultured weekly. The nematode suspensions also contained streptomycin (30 μg/ml) and chloramphenicol (30 μg/ml) to prevent bacterial growth. The microtitre plates were incubated at 28 °C for 24 h to determine the nematicidal activity. Nematode mortality was expressed as the mean percentage of dead *versus* live nematodes after substraction of the nematode mortality observed in the control wells. Each treatment was replicated three times.

For all experiments, the procedures described above were used, except when mentioned otherwise.

Firstly, spore-crystal mixtures of 128 *B. thuringiensis* isolates were tested for their nematicidal effect on juveniles and adults of *C. elegans.* The isolates were distributed over seven batches each consisting of fifteen to twenty isolates.

Effect of incubation time on nematicidal activity

The effect of incubation time on the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A and 958B and the nematicidal isolate NRRL repository No. B-18247 (obtained from Northern Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. – abbreviated as isolate 18247 further in the text; Barnes & Edwards, 1989) was determined. In a first experiment, the nematicidal bioassay was carried out as described above and nematode mortality determined after 1, 2, 4, 8, 16, 24, 32 and 48 hours. In a second experiment, 50-µl aliquots of the mixtures were added to microtitre plate wells containing juvenile and adult nematodes in either 50 µl distilled water or axenic culture medium. The microtitre plates were incubated at 28 °C and nematode mortality determined after 1, 3 and 6 days.

Effect of particle concentration on nematicidal activity

The effect of particle concentration on the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A, 958B and 18247 was evaluated by dose-response analysis. In a first experiment, 50-µl aliquots of the mixtures at a concentration of 10^7, 10^8, 2.5 10^8, 5 10^8, 7.5 10^8, 10^9, 1.5 to 2.5 10^9 (isolate 289A : 1.5 10^9; isolate 958B : 2 10^9; isolate 18247 : 2.5 10^9) or 10^{10} particles/ml were added to microtitre plate wells containing juvenile and adult nematodes in 50 µl distilled water. The microtitre plates were incubated at 28 °C and nematode mortality determined after 24 hours. In a second experiment, 50-µl aliquots of the mixtures at a concentration of 10^7 or 10^9 particles/ml were added to microtitre plate wells containing juvenile and adult nematodes in 50 µl axenic culture medium. The microtitre plates were incubated at 28 °C and nematode mortality determined after 1, 3 and 7 days.

Effect of bacterial culture age on nematicidal activity

The presence of the nematicidal activity during vegetative growth and sporulation of the *B. thuringiensis* isolates 289A, 958B and 18247 was investigated. Vegetative cells, spores and crystals were harvested daily during 9 days. The nematicidal activity of the spore-crystal mixtures was determined after incubation at 28 °C for 24 h. The appearance of vegetative cells, spores and crystals in the cultures was observed by phase-contrast microscopy.

Effect of storage time at 28 °C on nematicidal activity

The stability of the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A, 958B and 18247 at an ambient temperature of 28 °C was investigated. The mixtures were first treated with the enzyme inhibitor phenylmethylsulfonylfluoride (PMSF) at a final concentration of 0.1 mM and stored again at – 20 °C. After thawing, the mixtures were incubated at 28 °C for 0, 2, 5, 7, 9, 12 or 15 days. The nematicidal activity of the spore-crystal mixtures was determined after incubation at 28 °C for 24 h. As an additional control, juvenile and adult nematodes were also exposed to spore-crystal mixtures which were not pretreated with PMSF, not stored and only frozen once.

Effect of temperature on nematicidal activity

The effect of temperature on the stability of the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A, 958B and 18247 was investigated. The mixtures were first incubated at 4, 10, 19, 28, 37, 46, 60, 75 or 95 °C for 24 h or autoclaved at 120 °C for 20 min. After cooling to room temperature, the nematicidal activity of the spore-crystal mixtures was determined after incubation at 28 °C for 24 h. As an additional control, juveniles and adult nematodes were also exposed to untreated spore-crystal mixtures.

Effect of pH on nematicidal activity

The effect of pH on the stability of the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A, 958B and 18247 was evaluated. 250-µl aliquots of the isolates 289A, 958B and 18247 containing vegetative cells, spores and crystals were harvested by centrifugation (13 000 rpm) for 5 min. In a first experiment, the pelleted particles of all three isolates, mainly consisting of spores and crystals, were resuspended in 500 µl-aliquots of 20 mM citrate buffer (pH 2.5, 3, 4 or 5), 20 mM Tris-HCl buffer (pH 6, 7, 8 or 9) or 20 mM carbonate buffer (pH 10) and incubated at 4 °C for 3 days. After centrifugation (13 000 rpm) for 5 min, the pellets were first washed twice in 500 µl PBS and resuspended in 250 µl PBS. 50-µl aliquots of the mixtures were added to microtitre plate wells containing juvenile and adult nematodes in 50 µl axenic culture medium and the nematicidal activity determined after incubation at 28 °C for 24 h. As an additional control, the juvenile and adult nematodes were also exposed to spore-crystal mixtures incubated first in PBS at 4 °C for 3 days and to untreated spore-crystal mixtures. In the second experiment, the pelleted particles of the isolates 289A and 18247 were resuspended in 500 µl-aliquots of 20 mM citrate buffer (pH 2.5 or 5), with or without 0.1 mM PMSF, or 20 mM Tris-HCl buffer (pH 7 or 9), with or without 0.1 mM PMSF, and incubated at 4 °C for 3 days. After centrifugation (13 000 rpm) for 5 min., the pellets were first washed twice in 500 µl PBS and resuspended in 250 µl PBS. 50-µl aliquots of the mixtures were added to microtitre plate wells containing juvenile and adult nematodes in 50 µl axenic culture medium and the nematicidal activity determined after incubation at 28 °C for 24 h. As an

additional control, the juvenile and adult nematodes were exposed to untreated spore-crystal mixtures. In both experiments, the *B. thuringiensis* isolate 302AE, which shows no nematicidal activity against juveniles and adults of *C. elegans*, was also included as an additional control.

Effect of multiple freezing and thawing on nematicidal activity

The effect of multiple freezing and thawing on the stability of the nematicidal activity of the spore-crystal mixtures of the *B. thuringiensis* isolates 289A, 958B and 18247 was determined. The mixtures were thawed during 15 min. in a water bath at 28 °C and then frozen again at either – 20 °C or – 70 °C. This procedure was repeated 2, 4, 6, 8, or 10 times. After thawing, the nematicidal activity of the spore-crystal mixtures was determined after incubation at 28 °C for 24 h. As an additional control, juvenile and adult nematodes were also exposed to spore-crystal mixtures which were only once frozen and thawed.

Results

Nematicidal bio-assay

109 (85.2 %) of the 128 *B. thuringiensis* isolates tested caused no or less than 10 % mortality of the juveniles and adults of *C. elegans* while the spore-crystal mixtures of sixteen (12.5 %) isolates caused between 10 and 20 % mortality. Exposure to the isolates 289A, 18247 and 958B resulted in 21, 47 and 59 % mortality, respectively.

Effect of incubation time on nematicidal activity

In the first experiment, no mortality was observed during the first 4 h. Between 8 and 24 h, the mortality caused by isolate 18247 increased from 27 to 57 % while between 16 and 24 h, the mortality caused by the isolates 289A and 958B increased from 28 to 50 % and from 34 to 53 %, respectively. From 24 h onwards, no more significant increases in mortality were observed. In the second experiment, the mortality caused by the isolates 289A, 18247 and 958B increased between 1 and 6 days from 41 to 62 %, from 57 to 62 % and from 58 to 61 %, respectively, when tested in distilled water and from 44 to 59 %, from 64 to 71 % and from 59 to 68 %, respectively, when tested in axenic culture medium. The increase in mortality in both distilled water and axenic culture medium was higher in isolate 289A compared with the isolates 18247 and 958B, about 15-20 *vs* 6-7 % and 3-9 %, respectively. For all isolates, the mortality was usually somewhat higher (but less than 10 %) in axenic culture medium compared with distilled water. In the control wells containing either distilled water or axenic culture medium, the nematode mortality increased between 1 and 6 days from 10 to 28 % and from 5 to 10 %, respectively.

Effect of particle concentration on nematicidal activity

In the first experiment, no mortality was observed at a concentration of 10^7 particles/ml. At a concentration of 10^8 particles/ml, the mortality caused by isolate 958B was 30 %. Concentrations of 5 10^8 and 7.5 10^8 particles/ml of the isolates 18247 and 289A, respectively, were necessary to cause a mortality higher than 30 %. In the second experiment, all isolates caused less than 15 % mortality after 1 or 3 days at a concentration of 10^7 particles/ml. At a concentration of 10^9 particles/ml, the mortality was 45, 46 and 53 % for the isolates 18247, 289A and 958B, respectively, after 1 day. After 7 days, the mortality caused by the isolates 18247, 289A and 958B at a concentration of 10^7 particles/ml has increased to 9, 17 and 26 %, respectively, compared with 55, 67 and 78 %, respectively, at a concentration of 10^9 particles/ml.

Effect of bacterial culture age on nematicidal activity

After 1 day, all isolates caused no or less than 10 % mortality. At that time, the cultures consisted only of vegetative cells. 25 % of the cells of isolate 289A contained a spore. After 2 days, exposure to the isolates 289A, 958B and 18247 resulted in 39, 51 and 66 % mortality, respectively. At that time, the cultures consisted for about 50 % of vegetative cells, often containing a spore, and for about 50 % of a mixture of spores and crystals. From 2 days onwards, no more significant increases in mortality were observed.

Effect of storage time at 28 °C on nematicidal activity

The nematicidal activity of the isolates 18247 and 958B did not decline after storage at 28 °C for 15 days (Fig. 1). The mortality caused by the additional control mixtures was 56 % for both isolates 18247 and 958B. The nematicidal activity of isolate 289A declined from 30 to 5 % after storage at 28 °C for 7 days. The mortality caused by the additional control mixture of isolate 289A was 42 %.

Effect of temperature on nematicidal activity

Heating from 4 °C to 46 °C for 24 h had no effect on the nematicidal activity of any of the three isolates (Fig. 2). Heating to 75 °C and higher for 24 h or autoclaving at 120 °C for 20 min destroyed the nematicidal activity of all three isolates. The nematicidal activity of the isolates 958B and 289A was lost between 46 °C and 60 °C. In isolate 18247, the nematicidal activity was lost between 60 °C and 75 °C. Untreated spore-crystal mixtures of the isolates 289A, 958B and 18247 caused 34, 67 and 67 % mortality, respectively. Spore-crystal mixtures of isolate 289A, incubated from 4 °C to 37 °C

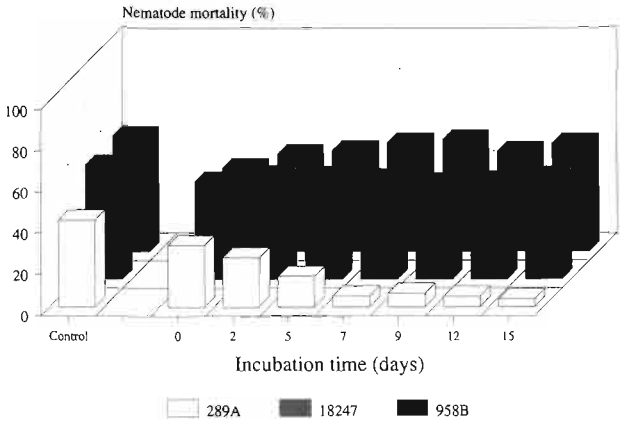

Fig. 1. *Stability of the nematicidal activity of the* Bacillus thuringiensis *isolates 289A, 958B and 18247 against hatched juveniles and adults of* Caenorhabditis elegans *after different storage times at 28 °C. Nematode mortality measured at 28 °C after 24 h incubation.*

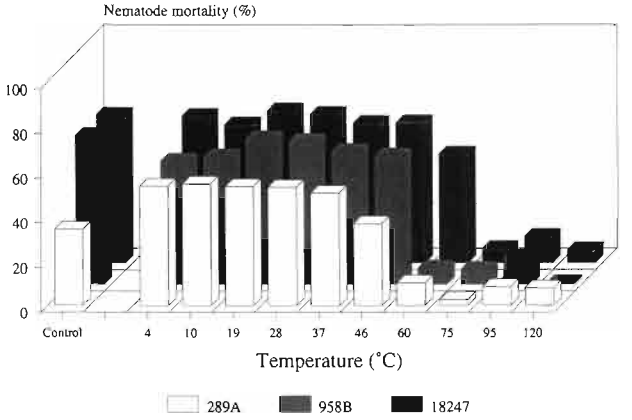

Fig. 2. *Effect of temperature on the stability of the nematicidal activity of the* Bacillus thuringiensis *isolates 289A, 958B and 18247 against hatched juveniles and adults of* Caenorhabditis elegans. *Nematode mortality measured at 28 °C after 24 hours incubation.*

caused 16-21 % higher mortality compared with the untreated mixtures.

Effect of pH on nematicidal activity

In the first experiment, the nematicidal activity of isolate 18247 remained unaffected after incubation in Tris buffer at pH 6 to 9 (Fig. 3). Incubation in citrate buffer at pH 2.5 to 5 and in carbonate buffer at pH 10 caused a 20 to 43 % and 50 % decrease, respectively, in mortality compared with the untreated spore-crystal mixtures. The nematicidal activity of isolate 289A decreased with increasing pH. From pH 7 onwards, isolate 289A no longer caused any mortality. The nematicidal

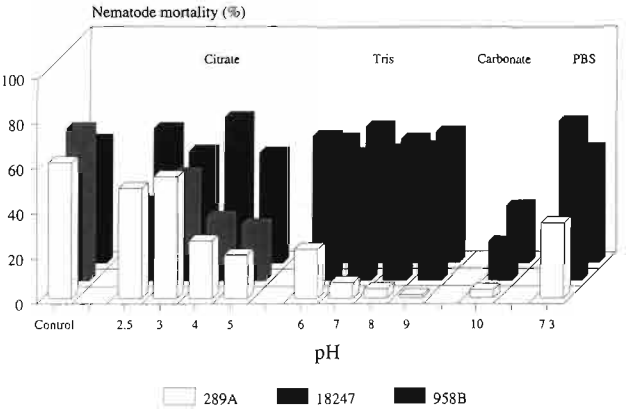

Fig. 3. *Effect of pH on the nematicidal activity of the* Bacillus thuringiensis *isolates 289A, 958B and 18247 against hatched juveniles and adults of* Caenorhabditis elegans. *Nematode mortality measured at 28 °C after 24 h incubation.*

activity of isolate 958B was not affected after incubation in the citrate and Tris buffers at pH 2.5 to 9. Only incubation in carbonate buffer at pH 10 resulted in a 29 % decrease in mortality compared with the untreated spore-crystal mixture. Incubation of the isolates 18247 and 958B in PBS remained without effect on their nematicidal activity while incubation of isolate 289A in PBS resulted in a 27 % decrease in mortality compared with the untreated mixture. In the second experiment, incubation of isolate 18247 in citrate buffer at pH 2.5 and 5 resulted in about 20 % loss of mortality compared with the untreated spore-crystal mixtures (Fig. 4). Addition of PMSF prevented the loss of mortality at pH 2.5 but not at pH 5. Mortality caused by isolate 18247 was not affected after incubation in Tris buffer at pH 7 and 9. Incubation of isolate 289A in the buffers caused a

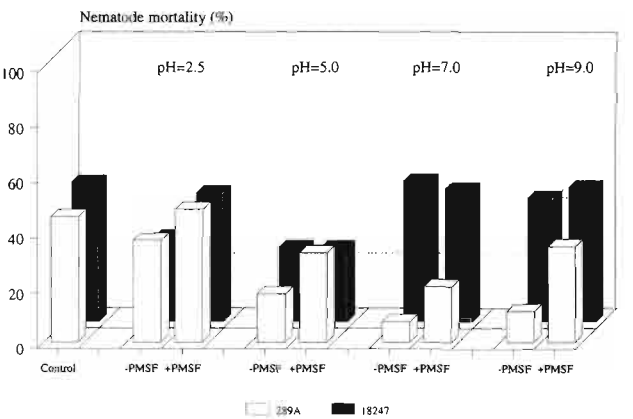

Fig. 4. *Effect of pH on the nematicidal activity of the* Bacillus thuringiensis *isolates 289A and 18247 against hatched juveniles and adults of* Caenorhabditis elegans. *Spore-crystal mixtures were resuspended in the buffers with or without PMSF. Nematode mortality measured at 28 °C after 24 h incubation.*

decrease in mortality with increasing pH. Addition of PMSF prevented the loss of mortality at all pH levels. In both experiments, isolate 302AE caused no mortality after incubation of the spore-crystal mixtures in the buffers at all the pH levels tested.

Effect of multiple freezing and thawing on nematicidal activity

Multiple freezing at – 20 °C or – 70 °C and thawing had no effect on the nematicidal activity of the isolates 289A and 958B but decreased the nematicidal activity of isolate 18247. After ten times freezing, either at – 20 °C or – 70 °C, and thawing, the mortality caused by isolate 18247 was 22 and 25 %, respectively, compared with 44 and 63 %, respectively, after one time freezing and thawing.

Discussion

Our data confirm the existence of *B. thuringiensis* isolates with nematicidal activity against hatched juvenile and adult nematodes. Previously, nematicidal activity of *B. thuringiensis israelensis* against 1st-stage juveniles of *T. colubriformis* that had hatched from their egg-shells (Bone *et al.*, 1988), of *B. thuringiensis kurstaki* against 1st-, 2nd- and 3rd- stage juveniles of *T. colubriformis* (Meadows *et al.*, 1989 *a*) and of *B. thuringiensis israelensis, B. t. kurstaki* and *B. t. morrisoni* against juveniles and adults of *Turbatrix aceti* (Meadows *et al.*, 1990) has been reported.

The frequency of occurrence of *B. thuringiensis* isolates with nematicidal activity against hatched juveniles and adults of *C. elegans* was low. Apparently, *B. thuringiensis* isolates with ovicidal activity occur more frequently : all 30 *B. thuringiensis* isolates tested by Bottjer *et al.* (1985) showed ovicidal activity against eggs of *T. colubriformis*.

In our experiments, a differential susceptibility between different nematode stages for the nematicidal activity of *B. thuringiensis* isolates was observed : only hatched juvenile and adult nematodes were killed; no ovicidal activity was observed (data not presented). A similar differential susceptibility was also reported by Bottjer *et al.* (1985) who observed activity of *B. t. israelensis* against the eggs of *T. colubriformus* and *N. brasiliensis* but not against the 3rd-stage juvenile and adult nematodes. In contrast, no such differential susceptibility was observed by Bone *et al.* (1988) and Meadows *et al.* (1989 *a*) who reported that *B. t. israelensis, B. t. kurstaki* and *B. t. morrisoni* were lethal for as well the eggs as the 1st-, 2nd- and 3rd-stage juveniles of *T. colubriformis*. The ovicidal activity of *B. t. israelensis, B. t. kurstaki* and *B. t. morrisoni* against *T. aceti* was not tested (Meadows *et al.*, 1990).

The presence of the nematicidal activity during vegetative growth and sporulation of *B. t. israelensis* against eggs of *T. colubriformis* (the 1st-stage juveniles were not included in this test) was also investigated by Bone *et al.* (1988) but only from day two onwards. As in our experiments, the nematicidal activity remained similar during the 7 days of culturing.

In our experiments, the antibiotics streptomycin and chloramphenicol had no effect on the nematicidal activity of the *B. thuringiensis* isolates tested. In contrast, the same antibiotics reduced or eliminated the ovicidal activity of, respectively, the *B. t. israelensis* and the *B. t. kurstaki* toxins (Bone *et al.*, 1988; Meadows *et al.*, 1989 *a*).

Analysis of the effects of the different conditions tested on the nematicidal activity of the three *B. thuringiensis* isolates revealed differences between the isolates which may indicate the presence of different toxins. Usually, the hatched juvenile and adult nematodes were more susceptible to the nematicidal activity of the *B. thuringiensis* isolates 18247 and 958B compared with isolate 289A. A similar differential susceptibility within a nematode species was also reported by Meadows *et al.* (1990) who observed that the population growth of *T. aceti* was reduced more by toxin(s) from *B. t. israelensis* compared with those from *B. t. kurstaki* and *B. t. morrisoni*. Also, compared with the other *B. thuringiensis* isolates tested, the nematicidal activity of isolate 18247 acted somewhat faster while the nematicidal activity of isolate 958B occurred at slightly lower particle concentrations. More obvious, however, were the observed differences in stability of the nematicidal activity between the *B. thuringiensis* isolates tested : the nematicidal activity of isolate 18247 was on the one hand the least sensitive to heating but on the other hand the only one which was decreased following multiple freezing and thawing; only the nematicidal activity of isolate 289A was not stable after storage at 24 °C for 7 days. Finally, pH changes resulted in differences in stability of the nematicidal activity between all *B. thuringiensis* isolates tested. Effects of temperature and multiple freezing and thawing on the stability of the nematicidal activity of *B. t. kurstaki* against 1st-, 2nd- and 3rd-stage juveniles of *T. colubriformis* were reported by Meadows *et al.* (1989 *a*). As in our experiments, autoclavation destroyed the nematicidal activity of *B. t. kurstaki* while it remained unchanged when frozen at 0 °C for 3 months but was lost when held at 22 °C for 2 weeks. The effect of temperature on the stability of the nematicidal activity of *B. t. israelensis* against 1st-stage juveniles of *T. colubriformis* is unclear : heating at 100 °C for 1 h only slightly reduced the toxicity while autoclaving destroyed it (Bone *et al.*, 1988). Loss of the nematicidal activity of *B. t. israelensis* against juveniles and adults of *T. aceti* when held at 22-24 °C for 10 days was reported by Meadows *et al.* (1990).

In our experiments, the mortality levels of juvenile and adult *C. elegans* increased with increased particle concentrations while the highest mortality levels were obtained within 24 hours. Similar results were reported on the nematicidal activity of *B.t. israelensis* against 1st-stage juveniles of *T. colubriformis* (Bone *et al.*, 1988).

However, in our experiments, the nematode mortality never reached 100 %. In contrast, all 1st-stage juveniles of *T. colubriformis* were killed when exposed to the highest toxin levels produced by *B. t. israelensis*. Preliminary observations indicate a differential susceptibility between the various juvenile and adult stages of *C. elegans* for the nematicidal activity of the *B. thuringiensis* isolates. The mortality levels of juvenile and adult *T. aceti* also increased with increased toxin levels produced by *B. t. israelensis* but the highest mortality levels were observed after 10 days of exposure (Meadows *et al.*, 1990). In contrast with *C. elegans* and *T. colubriformis*, only a decrease in population growth of *T. aceti* was observed, not a reduction of the population.

Since in our experiments mixtures of spores and crystals were used, the source of the nematicidal activity is unknown. In most previous studies, crystal-rich fractions were used and the observed ovicidal activity was attributed to a crystal toxin (Bottjer *et al.*, 1985; Bone *et al.*, 1985; Meadows *et al.*, 1989 *a*, 1990). Although in those studies the role of a δ-endotoxin could not be confirmed, δ-endotoxins are considered to be the origin of the nematicidal activity (Narva *et al.*, 1991).

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