Vol. 21, No. 2, pp. 169-180 International Journal of Nematology December, 2011

Screening of the nematicidal activity and sub-lethal effects of the bacterium *Bacillus thuringiensis* on the model nematode *Caenorhabditis elegans*

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Abstract. The nematicidal activity and two sub-lethal effects of 90 *Bacillus thuringiensis* Berliner strains from 83 serovars were assayed on *Caenorhabditis elegans* (Rhabditidae) 1st stage juveniles. The sub-lethal effects studied were (1) inhibition of the development of *C. elegans* 1st stage juveniles into adults, and (2) inhibition in the production of progeny after 3 days. Three different sets of β-exotoxin-minus *B. thuringiensis* preparations were tested for each strain: (1) whole-culture extracts, (2) alkali-solubilised cultures and (3) alkali-solubilised trypsin-digested cultures. Whole-culture extracts of *B. thuringiensis* var. *coreanensis, kenyae, jegathesan, israelensis, indiana* and *andaluciensis* caused the highest nematicidal activity. A total of 17 *B. thuringiensis* varieties inhibited the juvenile development into adults and 15 inhibited completely the production of progeny. Alkali-solubilised cultures of β-exotoxin-minus *B. thuringiensis* var. *alesti, konkukian, xiaguangiensis, entomocidus, sotto* and *rongseni* inhibited both *C. elegans* juvenile development into adults and the production of progeny. In addition, var. *cameroun, kumamotoensis, roskildiensis, dendrolimus, andaluciensis, londrina* and *huazhongensis* inhibited significantly the production of progeny. Alkali-solubilised trypsin-digested cultures of *B. thuringiensis* var. *israelensis, xiaguangiensis* and *andaluciensis* inhibited both *C. elegans* juvenile development into adults and the production of progeny. In addition, var. jegathesan, neoleonensis and londrina inhibited significantly the production of progeny. The *B. thuringiensis* serovars identified here, could next be assayed, along with their purified Cry and Cyt proteins on human-, animal-, and plant-parasitic nematodes. This could lead to the development of *B. thuringiensis*-based complements or alternatives to anthelmintics, fumigants and nematicides.

Keywords. *Bacillus thuringiensis*, *Caenorhabditis elegans*, Cry proteins, Cyt proteins, nematicidal activity.

INTRODUCTION

Nematodes are among the most abundant multicellular organisms on Earth. More than 20,000 species have been described and it is believed that the phylum may contain up to 500,000 species. It encompasses a wide variety of free-living species that feed on bacteria, fungi, and other organisms, and plant-, animal- and human-parasitic nematodes (Lee, 2002). Plant parasitic nematodes such as the species of the genera *Meloidogyne* Goeldi, 1892 (root-knot nematodes) (Tylenchida: Heteroderidae), *Aphelenchoides* Fischer, 1894 (foliar nematodes) (Tylenchida: Aphelenchoididae), *Pratylenchus* Filipjev, 1936 (root lesion nematodes) (Tylenchida: Pratylenchidae), *Heterodera* Schmidt, 1871 (cyst nematodes) (Tylenchida: Heteroderidae) and *Globodera* Skarbilovic, 1959 (cyst nematodes) (Tylenchida: Heteroderidae) cause mechanical damage to the roots, stems, leaves and flower structures of many plants in agriculture and horticulture (Perry and Moens, 2006; Bridge and Star, 2007; Khan, 2008, Perry *et al.*, 2009). The global economic losses to agricultural production from plant parasitic nematodes are estimated at $125 billion annually (Chitwood, 2003). Human parasitic nematodes such as *Ascaris lumbricoides* L., 1758

(Ascaridida: Ascarididae), *Wuchereria bancrofti* Cobbold, 1877 (Spirurida: Filariidae) and *Onchocerca* spp. Diesing, 1841 (Spirurida: Filariidae) are respectively parasites of the small intestine or cause lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness) (Anderson *et al.*, 2009). The number of humans infected with *Ascaris* species is estimated at 1.4 billion. In addition to these, more specific animal parasitic nematodes such as *Dirofilaria immitis* Leidy, 1856 (Spirurida: Filariidae) and *Trichostrongylus axei* Cobbold, 1879 (Trichostrongyloidea: Trichostrongylidae) either cause the dog heartworm or parasitize the small intestine of ruminants and the stomach of horses, respectively (Anderson *et al.*, 2009).

Control of plant parasitic nematodes relies on volatile chemical fumigants and non-volatile nematicides (Khan, 2008). Control of human and animal parasitic nematodes rests on the use of chemical anthelmintics (Anderson *et al.*, 2009). There is clearly a need for complements or alternatives for the control of parasitic nematodes.

Bacillus thuringiensis Berliner, 1915 is an ubiquitous Gram-positive, rod-shaped, aerobic, endospore-forming bacterium, further characterized by the production of a parasporal inclusion body (Logan and De Vos, 2009). The parasporal inclusion body is composed of one or several proteins, called the δ-endotoxins, which are encoded by two classes of genes, the crystal genes, *cry*, and the cytolytic genes, *cyt* (Aronson, 2010).

Some δ-endotoxins from selected *B. thuringiensis* strains have been shown to exhibit specific insecticidal activities against some lepidopteran, dipteran or coleopteran pest larvae (Aronson, 2010). The mode of action of the δ-endotoxins in susceptible insect larvae has been partly deciphered (Bravo *et al.*, 2007). To be active, the δ-endotoxins must first be solubilised in the alkaline environment of the insect midgut. Inactive protoxins are released and are proteolytically cleaved by midgut proteases. The activated toxins bind to specific, high-affinity receptors on the midgut epithelial cells, insert irreversibly into the membranes to form pores leading to colloid osmotic lysis and, eventually, insect death. In addition to the Cry and Cyt proteins, some *B. thuringiensis* strains produce a heat-stable β-exotoxin. It is an adenine analog and interferes with RNA biosynthesis thus resulting in broad-spectrum toxicity against invertebrates and vertebrates (Lecadet and de Barjac, 1981).

The wide diversity of *B. thuringiensis* strains has been classified based on H-serotyping, the immunological reaction to the bacterial flagellar antigens, into at least 82 serovars (Lecadet *et al.*, 1999). Ribotyping (Joung and Côté, 2001a; 2001b) and house-keeping genes (Soufiane and Côté, 2009) have revealed the phylogenetic relationships between all serovars. The molecular basis of H-serotyping have been deciphered in *B. thuringiensis* (Xu and Côté, 2006; Soufiane *et al.*, 2007; Xu and Côté, 2008). More than 250 Cry proteins, grouped into at least 116 holotypes, have now been characterized (Crickmore *et al.*, 1998; Crickmore *et al.*, 2011).

Some studies have proven the toxic activity of the *B. thuringiensis* β-exotoxin against selected plant parasitic nematodes (Prasad *et al.*, 1972; Ignoffo and Dropkin, 1977; Devidas and Rehberger, 1992), animal parasitic nematodes (Bone, 1989) and free-living nematodes (Ignoffo and Dropkin 1977; Devidas and Rehberger 1992). Other studies have demonstrated that δ-endotoxins from some *B. thuringiensis* strains exhibited nematicidal activity against eggs or young larval stages of animal parasitic nematodes (Bottjer *et al.* 1985) and free-living nematodes (Bottjer *et al.*, 1985; Meadows *et al.*, 1990). Furthermore, in 1995, Leyns *et al.* isolated two *B. thuringiensis* strains, from a collection of 128 isolates, with no mention of serovars diversity, which showed nematicidal activity against *C. elegans*. In the last decade, some Cry protein sub-families, Cry5A, Cry5B, Cry6B, Cry12A, Cry13A, Cry14A and Cry21 (Bravo et al., 1998; Marroquin et al. 2000; Wei *et al.* 2003; Kotze et al., 2005) have been shown to express nematicidal activities on selected parasitic and free-living nematodes.

Caenorhabditis elegans (Rhabditida: Rhabditidae) is a small free-living nematode. It was first used as a model for biological research in the early 1960s (Wood, 1988; Riddle et al., 1997). It is easy to use, has simple growth conditions, feeds on bacteria, has a rapid generation time and produces a large progeny with an invariant cell lineage (Wood, 1988). *C. elegans* has proven especially useful for the study of neurobiology and cellular differentiation (Wood, 1988; Riddle *et al.*, 1997) and was the first multicellular eukaryote to have its genome completely sequenced (*C. elegans* Sequencing Consortium 1998). Of interest, *C. elegans* was proposed as a model for parasitic nematodes (Bürglin *et al.*, 1998).

The aim of the present study was to assay the potential nematicidal activity and sub-lethal effects of a large collection of *B. thuringiensis* strains on the model nematode *C. elegans*. In addition to the reasons mentioned above, *C. elegans* is easily amenable to thorough screening programs, such as the one presented in this study with a large number of *B. thuringiensis* strains, serovars and assay conditions. (1) Whole-culture extracts, (2) alkali-solubilised cultures and (3) alkali-solubilised trypsin-digested cultures from 90 *B. thuringiensis* strains from 83 serovars were assayed on *C. elegans* 1st stage juvenile. In addition to their nematicidal activity, two sub-lethal effects were studied: (1) inhibition of the development of *C. elegans* 1st stage juveniles into adults, and (2) inhibition in the production of progeny.

MATERIALS AND METHODS

Caenorhabditis elegans

C. elegans *strain and culture condition*

The *C. elegans* strain BC842 was obtained from D. L. Baillie, Simon Fraser University, BC, Canada. Nematodes were maintained in culture on NG agar plates (3 g NaCl,

2.5 g Bacto peptone, 1 ml 5 mg ml^{-1} cholesterol, 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4 \cdot 7H_2O$, 25 ml 1M potassium phosphate pH·6, 17 g Bacto agar, ddH_2O to 1l) covered with a lawn of *Escherichia coli* strain HB101 and incubated at 15°C as described by Brenner (1974).

Synchronization of developmental stages

Eggs of *C. elegans* were collected for use in toxicity assays by treating samples with alkaline hypochlorite to remove all other life stages (Sulston and Hodgkin, 1988). Eggs were incubated at room temperature overnight. The emerging synchronous and axenic *C. elegans* 1st stage juvenile progeny was used to study the nematicidal activity of the 90 *B. thuringiensis* strains, thus avoiding *E. coli* contamination.

Bacillus thuringiensis

B. thuringiensis *strains and culture condition*

The 90 *B. thuringiensis* strains from 83 serovars used in this study and their provenance are listed in Table 1. They were obtained from H. de Barjac and M.-M. Lecadet, International Entomopathogenic *Bacillus* Centre, Institut Pasteur, Paris, France; from the *Bacillus* Genetic Stock Center, the Ohio State University, Columbus, OH, USA; and from the U.S. Department of Agriculture, National Center for Agriculture Utilization Research, formerly the Northern Regional Research Laboratory (NRRL), Peoria, IL, USA. *B. thuringiensis* var. *san diego* was purified from M-One™, a commercial formulation developed by Mycogen Corp., San Diego, CA, USA. The *B. thuringiensis* strain NRRL B-18765 obtained from U.S. Department of Agriculture – Agricultural Research Service Culture Collection, National Center for Agriculture Utilization Research, Peoria, was selected as a positive, toxic control in the toxicity assay because of its significant nematicidal activity against *C. elegans* population (Marroquin et al. 2000).

Each *B. thuringiensis* strain was inoculated in 50 ml of T3 broth (3 g Bacto tryptone, 1.5 g Bacto yeast extract, 50 ml 1M Na_2HPO_4 pH 6·8, 1 ml 0.05M $MnCl_2 \cdot 4H_2O$, ddH_2O to 1 l) and incubated on a rotary shaker (New Brunswick Scientific Model C-25, Edison, NJ, USA) at 180 rpm, 30°C for 5 d or until cell autolysis. Apparition of spores, parasporal inclusion bodies and cell lysis was monitored by phase-contrast microscopy.

Preparation of B. thuringiensis *whole-culture extracts for toxicity assay*

Upon lysis of the bacterial cells, *B. thuringiensis* spores and parasporal inclusion bodies were harvested by centrifugation at 4000 rpm in a Beckman Coulter Inc. AccuSpinFr™ (Fullerton, CA, USA) for 20 min at room temperature. The pellet was resuspended in a phosphate-buffered saline (8.76 g NaCl, 6.05 g K_2HPO_4, 1.70 g KH_2PO_4, ddH_2O to 1 l pH 7·2). The concentration of spores and parasporal inclusion bodies was adjusted to 1 X 10^9 spores-parasporal inclusion bodies ml^{-1} using an hemacytometer Neubaeur improved (VWR International Ltd., Montréal, QC, Canada).

Preparation of alkali-solubilised B. thuringiensis *for toxicity assay*

The *B. thuringiensis* lysed cultures were centrifuged at 4000 rpm in a Beckman AccuSpinFr™ (Palo Alto, CA, USA) for 20 min at room temperature. Each pellet was suspended in 50 $mmol^{-1}$ Na_2CO_3 (pH10), 10 $mmol^{-1}$ dithiothreitol (DTT), 1 $mmol^{-1}$ Na_2EDTA and incubated at 37°C for 2 h with continuous shaking. The extent of alkali-solubilisation of *B. thuringiensis* parasporal inclusions bodies was assessed under phase-contrast microscopy, and the results were compared to control samples in which the bacterial pellet was suspended in ddH_2O and incubated as above. The resultant alkali-solubilised suspension was centrifuged as above to remove spores and insoluble materials. The supernatant was dialysed at 4°C, overnight, against 100 volumes of sterile tap H_2O. The protein concentration of the supernatant fluid was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Alkali-solubilised B. thuringiensis samples were stored at -20°C until used.

Preparation of alkali-solubilised trypsin-digested B. thuringiensis *cultures for toxicity assay*

Alkali-solubilised *B. thuringiensis* cultures were activated with trypsin (1 mg ml^{-1}) (T-4665 from bovine pancreas, Sigma, Oakville, ON, Canada) to a final ratio trypsin/protoxins of 1/10 (v/v) and incubated at 37°C for 1 h.

Toxicity assay

Nematicidal activity and sub-lethal effects of (1) whole-culture extracts (2) alkali-solubilised and (3) alkali-solubilised trypsin-digested *B. thuringiensis* cultures were determined by a single-well assay using a 96-well microtiter plate according to Marroquin et al. (2000). Each well of the microtiter plate contained S-medium (1 l S basal [0.1 M NaCl, 0.05 M potassium phosphate pH 6, 1 ml l^{-1} cholesterol (5 mg ml^{-1} in EtOH)], 10 ml 1M potassium citrate pH 6, 10 ml trace metals solution [5 mM Na_2EDTA, 2.5 mM $FeSO_4 \cdot 7H_2O$, 1 mM $MnCl_2 \cdot 4H_2O$, 1 mM $ZnSO_4 \cdot 7H_2O$, 0.1 mM $CuSO_4 \cdot 5H_2O$, H_2O to 1 l], 3 ml 1M $CaCl_2$, 3 ml 1M $MgSO_4$), 3 µl of an *E. coli* strain HB101 culture as a food source, tetracycline (30 µg ml^{-1}) and chloramphenicol (30 µg ml^{-1}) to prevent spore germination and bacterial growth, respectively. *B. thuringiensis* culture was added to the S-medium according to the 3 different treatments: (1) 6 µl of *B. thuringiensis* whole-culture extract ($1 \cdot 10^9$ spores-parasporal inclusion bodies ml^{-1}), (2) 100 µg ml^{-1} of alkali-solubilised *B. thuringiensis* culture and (3) 100 µg ml^{-1} of alkali-solubilised trypsin-digested *B. thuringiensis* culture. The final volume in each well was 120 µl. *B. thuringiensis* strain NRRL B-18765 (1) whole-culture extract, (2) alkali-solubilised and (3) alkali-solubilised trypsin-digested culture was used as toxic, positive control (Marroquin et al., 2000). Two non-toxic

Table 1. List of *Bacillus thuringiensis* strains used in this study.

Serotypes	Serovars	Source	Strains	Serotypes	Serovars	Source	Strains
1	*thuringiensis*	IEBC*	T01 001	28a, 28c	*jegathesan*	IEBC	T28A001
2	*finitimus*	IEBC	T02 001	29	*amagiensis*	IEBC	T29 001
3a, 3c	*alesti*	IEBC	T03 001	30	*medellin*	IEBC	T30 001
3a, 3b, 3c	*kurstaki*	IEBC	T03A001	31	*toguchini*	IEBC	T31 001
3a, 3b, 3c	*kurstaki* HD-1	IEBC	T03A005	32	*cameroun*	IEBC	T32 001
3a, 3d	*sumiyoshiensis*	IEBC	T03B001	33	*leesis*	IEBC	T33 001
3a, 3d, 3e	*fukuokaensis*	IEBC	T03C001	34	*konkukian*	IEBC	T34 001
4a, 4b	*sotto*	IEBC	T04 001	35	*seoulensis*	IEBC	T35 001
4a, 4b	*dendrolimus*	USDA†	NRRL HD-7	36	*malaysiensis*	IEBC	T36 001
4a, 4c	*kenyae*	IEBC	T04B001	37	*andaluciensis*	IEBC	T37 001
5a, 5b	*galleriae*	IEBC	T05 001	38	*oswaldocruzi*	IEBC	T38 001
5a, 5c	*canadensis*	IEBC	T05A001	39	*brasiliensis*	IEBC	T39 001
6	*entomocidus*	USDA	NRRL HD-9	40	*huazhongensis*	IEBC	T40 001
6	*subtoxicus*	USDA	NRRL HD-10	41	*sooncheon*	IEBC	T41 001
7	*aizawai*	IEBC	T07 001	42	*jinghongiensis*	IEBC	T42 001
8a, 8b	*morrisoni*	IEBC	T08 001	43	*guiyangiensis*	IEBC	T43 001
8a, 8b	*sandiego*	Mycogen‡	M-One™	44	*higo*	IEBC	T44 001
8a, 8b	*tenebrionis*	IEBC	T08 017	45	*roskildiensis*	IEBC	T45 001
8a, 8c	*ostriniae*	IEBC	T08A001	46	*chanpaisis*	IEBC	T46 001
8b, 8d	*nigeriensis*	IEBC	T08B001	47	*wratislaviensis*	IEBC	T47 001
9	*tolworthi*	IEBC	T09 001	48	*balearica*	IEBC	T48 001
10a, 10b	*darmstadiensis*	IEBC	T10 001	49	*muju*	IEBC	T49 001
10a, 10c	*londrina*	IEBC	T10A001	50	*navarrensis*	IEBC	T50 001
11a, 11b	*toumanoffi*	IEBC	T11 001	51	*xiaguangiensis*	IEBC	T51 001
11a, 11c	*kyushuensis*	IEBC	T11A001	52	*kim*	IEBC	T52 001
12	*thompsoni*	IEBC	T12 001	53	*asturiensis*	IEBC	T53 001
13	*pakistani*	IEBC	T13 001	54	*poloniensis*	IEBC	T54 001
14	*israelensis*	IEBC	T14 001	55	*palmanyolensis*	IEBC	T55 001
15	*dakota*	IEBC	T15 001	56	*rongseni*	BGSC§	4BT1
16	*indiana*	IEBC	T16 001	57	*pirenaica*	BGSC	4BU1
17	*tohokuensis*	IEBC	T17 001	58	*argentinensis*	BGSC	4BV1
18	*kumanotoensis*	IEBC	T18 001	59	*iberica*	BGSC	4BW1
18a, 18c	*yosoo*	IEBC	T18A001	60	*pingluonsis*	BGSC	4BX1
19	*tochigiensis*	IEBC	T19 001	61	*sylvestriensis*	BGSC	4BY1
20a, 20b	*yunnanensis*	IEBC	T20 001	62	*zhadongensis*	BGSC	4BZ1
20a, 20c	*pondicheriensis*	IEBC	T20A001	63	*bolivia*	IEBC	T63 001
21	*colmeri*	IEBC	T21 001	64	*azorensis*	BGSC	4CB1
22	*shandongiensis*	IEBC	T22 001	65	*pulsiensis*	BGSC	4CC1
23	*japonensis*	IEBC	T23 001	66	*graciosensis*	BGSC	4CD1

24a, 24b	*neoleonensis*	IEBC	T24 001	67	*vazensis*	BGSC	4CE1
24a, 24c	*novosibirsk*	IEBC	T24A001	68	*thaïlandensis*	IEBC	T68 001
25	*coreanensis*	IEBC	T25 001	69	*pahangi*	IEBC	T69 001
26	*silo*	IEBC	T26 001		*wuhanensis*	BGSC	4T1
27	*mexicanensis*	IEBC	T27 001			IP**	407 Cry⁻
28a, 28b	*monterrey*	IEBC	T28 001			USDA	B-18765

* International Entomopathogenic *Bacillus* Centre, Institut Pasteur, Paris, France.
† U. S. Department of Agriculture, National Center for Agriculture Utilization Research, Peoria, IL, USA.
‡ *B. thuringiensis* var. *sandiego* was purified from M-One™, a commercial formulation developed by Mycogen Corp., San Diego, CA, USA.
§ *Bacillus* Genetic Stock Center, the Ohio State University, Columbus, OH, USA.
Institut Pasteur, Paris, France.

negative controls, *B. thuringiensis* strain 407 Cry⁻ (Lereclus et al., 1989), a crystal-minus mutant, and ddH_2O were included. *C. elegans* 1st stage juvenile were individually pipetted in a 1 µl volume and transferred to each well of a microtiter plate. The plates were incubated at 25°C for 3 d. The experimental design was a randomized complete block with 1 nematode per well, 8 nematodes per microtiter plate, and 12 replicates (N = 96) for the *B. thuringiensis* whole-culture extracts toxicity assays, and with 1 nematode per well, 8 nematodes per microtiter plate, and 9 replicates (N = 72) for the alkali-solubilised and alkali-solubilised trypsin-digested *B. thuringiensis* cultures.

Lethal effects of the 90 *B. thuringiensis* strains on *C. elegans* were recorded 3 d after incubation, regardless of the bacterial cell treatment, by counting dead or live nematodes in each well. Organisms were considered dead when they no longer reacted after probing. Sub-lethal effects were also evaluated. First, to determine whether the treatments had an effect on the development of the nematodes, *C. elegans* developmental stages in each well were noted as juvenile (L1, L2 and L3) or adult (L4 and adult). Second, presence or absence of progeny was scored.

Statistical analysis

C. elegans mortality, given by the percent of dead nematodes over total nematodes, was corrected by Abbott's formula (1925). Inhibition of *C. elegans* in their developmental stages is given as the percent of adults over total live nematodes. *C. elegans* progeny is given as the percent of nematodes with brood over total live nematodes. Statistical analyses were performed on arcsine square root transformed data to normalize the distribution. The lethal and sub-lethal effects of (1) whole-culture extracts, (2) alkali-solubilised and (3) alkali-solubilised trypsin-digested *B. thuringiensis* cultures were analyzed separately and subjected to one-way analysis of variance (PROC ANOVA or PROC GLM; SAS Institute, 2003) followed by Waller-Duncan k-ratio t test to separate treatments means (SAS Institute, 2003).

RESULTS

C. elegans 1st stage juveniles fed either with the non-toxic, negative control, *E. coli* strain HB101, or with the non-nematicidal *B. thuringiensis* var. *colmeri* are, after 3 d of incubation, healthy, vigorous and yield progeny (data not shown). On the other hand, when *C. elegans* 1st stage juveniles were fed with the toxic, positive control, *B. thuringiensis* strain NRRL B-18765, they either died or their development were stopped at the 1st stage juvenile (data not shown). Also, *C. elegans* were considered intoxicated by a *B. thuringiensis* strain when their developmental stages were inhibited, appeared lethargic or did not yield progeny.

(1) *Nematicidal activity and sub-lethal effects of whole-culture extracts of* B. thuringiensis *cultures*

As shown in Fig. 1A, whole-culture extract of the positive control *B. thuringiensis* strain NRRL B-18765 caused the highest *C. elegans* larval mortality with 100% after 3 d of incubation. A total of six more *B. thuringiensis* varieties caused significantly higher percent mortality than the non-toxic negative control, *B. thuringiensis* strain 407 Cry⁻ (0.07%) (F = 10.82, df = 89,1072, $P < 0.0001$). These were var. *coreanensis*, *kenyae*, *jegathesan*, *israelensis*, *indiana* and *andaluciensis* with 62, 36, 36, 32, 30 and 29% mortality, respectively.

As shown in Fig. 1B, *C. elegans* 1st stage juveniles were significantly delayed in their development, when fed with seventeen *B. thuringiensis* varieties. These were var. *darmstadiensis* (20% of juveniles reaching the adult stage), *coreanensis* (24%), *kenyae* (29%), *indiana* (34%), *israelensis* (37%), *dendrolimus* (37%), *thuringiensis* (39%), *andaluciensis* (42%), *pakistani* (43%), *londrina* (45%), *jegathesan* (46%), *galleriae* (49%), *mexicanensis* (53%), *tolworthi* (59%), *finitimus* (62%), *cameroun* (66%) and *entomocidus* (67%). These differed significantly from

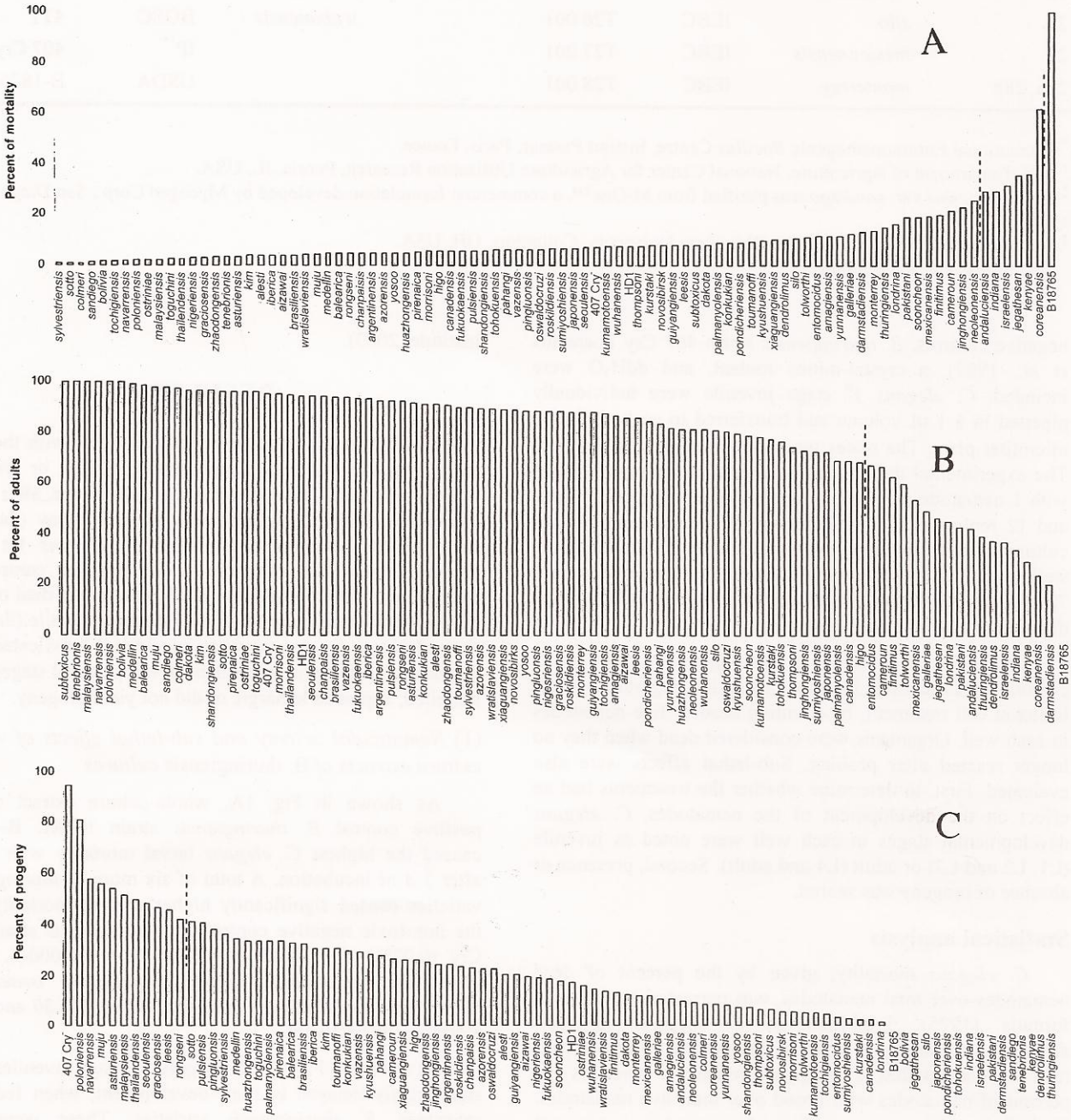

Fig. 1. Nematicidal activity (A) and sub-lethal effects (B and C) of whole-culture extracts of *Bacillus thuringiensis* cultures on *Caenorhabditis elegans* 1st stage juveniles after 3 d of incubation. Results are given as percent of wells per 96-well microtiter plate, containing dead *C. elegans* (A), adults (B) and presence of progeny (C) for each *B. thuringiensis* strain tested. The vertical dotted lines separate statistically different groups of *B. thuringiensis* strains; Fig. 1A ($F = 10.82$, df = 89,1072, $P < 0.0001$), 1B ($F = 13.20$, df = 89, 1068, $P < 0.0001$), 1C ($F = 6.88$, df = 89, 1070, $P < 0.0001$).

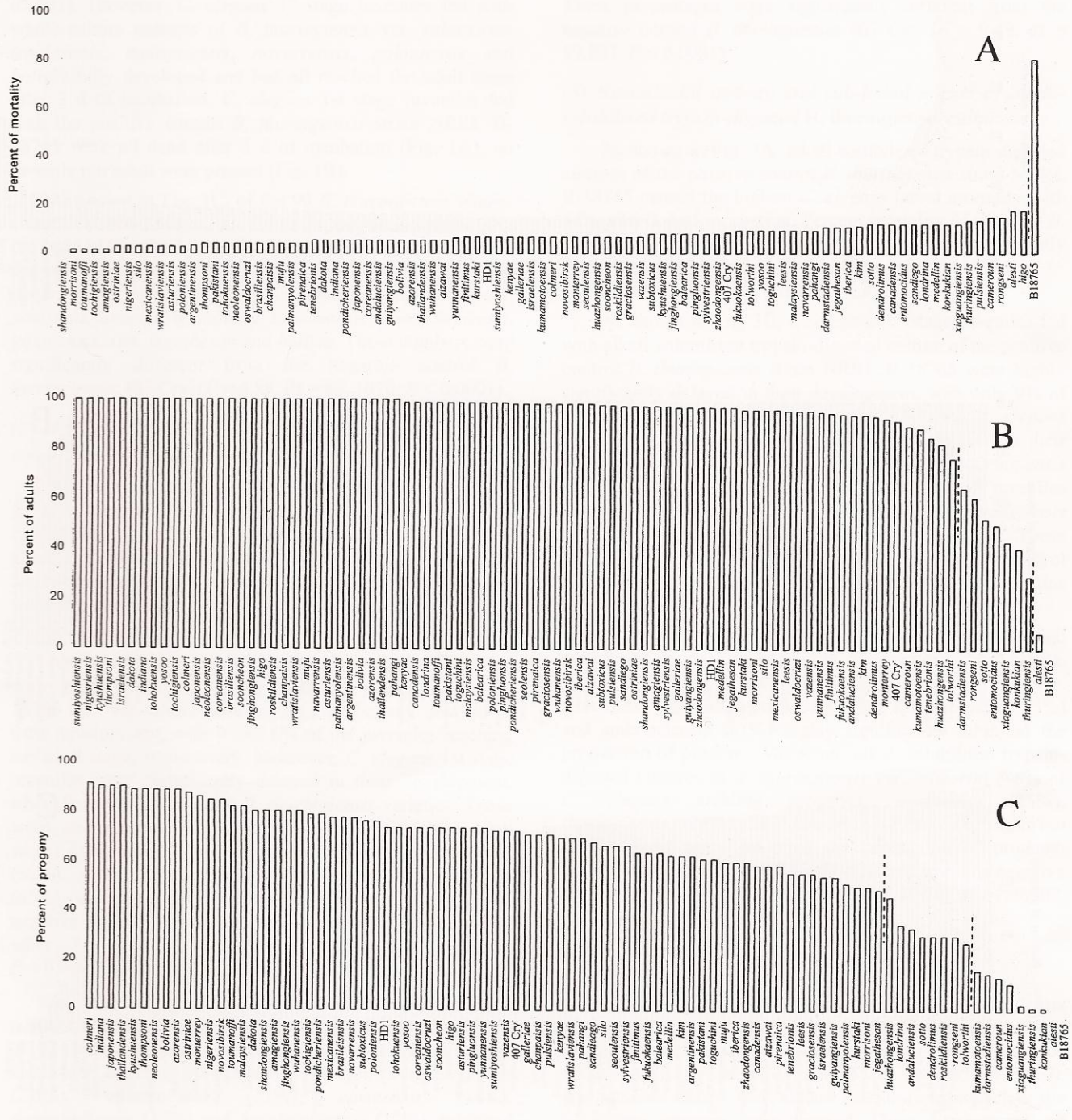

Fig. 2. Nematicidal activity (A) and sub-lethal effects (B and C) of alkali-solubilised *Bacillus thuringiensis* culture on *Caenorhabditis elegans* 1st stage juveniles after 3 d of incubation. Results are given as percent of wells per 96-well microtiter plate, containing dead *C. elegans* (A), adults (B) and presence of progeny (C) for each *B. thuringiensis* strain tested. The vertical dotted lines separate statistically different groups of *B. thuringiensis* strains; Fig. 2A ($F = 3.11$, df = 89,807, $P < 0.0001$), 2B ($F = 19.68$, df = 89,807, $P < 0.0001$), 2C ($F = 9.49$, df = 89,807, $P < 0.0001$).

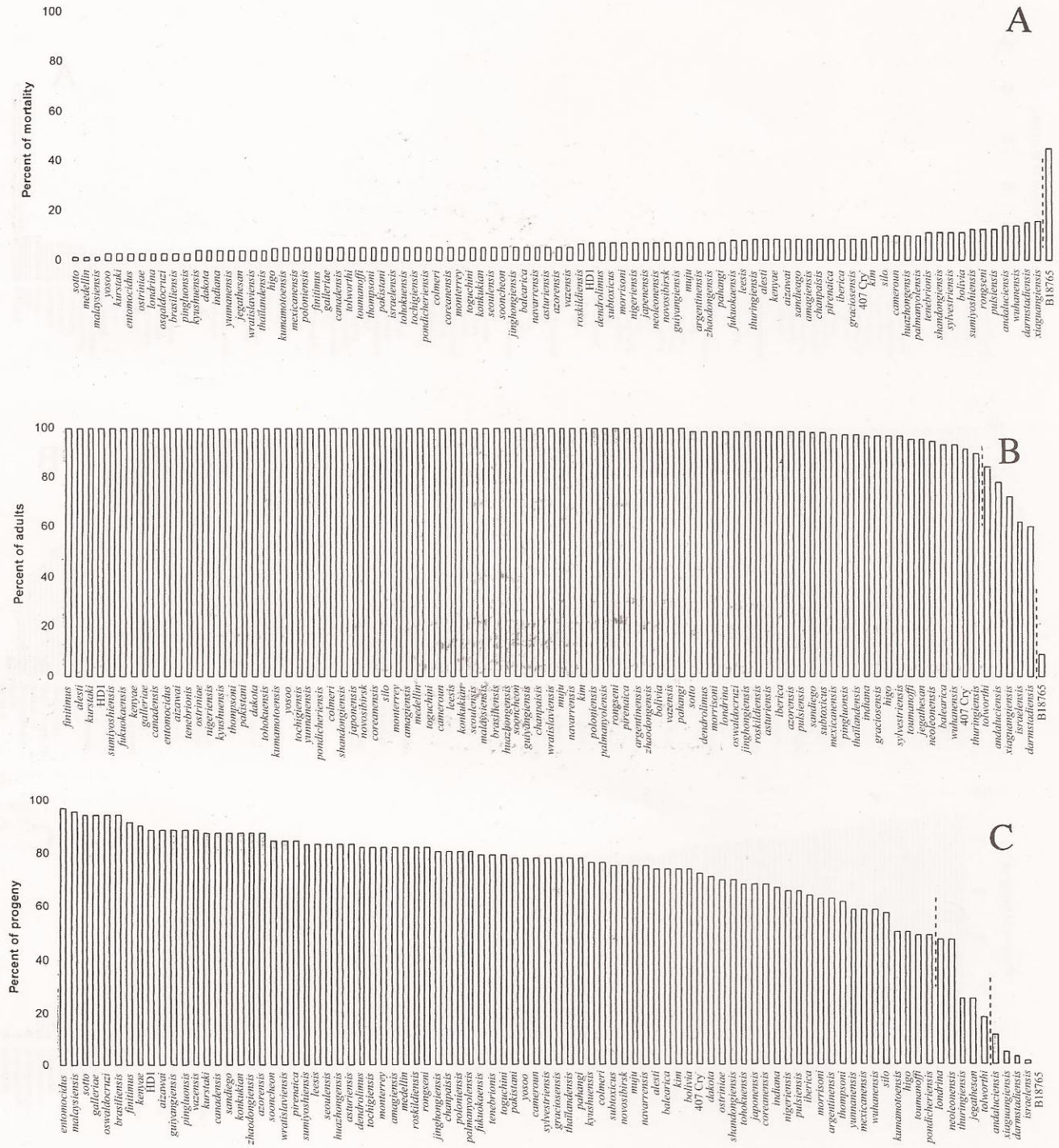

Fig. 3. Nematicidal activity (A) and sub-lethal effects (B and C) of alkali-solubilised trypsin-digested *Bacillus thuringiensis* culture on *Caenorhabditis elegans* 1st stage juveniles after 3 d of incubation. Results are given as percent of wells per 96-well microtiter plate, containing dead *C. elegans* (A), adults (B) and presence of progeny (C) for each *B. thuringiensis* strain tested. The vertical dotted lines separate statistically different groups of *B. thuringiensis* strains; Fig. 3A ($F = 1.73$, df = 89,807, $P < 0.0001$), 3B ($F = 22.71$, df = 89,807, $P < 0.0001$), 3C ($F = 10.14$, df = 89,807, $P < 0.0001$).

the negative control *B. thuringiensis* strain 407 Cry⁻ and the other 72 strains tested (F = 13.20, df = 89, 1068, P < 0.0001). However, *C. elegans* 1st stage juveniles fed with whole-culture extracts of *B. thuringiensis* var. *subtoxicus*, *tenebrionis*, *malaysiensis*, *navarrensis*, *poloniensis* and *bolivia* fully developed and had all reached the adult stage after 3 d of incubation. *C. elegans* 1st stage juveniles fed with the positive control *B. thuringiensis* strain NRRL B-18765 were all dead after 3 d of incubation (Fig. 1A), no juvenile nor adult were present (Fig. 1B).

As shown in Fig. 1C, of the 90 *B. thuringiensis* whole-culture extracts assayed, a total of 79 inhibited significantly the production of progeny. Of these, 15 inhibited completely the production of progeny. These were var. *thuringiensis*, *dendrolimus*, *kenyae*, *tenebrionis*, *sandiego*, *darmstadiensis*, *pakistani*, *israelensis*, *indiana*, *tohokuensis*, *pondicheriensis*, *japonensis*, *silo*, *jegathesan* and *bolivia*. These numbers were significantly different from the negative control *B. thuringiensis* 407 Cry⁻ (F = 6.88, df = 89, 1070, P < 0.0001).

(2) *Nematicidal activity and sub-lethal effects of alkali-solubilised* B. thuringiensis *cultures*

As shown in Fig. 2A, alkali-solubilised culture of the positive control *B. thuringiensis* strain NRRL B-18765 caused the highest *C. elegans* larval mortality with 81% after 3 d of incubation. Percent mortality of all other *B. thuringiensis* strains presented did not differ significantly from the negative control *B. thuringiensis* strain 407 Cry⁻ (F = 3.11, df = 89,807, P < 0.0001).

As shown in Fig. 2B, *C. elegans* 1st stage juveniles fed with alkali-solubilised *B. thuringiensis* strain NRRL B-18765 and var. *alesti* were highly significantly delayed in their development, with 0 and 6% of the juveniles reaching the adult stage, respectively. Moreover, *C. elegans* 1st stage juveniles were significantly delayed in their development, when fed with seven other *B. thuringiensis* varieties. These were *thuringiensis* (29% of juveniles reached the adult stage), *konkukian* (41%), *xiaguangiensis* (43%), *entomocidus* (50%), *sotto* (52%), *rongseni* (61%) and *darmstadiensis* (65%) cultures. These differed significantly from the negative control *B. thuringiensis* strain 407 Cry⁻ and the other 71 strains tested (F = 19.68, df = 89,807, P < 0.0001).

As shown in Fig. 2C, alkali-solubilised cultures of the positive control *B. thuringiensis* strain NRRL B-18765 (0% of *C. elegans* yielded progeny) and var. *alesti* (0%), *konkukian* (1.4%), *thuringiensis* (1.4%), *xiaguangiensis* (2.8%), *entomocidus* (11%), *cameroun* (14%), *darmstadiensis* (15%) and *kumamotoensis* (17%), inhibited highly significantly the production of progeny. Moreover, alkali-solubilised cultures of *B. thuringiensis* var. *tolworthi* (28% of *C. elegans* yielded progeny), *rongseni* (31%), *roskildiensis* (31%), *dendrolimus* (31%), *sotto* (31%), *andaluciensis* (33%), *londrina* (35%) and *huazhongensis* (46%) inhibited significantly the production of progeny. These percentages were significantly different from the negative control *B. thuringiensis* 407 Cry⁻ (F = 9.49, df = 89,807, P < 0.0001).

(3) *Nematicidal activity and sub-lethal effects of alkali-solubilised trypsin-digested* B. thuringiensis *cultures*

As shown in Fig. 3A, alkali-solubilised trypsin-digested cultures of the positive control *B. thuringiensis* strain NRRL B-18765 caused the highest *C. elegans* larval mortality with 44% after 3 d of incubation. Percent mortality of all other *B. thuringiensis* strains presented did not differ significantly from the negative control *B. thuringiensis* strain 407 Cry⁻ (F = 1.73, df = 89,807, P < 0.0001).

As shown in Fig, 3B, *C. elegans* 1st stage juveniles fed with alkali-solubilised trypsin-digested culture of the positive control *B. thuringiensis* strain NRRL B-18765 were highly significantly delayed in their development, with only 9% of the juveniles reaching the adult stage. Likewise, *C. elegans* 1st stage juveniles were also significantly delayed in their development, when fed with five other *B. thuringiensis* varieties. These were *darmstadiensis* (60% of juveniles reaching the adult stage), *israelensis* (62%), *xiaguangiensis* (72%), *andaluciensis* (78%) and *tolworthi* (84%). These differed significantly from the negative control *B. thuringiensis* strain 407 Cry⁻ and the other 84 strains tested (F = 22.71, df = 89,807, P < 0.0001).

As shown in Fig. 3C, alkali-solubilised trypsin-digested cultures of the positive control *B. thuringiensis* strain NRRL B-18765 (0% of *C. elegans* yielded progeny) and var. israelensis (1%), darmstadiensis (3%), xiaguangiensis (5%) and andaluciensis (11%) highly significantly inhibited the production of progeny. Moreover, alkali-solubilised trypsin-digested cultures of *B. thuringiensis* var. *tolworthi* (18% of *C. elegans* yielding progeny), *jegathesan* (25%), *thuringiensis* (25%), *neoleonenesis* (47%), and *londrina* (47%) significantly inhibited the production of progeny. These numbers were significantly different from the negative control *B. thuringiensis* 407 Cry⁻ (F = 10.14, df = 89,807, P < 0.0001).

DISCUSSION

The aim of our study was to identify *B. thuringiensis* strains and serovars which could exhibit nematicidal activity or sub-lethal effects on *C. elegans*, where *C. elegans* served as a model for parasitic nematodes. A total of 90 *B. thuringiensis* strains, with at least one strain from each of the 83 known serovars, were assayed. Three different sets of bacterial culture preparations were used: (1) whole-culture extracts, (2) alkali-solubilised, and (3) alkali-solubilised trypsin-digested cultures. Our results indicate that, next to the positive control, *B. thuringiensis* var. *coreanenesis*, and

to a lesser extent var. *kenyae, jegathesan, israelensis, indiana* and *andaluciensis*, whole-culture extracts caused the highest nematicidal activities. *Caenorhabditis elegans* 1st stage juveniles appear to possess, to some extent, the necessary complements for proper processing of the whole-culture extracts of these serovars: alkali-solubilisation, proteolytic activation and receptor binding. Besides the positive control and *B. thuringiensis* var. *coreanenesis*, the development of *B. thuringiensis*-based *C.elegans* control agents may have to be done by taking advantage of their sub-lethal effects, either without or with preliminary *in vitro* processing.

Three serovars, *B. thuringiensis* var. *thuringiensis, tolworthi* and *darmstadiensis*, each capable of producing the thermostable, wide-spectrum β-exotoxin, showed sub-lethal effects on *C. elegans* in at least one of the three culture preparations. The development of these *B. thuringiensis* serovars into commercial formulations for the control of parasitic nematodes, however, appears unlikely. Most countries will not register *B. thuringiensis* products containing β-exotoxin because of its wide-spectrum of action (Glare and O'Callaghan, 2000). Several *B. thuringiensis* β-exotoxin-minus strains, however, showed significant sub-lethal effects on *C. elegans* 1st stage juveniles. Most newly discovered *B. thuringiensis* serovars are routinely assayed on a limited number of targets, usually insects, mostly lepidopterans, sometimes dipterans or coleopterans, by their respective discoverers. In most cases, they are not assayed on *C. elegans* or parasitic nematodes. In light of our results on *C. elegans*, it would be very valuable to assay the *B. thuringiensis* serovars identified here on plant-, animal-, and human-parasitic nematodes. Certainly, some of these serovars may require prior *in vitro* processing: alkali-solubilisation and proteolytic activation. In some cases, their *cry* and *cyt* genes complements are known (e.g., *alesti, sotto, dendrolimus, entomocidus, israelensis, kumamotoensis, neoleonensis, jegathesan* and *cameroun*). Unfortunately, this is not the case for all serovars revealed above (e.g., *londrina, konkukian, andaluciensis, roskildiensis, xiaguangiensis* and *rongseni*). It would be interesting to further characterize the latters and to clone their *cry* and *cyt* genes. These serovars may harbor novel classes of Cry or Cyt proteins. Although the insecticidal activity of several Cry proteins has been characterized, the insecticidal activity of the Cyt proteins has long been thought to be restricted, *in vivo*, to dipteran pests (Höfte et Whiteley 1989). Recently, however, the Cyt proteins known toxicity spectrum was expanded to include coleopteran pests (Federici and Bauer, 1998). It is still unknown whether the Cyt proteins are toxic to nematodes. Directed screenings with purified Cry or Cyt proteins on *C. elegans*, and parasitic nematodes, appear warranted to better identify the source of the sub-lethal effects.

From a biological control perspective, *B. thuringiensis* varieties that showed sub-lethal effects might prove very valuable in presumably reducing, in the field, the nematode population of subsequent generations. In some cases, new formulations or even alternative delivery mechanisms may have to be researched. Various formulations may have to be developed to take into consideration the environment where the parasitic nematode is found and make it amenable to biological control with *B. thuringiensis*. Expressing the toxin gene into a different host, including, but not limited to, transgenic plants, will be an approach to control plant parasitic nematodes.

Interestingly, *B. thuringiensis* var. *coreanensis* whole culture extract was nematicidal to *C. elegans* juveniles but not the alkali-solubilised nor the alkali-solubilised trypsin-digested cultures. The possible solubilisation of *B. thuringiensis* var. *coreanensis* parasporal inclusion bodies in *C. elegans* juveniles and cleavage by gut proteases might generate a toxin different than the one generated with the *in vitro* solubilisation and activation protocol used here. One of the limitations of the experimental procedures used in this study rests on the choice of a single proteolytic enzyme, trypsin. Although our SDS-PAGE profiles showed that trypsin could efficiently cleave the protoxins, the use of other proteolytic enzymes (e.g. chymotrypsin, proteinase K, nematode gut proteases, etc.), different in their cleavage recognition site(s), could very well generate different toxins with presumably different biological activities. This work could also be followed by assaying, for periods longer than the 3 d presented here, the β-exotoxin-minus *B. thuringiensis* strains identified in this study which exhibited sub-lethal effects. Would the *C. elegans* inhibited in their development still be capable of reaching the adult stage? Would they reproduce? How would the progeny appear and behave? How would *C. elegans* appear or behave should the concentrations of alkali-solubilised or alkali-solubilised trypsin-digested cultures of *B. thuringiensis* be increased? To further characterize the *B. thuringiensis* strains presenting a nematicidal activity or sub-lethal effects on *C. elegans* juveniles identified here, and their Cry and Cyt proteins, it is necessary to develop or adapt to *B. thuringiensis* strains and toxins, a series of toxicity assays on and plant-, animal- and human-parasitic nematodes. These are some of the questions and issues we are planning to address in the near future.

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