Evaluation of strains of *Bacillus thuringiensis* as biological control agents of the adult stages of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae)

BY

Fabienne Eugenie Joseph Saadé

Department of Entomology
McGill University, Montreal

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

(March 1993)

Name Fabienne Eugenie Joseph Saadé

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

ENTOMOLOGY

SUBJECT TERM

0	3	5	3

SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984

Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

Suggested short title:

Bacterial control of the carrot weevil in Quebec

ABSTRACT

M.Sc. Fabienne E. J. Saadé Entomology

Evaluation of strains of *Bacillus thuringiensis* as biological control agents of the adult stages of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae)

Strains of *Bacillus thuringiensis* active against Coleoptera were evaluated for toxicity against the adult stage of the carrot weevil, *Listronotus oregonensis*. Mortality and frass bioassays using a suitable semi-artificial diet showed strains A30, A429 and BTI to be highly toxic. Mortality persisted after initial exposure to the bacteria with the survivors not resuming normal feeding. Attempts to reisolate *B. thuringiensis* from the insects revealed *B. thuringiensis* like organisms in the gut and in/on other structures. At the midgut pH of the insect (pH 8.0), the crystals of the toxic strains were significantly more soluble *in vitro* than were crystals of the less toxic strain A311. Proteolytic activation of the crystals with gut extracts yielded a protein band (66-67 kDa) for strains A30 and A429 which was similar to the apparent molecular weight of the toxin protein for BTI. Evidence suggests that the low toxicity of strain A311 might be due, in part, to the absence of the toxic moiety of the δ-endotoxin.

RESUME

M Sc. Fabienne E. J. Saadé Entomologie

L'évaluation de l'effet toxique de plusieurs souches de *Bacillus thuringiensis* contre les adultes du charançon de la carotte, *Listronotus oregonensis* (Coleoptera: Curculionidae)

Plusieurs souches de *Bacillus thuringiensis* connues d'être actives contre les Coléopteres ont été évalué pour leur effet toxique contre les adultes du charançon de la carotte, *Listronotus oregonensis*. C'est par le biais de bioessais de mortalité et de matiere fecale qu'on montre que les souches A30, A429 et BTI sont les plus toxiques contre les adultes du charonçon de la carotte se nourissant sur une nourriture semi-artificielle. les taux éleves de mortalité persistaient même après l'enlevement de la nourriture contaminée par les souches et le remplacement de cette nourriture par une non-contaminee. Les adultes survivant l'effet toxique des souches n'ont pas regagné leurs habitudes alimentaires normales Fait surprenant, l'isolation de *B. thuringiensis* des adultes a revele la presence de microorganismes apparentés à *B. thuringiensis* dans le tube digestif des insectes ainsi que dans d'autre structures. A un pH correspondant a celui du tube digestif des adultes (pH 8.0), la solubilite des crystaux des souches toxiques était significativement plus élevee *in vitro* que les crystaux de la souche peu toxique A311. L'activation proteolytique des crystaux par les extraits du tube digestif a donne une bande proteinique (66-67 kDa) pour les souches A30 et A429. le poids moleculaire de cette bande était similaire à celui de la toxine proteinique de BTI L'analyse proteinique des souches suggere que la faible toxicite de la souche A311 est dûe, en partie, a l'absence de la portion toxique de la δ-endotoxine.

Dedicated to Joe, Jenny and Eddy

ACKNOWLEDGEMENTS

I would like to thank my co-supervisor, Dr. Gary B. Dunphy, for his support and guidance and for the stimulating discussions regarding numerous aspects of this study. I also thank my co-supervisor, Dr. William N Yule, for his encouragement throughout the project.

I am indebted to Dr Roger Bernier for making this project possible by providing the bacterial strains and useful technical information.

I would like to express my gratitude to the Department of Entomology and the Faculty of Graduate Studies and Research for financial support in the form of scholarships , teaching assistantships and Summer research awards

Thanks also go to Dr Guy Boivin for supplying carrot weevils upon request

Many thanks, as well, to Pierre Langlois for his technical help, and to Diane King and Marie Kubecki for their secretarial assistance.

Thanks to Arnaud Jarlan and Andrea Schroeder for taking care of the insect cultures

I also thank the members of the faculty and graduate students of the department of Entomology who have contributed to the realization of this work In particular, I acknowledge the friendship and valuable discussions of Adla Halwani. Thanks to Tanya Searle, especially for her assistance with the insects, and to Graham Thurston for his good humour. Thanks also go to Dr Barry Jewell for the valuable technical tips.

I am also indebted to my parents and uncles for their encouragement and moral support

Finally, I wish to acknowledge the invaluable feedback, understanding, support and love, of my husband, Edward Awad, throughout this project.

TABLE OF CONTENTS

Chapter 3

LIST OF TABLES

LIST OF FIGURES

Chapter 3

GENERAL INTRODUCTION

The carrot weevil, *Listronotus oregonensis* (Le Conte), is a major pest of several crop plants in the northeastern states of the United States of America (Chandler, 1926; Pepper, 1942, Whitcomb, 1965) and Canada (Perron, 1971; Martel *et al.*, 1975, Stevenson, 1976). It attacks Umbelliferous plants, but its preferred host is the carrot, *Daucus carota*.

In Quebec, the carrot weevil is univoltine (Perron, 1971). The overwintered adults invade carrot fields in the spring and feed on the new foliage (Boivin and Belair, 1989). The adult females oviposit in the stalk or crown of young plants and the larvae, upon hatching, penetrate the carrot roots. As the larvae feed they make tunnels in the upper one third section of the root effectively reducing crop value (Whitcomb, 1965).

Since 1967, carrot root injuries caused by *L. oregonensis* have ranged from 2% to 22% in Quebec (Perron, 1971). When left uncontrolled, this insect pest can cause damage of up to 40% of the Quebec carrot crop (Boivin, 1985a) reducing the acceptability of the crop to the packing and canning industries which can tolerate no more than 5% root injury (Perron, 1971).

The only recommended control measure, to date, is the use of insecticides directed against the adults. The problems inherent with the use of chemical insecticides (i.e. environmental contamination, insect resistance, effects on non-target organisms), and the beginning of an integrated pest management (IPM) program for carrots have encouraged research into alternative control measures. Research consisted of the potential use of an egg parasitoid, *Anaphes sordidatus* and of three species of entomogenous nematodes, *Steinernema carpocapsae* [= *S. feltiae* (Poinar, 1989)], *S. feltiae* [= *S. bibionis* (Poinar, 1989)], and *Heterorhabditis bacteriophora* [= *H. heliothidis* (Poinar, 1990)] to keep the pest below the economic threshold (Belair and Boivin, 1985, Boivin, 1985a), but to date there are no definitive results. Carabid beetles have shown potential as predators of the different stages of the insect (Baines *et al.*, 1990). Strains of fungal pathogens of *Metarhizium anisopliae* and *Beauveria bassiana* against the larvae have been isolated (Searle and Tule,

1988) with efficacy studies continuing.

A study was started in the fall of 1990 to evaluate strains of the entomopathogenic bacterium, *Bacillus thuringiensis*, with activity against pest Coleoptera, as microbial agents against the carrot weevil To be effective, the bacterium must be ingested by the insect for protoxin to dissolve and transform into an active toxin by means of limited proteolysis by the insect gut proteases (Luthy *et al* , 1982) The larval stages occur within the carrot root making it difficult to incorporate the bacterium into the insect's feeding habitat Therefore, control should be aimed at the leaf feeding stages (adult) of the insect.

The experiments in this study were designed to:

1) Examine the pathogenicity of seven strains of *Bacillus thuringiensis* directed against the adult stage of the carrot weevil.
2) Examine the effect of intoxicated survivors upon discontinuing their exposure to the insecticidal crystals.
3) Determine the midgut pH of the adult carrot weevil and its relationship to crystal solubilization.
4) Examine the involvement of two key steps in the mode of action of the δ endotoxin, i.e. crystal solubilization *in vitro*, and proteolytic processing with gut juice extract.

This thesis is written in a manuscript format and the following excerpt from Guidelines Concerning Thesis Preparation, states the conditions under which the thesis must be written.

"The candidate has the option, subject to the approval of the department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation, (available at the Thesis Office). Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported. Abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introduction

and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted.

While the inclusion of manuscripts co-adhered by the Candidate and others is not prohibited by McGill, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is made more difficult in such cases, and it is in the Candidate's interest to make authorship responsibilities perfectly clear."

There are two Chapters presented as papers for submission to the Canadian Entomologist (Chapter 2) and Journal of Invertebrate Pathology (Chapter 3).

CHAPTER 1

LITERATURE REVIEW

THE CARROT WEEVIL

Taxonomy and distribution

The carrot weevil, *Listronotus oregonensis* (le Conte), (Coleoptera:Curculionidae) was first identified in 1860 as *Listroderes oregonensis* (Le Conte) and was subsequently renamed *Listronotus oregonensis* in 1876 (Henderson, 1939). Other synonyms used to denote this species include:

Listronotus latiusculus (Le Conte)
Listronotus impressifrons (Le Conte)
Listronotus rudipennis (Blatchley).

The carrot weevil is native to North America extending from Quebec to Florida and northwest to Manitoba (Buchanan, 1932).

Pest status

The first record of carrot weevil injuries was in 1902 in Virginia on parsley with subsequent sporadic reports from Illinois, Connecticut, New York, and Washington D.C. on crops of parsley and carrots (Pepper, 1942). In 1925, 50 to 90% of the carrot crop was injured in Illinois (Chandler, 1926) and the carrot weevil reached pest status in Iowa (Harris, 1926) Severe outbreaks on celery and carrots occurred in New Jersey in 1936 (Pepper and Hagmann, 1938), and on carrots in Massachussetts in 1956 (Whitcomb, 1965). Presently, the carrot weevil remains a pest in some parts of the northeastern United States of America (Grafius *et al* , 1983)

In Ontario, the carrot weevil was first identified at the turn of the century but did not achieve economic importance until 1969 (Stevenson, 1976). The first invasion of the carrot weevil was observed in 1956 in the Holland Marshes and Bradford (Harcourt, 1959) and by 1963, the carrot weevil was beginning to cause concern (Harcourt, 1963). The first sign of the carrot weevil in Quebec was in 1967 in Ste. Clotilde (Perron, 1971) It gained economic importance by 1970 when damage ranged between 2 and 22%

root injuries of the carrot crop (Perron, 1971). In both provinces, the carrot weevil attacks are restricted to the carrot crop. To date, the carrot weevil continues to cause problems to carrot growers in both Ontario and Quebec (Boivin and Sauriol, 1984; Stevenson, 1985).

Description of stages

Adult

Adult weevils are oblong, black, with cupreous tinged scales (Whitcomb, 1965). The antennae are clubbed and reddish brown in colour. The stripes on the thorax and the mottling on the elytra are a result of the presence of a layer of dark scales. The elytra are striated and outlined by rows of grey setae. They have well developed wings but are rarely seen in flight (Boyce, 1929).

The adults vary considerably in size with the males being the smallest (Table 1) (Martel *et al.*, 1976). However, the sexual dimorphism in size is not an accurate means of determining sex. According to Whitcomb (1965) males can be separated from females by examining their first ventral abdominal segment, this segment having an oblong depression in males and being swollen with a small oval depression in females.

Egg

The eggs are elliptical and uniform in size (Table 1) (Martel *et al.*,1976). Both fertilized and unfertilized eggs are initially light yellow in colour with fertilized eggs becoming darker with age and almost black just prior to eclosion (Baudoin and Boivin, 1985). The position of the larva is easily discernible in the final stages of development. The larva uses its mandibles to puncture the egg membrane and leave the egg.

Larva

The larvae are slightly curved, legless and creamy white in colour vermiform larval instars (Harris, 1926; Martel *et al.*, 1976). Larval

Table 1. Dimensions of adult and egg stages of the carrot weevil, *Listronotus oregonensis*

	Length (mm)	Width (mm)
Adult		
male	6.00 ± 0.30	2.20 ± 0.10
female	6.50 ± 0.30	2.40 ± 0.10
Egg	0.80 ± 0.10	0.50 ± 0.03

(Summary data from Martel *et al.*, 1976).

with a sclerotized amber head capsule (Whitcomb, 1965). There are four instars that can be differentiated according to head-capsule width which exhibits exponential growth (Table 2) (Martel *et al.*, 1976). The mouthparts are functional throughout the instars and the spiracles are prominent. A consistent pattern of setae is present on the head and body segments (Whitcomb, 1965).

Prepupa and pupa

The prepupa is a nonfeeding stage of the fourth instar (Martel *et al.*, 1976) and measures 7 mm long and 2 mm wide (Whitcomb, 1965). The larvae cease to feed and enter the prepupal stage without moulting (Martel *et al.*, 1976)

The pupae are exarate, creamy white, 5-7 mm long, and have a typical curculionid form. The head, thorax, legs, and abdominal segments are lined with stiff spines (Whitcomb, 1965). If disturbed, the pupae flex their bodies (Pepper, 1942).

Life cycle

Although there are 2-3 generations of the carrot weevil in the United States of America per year, this insect is usually univoltine in Canada (Whitcomb, 1965, Perron, 1971; Stevenson, 1976). Adult weevils overwinter both in the field borders and in incompletely harvested carrot fields (Grafius and Collins, 1986, Pepper, 1942; Stevenson, 1976). The development of the carrot weevil is temperature-dependent (Martel *et al.*, 1976, Simonet and Davenport, 1981). The overwintered adults have an obligatory preoviposition period (Whitcomb, 1965) which averages 17 days at 21°C and 10 days at 27°C (Martel *et al.*, 1976). Pepper (1942) reported that newly emerged females mated prior to overwintering but, the usual trend is for overwintered adults to mate in Spring (Whitcomb, 1965). In Canada, oviposition starts in early May (Stevenson, 1985, Boivin, 1985a). According to Baudoin and Boivin (1985), total fecundity is neither affected by the number of matings per female nor by the number of females mated by a single male. The female chews a cavity in the petiole or crown

Table 2. Head-capsule width of the larval instars of the carrot weevil, *Listronotus oregonensis*

Instar	Head width (mm)
I	0.31 ± 0.002
II	0.44 ± 0.004
III	0.65 ± 0.004
IV	0.99 ± 0.004

(Summary data from Martel, *et al.*, 1976).

of the carrot, deposits 2 - 3 eggs and seals the site with a black exudate from her genital segments (Harris, 1926; Pepper, 1942). The daily number of eggs laid increases as the temperature increases from 18.3 °C and 29 4 °C, the eggs requiring 12.8 and 4.8 days for eclosion, respectively (Simonet and Davenport, 1981). The first instar larvae immediately start feeding in the root. The larvae in the petioles penetrate the roots either by tunnelling down into them or by leaving the stalk and invading from the soil (Stevenson 1981). The average development time for the first three instars is 13.1 days at 23.9°C (Simonet and Davenport 1981). The development time for fourth-instar larvae in the field is double that in the laboratory taking 6.5 days at 23.9°C (Stevenson, 1976, Simonet and Davenport, 1981). The fourth-instar larvae leave the carrots and enter the prepupal stage in the soil (Pepper, 1942) They construct an earthen cell by bending their bodies back and forth rapidly, compacting the surrounding soil (Harris 1926, Martel *et al.*, 1976). At the same temperature, pupae require an average of 6.8 days to develop (Simonet and Davenport, 1981). Teneral adults remain in their earthen cells for a short time, emerge from the ground and feed on foliage until they locate suitable overwintering sites (Harris, 1926) . Adults feed and oviposit on several Umbelliferous host plants: cultivated and wild carrots, celery, patience dock, broad-leaf plantain, parsley, and dill (Pepper and Hagmann, 1938). Laboratory experiments of Simonet and Davenport (1981) established that at 18.3°C (in early Spring) and 23.9°C for (Summer), a full cycle from egg to adult would require a minimum of 38.2 days. Adding 17 days as a minimum preoviposition period brings the total number of days to complete a single generation to 55.2.

Damage

Damage to the crop is caused by both the adults and larvae. However, the injuries caused by the larvae are of a more serious nature.

Adults

The adult carrot weevil can feed on all exposed parts of the carrot,

the feeding marks appearing on the foliage, the petioles and the crown. Similarly, oviposition traces are left on exposed regions of the plant. Such damage is not of major importance because it does not hinder the development and marketability of the plant.

Larvae

The larvae eat their way into the upper one third of the carrot root resulting in galleries lined with damp sticky frass (Pepper, 1942). There are usually more than one larva per carrot and these feed in the root forming dark zig-zag tunnels which often join together to produce a broad lesion (Anon, 1977)

It is believed that in the past, the carrot rust fly, *Psila rosae*, was misidentified as the agent responsible for carrot weevil damage. This confusion was overcome when it came to be known that carrot rust flies form tunnels in the lower two-third sections of the carrot root and have smaller dimensions than those of the carrot weevil (Stevenson, 1981)

When left uncontrolled, the carrot weevil can damage up to 40% of the Quebec carrot crop (Boivin, 1985a) Since the packing and canning industries can tolerate no more than 5% root injury (Perron, 1971), it is evident that some pest control is usually necessary.

Types of control

Chemical control

In the past, *L. oregonensis* has been controlled with insecticides (Martel *et al.*, 1975). Systemic insecticides are required to reach the eggs and the larvae, while the adults are targeted with foliar applications of contact insecticides. Organochlorines (heptachlor and aldrin) (Whitcomb, 1965), and arsenicals (calcium arsenate) (Pepper, 1942) have been used. The organochlorines were most effectively applied as granules in the soil followed by foliage applications. However, the toxicity of the more persistent insecticides on the carrot root has led to

the use of foliar applications against the overwintered adults prior to oviposition as the only means of chemical control (Brodeur, 1985; Boivin, 1985a). Presently, phosmet (Imidan^{R}) is registered for this purpose in Quebec at the rate of 2.25 Kg/ha twice seasonally (CPVQ, 1987).

Cultural control

Annual crop rotation effectively controls the carrot weevil (Anon, 1977). Since the adults rarely fly , the presence of this insect in the field is a warning for an infestation the following year if the farmer attempts to grow carrots there once again (Stevenson, 1981). However, detection of the adults requires efficient monitoring which, in turn, is time consuming and costly Monitoring programs for this pest insect have begun in Michigan (Grafius *et al.*, 1983), Ontario (Stevenson, 1985), and Quebec (Boivin and Sauriol, 1984). As an alternative to monitoring insect population densities, a trap crop surrounding a carrot field was sprayed with insecticides which successfully reduced the level of carrot weevil infestation on the carrot (Whitcomb, 1965)

Another cultural practice used to escape severe damage by the carrot weevil is to delay the date of planting (Boivin, 1985a, Stevenson, 1976; Chandler, 1926). A significant inverse relation exists between the date of sowing and the number of eggs laid. Although 70% of the eggs are laid during the fourth through the seventh true-leaf-stage of the carrot, most carrot weevil damage was eliminated when carrots were sown after 400-450 DD (degree days, base 7°C) However, this practice is not usually followed, since it tends to reduce the yield of the crop.

Biological control

Although biological control is another possibility to minimize damage caused by the carrot weevil, surprisingly little information is offered in the literature

The mymarid wasp *Anaphes sordidatus* (Hymenoptera) is an egg parasitoid of *L. oregonensis* (Collin and Grafius, 1986a,b,c) parasitizing 50% of carrot weevil eggs in Quebec (Boivin, 1985b). *Aliotus curculionis*,

a braconid wasp, was found to parasitize 2% of the carrot weevil larvae in Massachusetts (Whitcomb, 1965). On the other hand, predatory carabid beetles (Coleoptera: Carabidae), which are abundant in carrot fields of southwestern Quebec have been shown to consume different stages of the insect (Baines *et al.*, 1990). However, like most parasites and predators, they would require simple and cheap mass rearing techniques to be effective, and these have yet to be designed.

Laboratory studies on the susceptibility of the carrot weevil stages to three entomogenous nematodes *Steinernema carpocapsae* [*S. feltiae* (Poinar, 1989)], *S. feltiae* [=*S. bibionis* (Poinar, 1989)], and *Heterorhabditis bacteriophora* [=*H. heliothidis* (Poinar, 1990)] have shown the larval stage to be the most susceptible stage and the former two species of nematodes to be the most aggressive species (Bélair and Boivin, 1985). Boivin and Bélair (1989), also studied the infectivity of two strains of *S. carpocapsae* (=*S. feltiae*) in relation to temperature, sex, and age of the carrot weevil adults.

With regard to fungal pathogens, Searle and Yule (1988) have selected virulent strains of *Metarhizium anisopliae* and *Beauveria bassiana* by means of laboratory bioassays against the larval stages of the carrot weevil. At this point, field tests would have to be carried out to determine the effectiveness of these strains on *I. oregonensis* under natural conditions and local muck soils.

A new contribution to the Quebec Integrated Pest Management Program for the control of the carrot weevil would be the use of strains of an entomopathogenic bacterium, *Bacillus thuringiensis*, that have shown activity against Coleopteran species.

BACILLUS THURINGIENSIS

Historical background

Bacillus thuringiensis was isolated at the turn of the century by a Japanese bacteriologist, S. Ishiwata (Ishiwata, 1901). The pathogen was isolated from the silkworm, *Bombyx mori*, and was named Sottokin which

means " sudden death bacillus " (Ishiwata, 1905). At the time, this organism was considered a threat to a very important industry, silk production (Luthy *et al.*, 1982) It was not until decades later that its potential use for insect control was realized.

The second isolation was made by E. Berliner in Thuringia, Germany from diseased larvae of the Mediterranean flour moth, *Anagasta kuehniella*, (Zeller) and it was named *Bacillus thuringiensis* (Berliner, 1911). Berliner described it and showed that it fulfilled Koch's postulates, thus validating his naming of the isolate (Luthy *et al.*, 1982). Within the present taxonomy, the strain described by Berliner has been designated as subspecies *thuringiensis* (Luthy *et al.*, 1982).

Between 1920 and 1950, several field applications with *B. thuringiensis* were undertaken with various degrees of success. Sporeine, the first commercial product of *B. thuringiensis* was manufactured in France around 1938 (Lambert and Peferoen, 1992). In the 1950s, Hannay (1953) discovered the active ingredient of *B. thuringiensis* and in 1956, Angus (1956) proved that the insecticidal activity was located in the parasporal inclusion

During the 1960s, a number of industrial formulations of *B. thuringiensis* were produced in the United States of America, the Commonwealth of Independent States (former Soviet Union) ,France, and Germany (Lambert and Peferoen, 1992). Based on *B. thuringiensis* subspecies *thuringiensis*, the insecticides had low activity and could not compete with chemical insecticides in either efficacy or cost (Beegle and Yamamoto, 1992).

In 1962, I Kurstak isolated a new subspecies of *B. thuringiensis* from *A. kuehniella* larvae in France and in 1970, Dulmage isolated the same subspecies (which they named *kurstaki*) from the pink bollworm, *Pectinophora gossypiella*. The isolate proved to be more potent than the subspecies *thuringiensis* and was competitive with chemical insecticides (Dulmage, 1970) Presently, several million kilograms of *kurstaki*-based products are manufactured annually in the United States of America with usage registered for approximately 30 crops and against more than 90 pest insects worldwide (Beegle and Yamamoto, 1992).

Until the 1970s, most of the described *B. thuringiensis* strains had

demonstrated activity against lepidopteran insects. In Israel in 1976, Goldberg and Margalit (1977) isolated a *B. thuringiensis* with mosquito and blackfly larvicidal activity. It was named subspecies *israelensis* (de Barjac, 1978) and had an outstanding level of activity and speed of kill.

The most recent *B. thuringiensis* isolate to have commercial promise is subspecies *tenebrionis*. In 1982, Krieg *et al* isolated from *Tenebrio molitor* a new strain of *B. thuringiensis* pathogenic against certain coleopteran insects and named it *B thuringiensis* subsp *tenebrionis* (Krieg *et al.*, 1983) In San Diego, in 1986, a strain of *B thuringiensis* with similar activity was described by Herrnstadt *et al* , and was referred to as *B. thuringiensis* subsp. *san diego* However, it was subsequently demonstrated that both strains were identical (Krieg *et al.*, 1987a).

The susceptibility of several coleopteran species including economically important pests (eg., Colorado potato beetle *Leptinotarsa decemlineata*, and the cotton boll weevil, *Anthonomus grandis*) to *B thuringiensis* subsp *tenebrionis* (Herrnstadt *et al* , 1986) has attracted attention to the subspecies in terms of its effectiveness as a biocontrol agent (Herrnstadt *et al.*, 1986; Riethmuller and Langenbruch, 1989, Ferro and Gelernter, 1989, Zehnder and Gelernter, 1989), its biochemistry (Bernhard, 1986, Krieg *et al.*, 1987b; Li *et al.*, 1988; Carroll *et al.*, 1989); as well as its molecular biology and genetics (Herrnstadt *et al.*, 1987; Jahn *et al.*, 1987, McPherson *et al* , 1988, Sekar, 1988)

Most studies of the effectiveness of *B. thuringiensis* subsp.*tenebrionis* have been for the control of *L. decemlineata*, the most destructive pest of potatoes in northeastern United States of America (Ferro and Gelernter, 1989). Both laboratory and field tests indicated that the pathogen effectively controlled the larval population of this pest on leaves It was also noted that the toxin is biodegradable and has no known effect on non-target organisms (Herrnstadt *et al* , 1986) Screening of *B. thuringiensis* subsp *tenebrionis* isolates for insecticidal activity revealed that families in the order Coleoptera differed in their susceptibility to the strain (Table 3).
There are numerous formulations of *B. thuringiensis* subsp. *tenebrionis* (Table 4) (Beegle and Yamamoto, 1992).

Table 3. Insects evaluated for susceptibility to *Bacillus thuringiensis* subspecies *tenebrionis* (= subsp. *san diego*).

Order	Family	Species	Stages tested	Activity
Coleoptera	Chrysomelidae	-*Diabrotica undecimpunctata*	A, L1, L2	+
		-*Haltica tombasina*	A, L2, L3	+++
		-*Leptinotarsa decemlineata*	L1, L2	+++
		-*Pyrrhalta luteola*	A, L1, L2, L3	++++
	Curculiondae	-*Anthonomus grandis*	A, L2, L3	+++
		-*Otiorhynchus sulcatus*	L2, L3	++
	Dermestidae	-*Attagenus unicolor*	L3	-
	Ptinidae	-*Gibbium prylloides*	A	-
	Tenebrionidae	-*Tenebrio molitor*	L1, L2, L3	++
		-*Tribolium castancum*	A, L3	-

Assays against *Diabrotica undecimpunctata*, *Leptinotarsa decemlineata*, and *Pyrrhalta luteola* were carried out by spraying spore/crystal preparations, or purified crystals on to leaf discs from appropriate host plants. All other assays were carried out by incorporating spore/crystal preparations or purified crystals into an appropriated diet mixture. Insecticidal activity was arbitrarily classified from weak (+) to very strong (++++). A, adults; L, larvae. Assays against *Otiorhynchus sulcatus*, *Attagenus unicolor*, *Tenebrio molitor* and *Tribolium castancum* were carried out for 30 days. All other assays were carried out for 7 days. (From Herrnstadt *et al.*, 1986).

Table 4. *Bacillus thuringiensis* subspecies *tenebrionis* based commercial products.

Product	Registration date	Manufacturer
M-One	1988	Mycogen
M-Trak	1991	Mycogen
Trident	1988	Sandoz Agro, Inc.
Trident II	1990	Sandoz Agro, Inc.
Foil*	1990	Ecogen
Di Terra	1991	Abbott Laboratories
Novodor	Pending	Novo Industries

* Combination of subsp. *kurstaki* and *tenebrionis*

(from Beegle and Yamamoto, 1992).

Nomenclature

B. thuringiensis is a Gram-positive, endospore-forming bacterium of the family Bacillaceae. Although it is similar to *Bacillus cereus*, it is considered a separate species largely due to the presence of parasporal crystals in *B. thuringiensis* (Lambert and Peferoen, 1992). The need for a method to identify and classify *B. thuringiensis* subspecies became necessary as different isolates were discovered to have different activity spectra, and *B. thuringiensis* became increasingly commercially important (Beegle and Yamamoto, 1992). Initially, the method was based on morphological and biochemical characteristics (Heimpel, 1967; Heimpel and Angus, 1958, 1960) de Barjac (1981) and de Barjac and Bonnefoi (1962, 1968, 1973) introduced a classification scheme based on serological analysis of vegetative cell flagellar (H) antigens and biochemical characteristics. Several other identification and classification methods were also developed, such as esterase analysis of vegetative cells (Norris, 1964), crystal serology (Krywienczk and Angus, 1960, Krywieczyk *et al.*, 1978, Smith, 1987), and susceptibility of *B thuringiensis* to phage (Jones *et al* ,1983) However, flagellar serotyping became the method of choice mainly because a flagellar serotyping service was made available by the Institut Pasteur. Presently, there are 36 serotypes of *B. thuringiensis* (Beegle and Yamamoto, 1992).

The most recent taxonomic procedures used include High Pressure Liquid Chromatography (HPLC) (which can accurately distinguish crystal types within a serotype) and plasmid mapping, and gene cloning and sequencing of the crystal toxin (Beegle and Yamamoto, 1992). A new taxonomic system has been proposed for *B thuringiensis* crystal genes (Cry) based on the types of crystal proteins produced and the activity spectrum of the insecticidal toxins (Table 5) (Hofte and Whiteley, 1989). Since the primary interest in *B. thuringiensis* is in its ability to kill pest insects, it seems likely that the identification/classification scheme based on structural similarities and insecticidal spectra of the encoded proteins will replace that based on flagellar serotypes

Table 5. Host specificity of crystal proteins

Gene type	Host range
CRY I	Lepidoptera
CRY II	Lepidoptera and Diptera
CRY III	Coleoptera
CRY IV	Diptera

(Summarized from Hofte and Whiteley, 1989).

Sporulation and crystal production

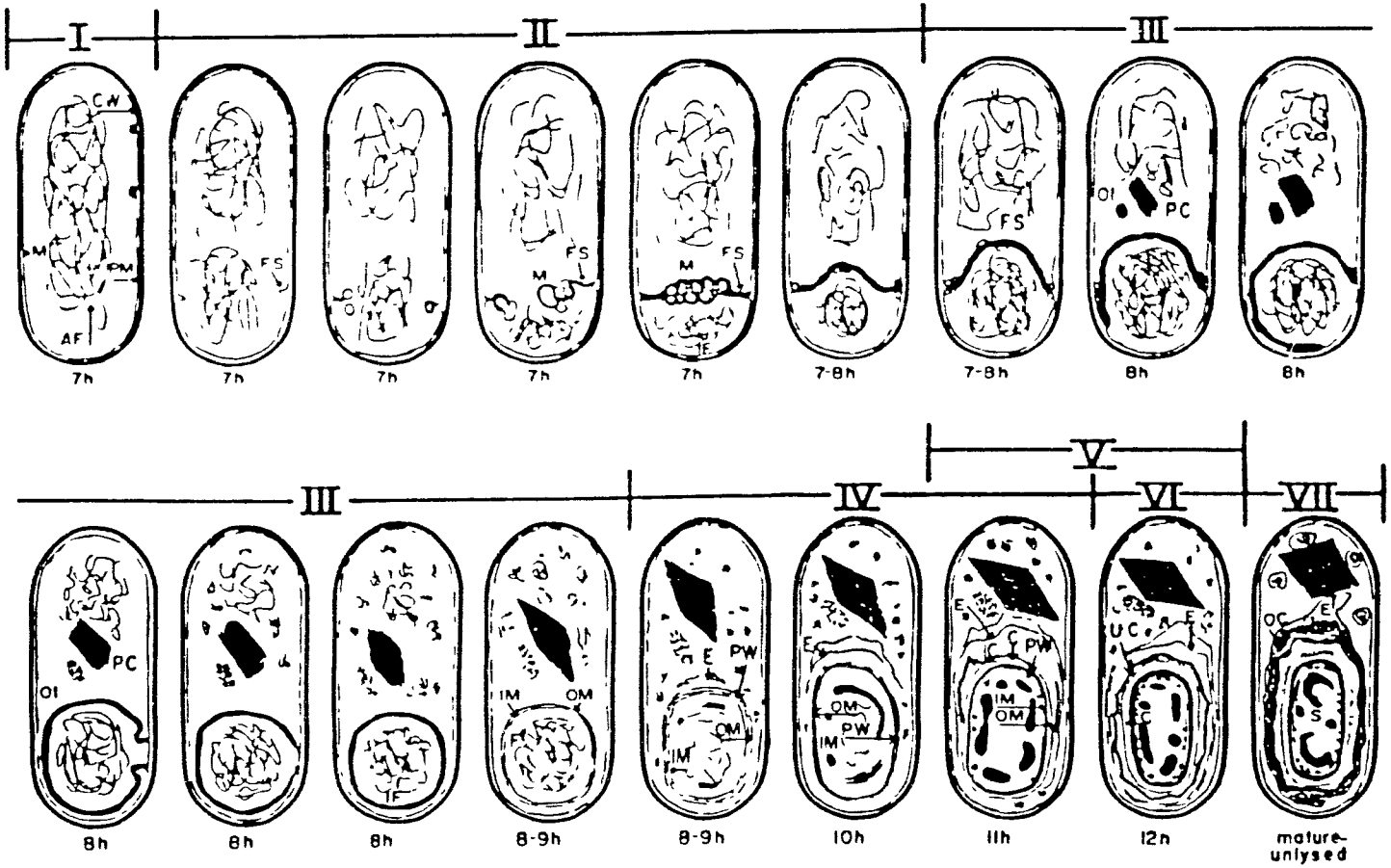
St. Julian *et al.* (1971) documented the different morphological stages from the dormant spore to the vegetative cell of *B. thuringiensis*. A brief description follows.

The vegetative cells of *B. thuringiensis* are rod-shaped (average dimension of 3 5μm x 1.2-1.5μm) peritrichously flagellated, and usually occur as filaments of at least four cells. Vegetative cells are known to produce water soluble, insecticidal toxins (see section "Types of toxins other than the δ-endotoxin")

B thuringiensis is commonly found in natural soils and in environments with high densities of insects such as grain storage and insect rearing facilities and sericulture farms (Lambert and Peferoen, 1992) The dormant spores are able to survive unfavourable conditions over a long period of time (Luthy *et al* , 1982). Their resistance to heat, chemicals, irradiation and desiccation is attributed to the dehydration of the spore cytoplasm during sporulation. In nature, the spores undergo ageing, a slow process of activation which causes the breakage of disulfide linkages of spore coat cystine thus causing a change in the tertiary structure of the proteins which is originally responsible for the dormant state of the spores. The initiation of germination is an irreversible process (Luthy *et al.*, 1982) A series of degradative reactions occur during germination and are followed by a period of biosynthetic activities necessary for the outgrowth of the vegetative cell.

The exhaustion of the medium especially of their source of C, N, and P induces the initiation of sporulation (Luthy *et al.*, 1982). The sporulation process is subdivided into seven stages (Fig. 1). During stage I, an axial chromatin filament is formed. In stage II, the chromatin filament separates into two chromosomes and a subpolar forespore septum is formed delimiting the site of the future spore. During stage III, biogenesis of a crystal begins. The crystal is a proteinaceous parasporal inclusion known as the δ-endotoxin and is lethal to specific groups of insects. It accounts for 20-30% of the total protein of the sporangium (Aronson *et al.*, 1986). Stage III is also morphologically

Figure 1. Diagrammatic scheme of sporulation in *B. thuringiensis*. Abbreviations: M, mesosome; CW, cell wall; PM, plasma membrane; AF, axial filament; FS, forespore septum; IF, incipient forespore; OI, ovoid inclusion; PC, parasporal crystal; F, forespore; IM, inner membrane , OM, outer membrane; PW, primordial cell wall; E, exosporium, LC, lamellar spore coat; C, cortex; UC, undercoat; OC, outer spore coat; S, mature spore in an unlysed sporangium (from Faust and Bulla, 1982).


I
II
III
III
IV
V
VI
VII
CW
M
OM
AF
FS
OI
PC
IM
OM
PW
IF
OC
UC
S
7h
7h
7h
7h
7h
7-8h
7-8h
8h
8h
8h
8h
8h
8-9h
8-9h
10h
11h
12h
mature-
unlysed

characterized by the formation of a free forespore with an inner and outer membrane. The cortex and the primordial cell wall are formed between the inner and outer membranes during stage IV. In the course of stage V, the layers of the sporecoat develop and the exosporium which surrounds the spore is completed. During stage VI, the spore maturation proceeds and is completed during stage VII At this stage lytic enzymes release the spores and crystals from the sporangium The events of sporulation and crystal formation have been discussed in detail in reviews presented by Ribier and Lecadet (1973), and Bechtel and Bulla (1976).

The predominent crystals of most *B thuringiensis* subspecies so far identified are comprised of 130-140 kilodalton polypeptides (Aronson *et al.*, 1986). There are, however, differences in number, shape, and composition of these parasporal inclusions While the numbers can range from 1-5 crystals/ bacterial cell, the shape that they can assume is bipyramidal, cuboid, ovoid, or amorphous (Aronson *et al.*, 1986)

Types of toxins other than the δ-endotoxin

α-exotoxin

Also identified as possibly lecithinase C (phospholipase C), α exotoxin is a heat-labile protein that is produced by vegetative cells (Toumanoff, 1954). However, Krieg (1971b) and Ivinskiene (1978) determined that this toxin was not a lecithinase. α exotoxin was shown to be toxic to the diamond-back moth, *Plutella xylostella* It is also toxic to mice *per os* (Krieg, 1971a), hence the synonym "mouse factor" The presence of the toxin is only demonstrable by bioassay and cannot be readily isolated or purified (Krieg and Lysenko, 1979) Its size is 45 50 kilodaltons (Krieg, 1986).

ß-exotoxin

The heat stable ß-exotoxin was discovered by McConnell and Richards (1959). It is also known as a fly factor, a heat-stable toxin, a thermostable toxin, and thuringiensin (Beegle and Yamamoto, 1992). It is

produced by vegetative cells as a water-soluble nucleotide analog of adenine, ribose, glucose, and allaric acid with a phosphate group (Farkas *et al.*, 1969). The ß-exotoxins produced by different subspecies of *B. thuringiensis* are not all identical (Mohd-Salleh *et al.*, 1980; Gingrich *et al.*, 1992a, 1992b). Type II ß-exotoxin was found to be more specific than type I and is very active against *L. decemlineata* (Levinson *et al.*, 1990). Type I ß-exotoxin has a very wide host range and acts by inhibiting the DNA-dependent RNA polymerase (Sebesta and Horska, 1970). In the Commonwealth of Independent States, commercial products are used effectively against species of red mites (Acari), larvae of house flies (Diptera, Muscidae) and blowflies (Diptera, Calliphoridae) (Beegle and Yamamoto, 1992)

δ-endotoxin

The parasporal crystals (δ-endotoxins) are divided into three pathotypes according to their activity spectra (Krieg *et al.*, 1983):

Pathotype A: The crystals are active against lepidopteran larvae and are usually bipyramidal. The crystal is composed of 230 kilodalton dimers (Holmes and Monroe, 1965) which, under alkaline conditions dissociate into 135 kDa protoxins that become toxic only after partial enzymatic digestion. Yamamoto and McLaughlin (1981) isolated from *B. thuringiensis* subsp *kurstaki* a 65 kDa protein different from the 135 kDa protein It was termed P2 and was found to be toxic to both Lepidopterans and Dipterans

Pathotype B: The crystals are highly active against mosquito (Culicidae) and blackfly (Simuliidae) larvae and are presently found in subsp. *israelensis* and a dipteran-active subsp. *morrisoni* isolate (Beegle and Yamamoto, 1992) Several irregularly shaped crystals are produced per sporangium. These crystals are composed of three groups of proteins: 128-135 kDa, 72-78 kDa, and 27 kDa Although the latter has little insecticidal activity, it may have synergistic action with the former toxin proteins (Wu and Chang, 1985).

- Pathotype C: These crystals are active against coleopterans and are located in subsp. *tenebrionis*. They are unique in shape and

protein characteristics in that the crystal is square and flat, and is made up of 67 kDa protein molecules (McPherson *et al.*, 1988). Unlike other *B. thuringiensis* subspecies, *tenebrionis* crystals do not have disulfide bonds (Bernhard, 1986). In addition, unlike other crystal types, NaBr- or alkali-dissolved crystals recrystallize when NaBr is removed or the pH is lowered (Beegle and Yamamoto, 1992).

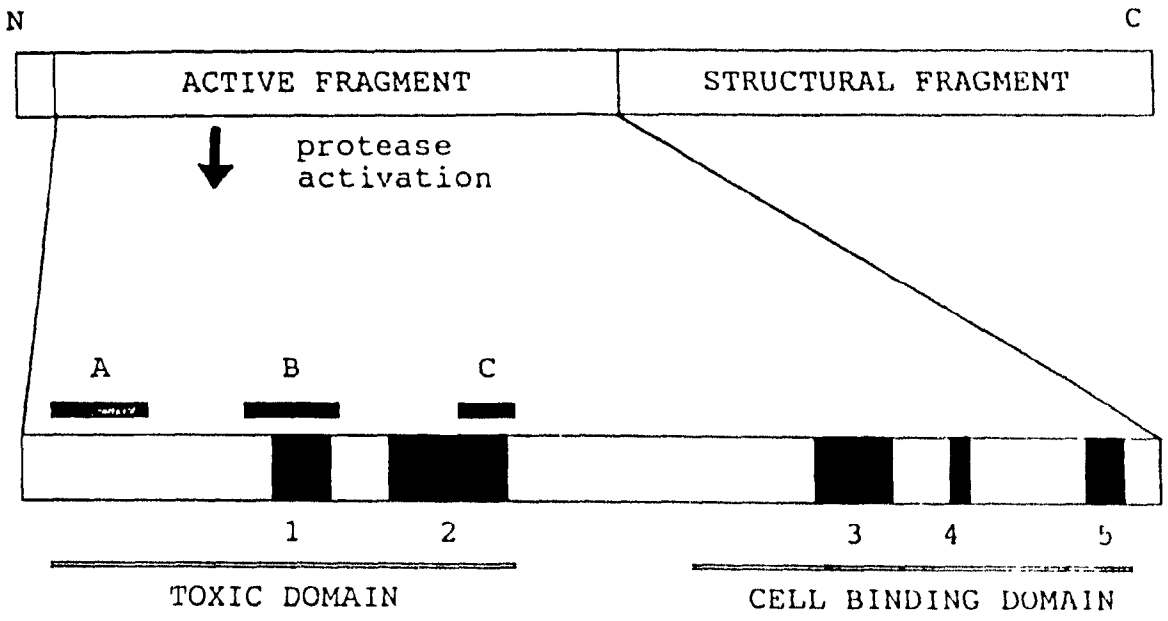
Mode of action

The mode of action of the δ-endotoxin is partially attributed to its three-dimentional structure. The domains of a typical pathotype A protoxin derived from *B. thuringiensis* is illustrated in Figure 2. The protoxin is cleaved by insect midgut proteases releasing the C terminal structural fragment. The remaining N-terminal half forms the active fragment which consists of at least 2 domains, the toxic and the cell binding domains. There are five conserved regions within the active fragment. The toxic domain contains hydrophobic regions (solid dashes) and the putative ion pore formed by the Cry toxin is derived from α-helices found within the toxin domain. The cell-binding domain is important in receptor binding and structural conformation (Gill *et al.*, 1992).

Recently, Li *et al* (1991) published the structure of subsp *tenebrionis* toxin called CRY IIIA. This toxin is made of 644 amino acid residues which are divided into three domains: I, II, and III. Domain I is probably involved in pore formation. Domain II is the receptor binding domain. And domain III is thought to protect the other domains from proteinase digestion. The determination of the structure makes it possible to determine the involvement of each amino acid residue in the insecticidal activity.

In general, the crystal is an aggregate of a relatively large protein subunit that can be dissociated under specific conditions (Faust and Bulla, 1982). It is insoluble in water and usually dissolves under alkaline conditions of the insect gut to release the protein subunits (Carroll *et al.*, 1989). Many of the larger crystals are held together by intermolecular disulfide bonds which require very high pH or reducing conditions to initiate dissolution of the crystals (Nickerson, 1980). The

Figure 2. Domains of activated *Bacillus thuringiensis* toxin (from Gill *et al.*, 1992).


N
C
ACTIVE FRAGMENT
STRUCTURAL FRAGMENT
protease
activation
A
B
C
1
2
3
4
5
TOXIC DOMAIN
CELL BINDING DOMAIN

crystal proteins of *B. thuringiensis* subsp. *tenebrionis* are smaller, and are rendered insoluble by salt bridges (Asp 142-Asp 165, Glu 223-Lys 293) (Li *et al.*, 1991) that break down in concentrated salt solutions (NaBr) *in vitro* (Bernhard, 1986) It has been reported that some insects have a low susceptibility to specific crystal proteins due to the inefficient solubilization of the crystals which, once solubilized *in vitro*, significantly enhance the toxic activity (Jaquet *et al.*, 1987).

Once solubilized, the subunits of most crystals of pathotype A are proteolytically converted into an active fragment (Figure 2) by the midgut proteases The size of the smallest active fragment, the protease-resistant core, varies with the *B. thuringiensis* strain (Gill *et al.*, 1992) (*e.g.* only limited proteolysis occurs at the N-terminal for CRY I protoxins while approximately 500 amino acid residues are removed from the C terminal) In contrast, CRY II and III proteins appear to be naturally truncated and do not undergo protease-mediated C-terminal cleavage. In fact, even a small C-terminal truncation of eleven amino acids may result in a loss of activity in CRY IIA (Widner and Whiteley, 1989).

The insecticidal specificity of the toxins is due to high-affinity binding to specific membrane receptors (Li *et al.*, 1991). The toxin has been shown to bind specifically to the brush border membrane vesicles of the midgut epithelium (Gill *et al* , 1992) The crystal toxins require a specific plasma membrane receptor on the midgut epithelial cells. The affinity of the crystal toxins varies from relatively low to high. An active area of research involves the characterization of the receptor which, in some cases, is believed to be a glycosylated protein (Knowles and Ellar , 1986, Hofmann *et al.*, 1988; Ellar, 1990). It has been suggested that receptor glycosylation is essential for toxin binding and toxicity against pathotype A or Cry I gene product the toxin acting like a lectin (Ellar, 1990)

The δ endotoxin eventually causes the epithelial cells to disrupt by forming lytic pores in the insect membrane (Li *et al.*, 1991). The trigger for the pore formation may be due to receptor binding and the consequent interaction of the toxin with the membrane bilayer. The formation of pores in the membrane of susceptible insect cells causes a net influx of ions and an accompanying inflow of water which forces the cells to swell and

lyse (Hofte and Whiteley, 1989).

Variability in insecticidal activity is also attributed to the susceptibility of the insect, with three types being identified (Milne *et al.*, 1990):

- Type I: Insects which suffer general paralysis and show blood pH change to crystal protein alone (*Bombyx mori* and *Manduca sexta*).

- Type II: Insects which are susceptible to crystals alone, exhibit gut paralysis but no leakage, no change in blood pH, and no general paralysis (most Lepidoptera).

-Type III. Insects which require both crystals and spores for pathogenesis (*Anagasta kuehniella* and *Lymantria dispar*).

REFERENCES

Angus, T. A. 1956. Extraction, purification and properties of *Bacillus sotto* toxin. *Can J. Microbiol.* **2**: 416-426.

Anon., 1977. The carrot weevil. Agriculture Canada Insect Identification. Sheet no. 28.

Aronson, A. I., W. Beckman, P. Dunn. 1986. *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.* **50**: 1-24.

Baines, D., R Stewart and G. Boivin. 1990. Consumption of carrot weevil (Coleoptera: Curculionidae) by five species of carabids (Coleoptera: Carabidae) abundant in carrot fields in southwestern Quebec. *Environ. Entomol* **19**: 1146-1149.

Beaudoin, G. and G Boivin. 1985. Effets d'accouplements répétés sur l'oviposition du charançon de la carotte, *Listronotus oregonensis* (Coleoptera: Curculionidae) au laboratoire. *Rev. Entomol. Québec.* **30**: 23-27.

Bechtel, D. B. and L A. Bulla. 1976. Electron mocroscopic study of sporulation and parasporal crystal formation in *Bacillus thuringiensis*. *J. Bacteriol.* **127**: 1472-1481.

Beegle, C. C. and T. Yamamoto 1992. Invitation paper (c. P. Alexander Fund)· Histology of *Bacillus thuringiensis* Berliner, research and development. Can Entomol. 124· 587-616.

Belair, G and G Boivin 1985 Susceptibility of the carrot weevil (Coleoptera Curculionidae) to *Steinernema feltiae*, *Steinernema bibionis* and *Heterorhabditis. heliothidis. J. Nematol.* **17**: 363-366.

Berliner, F 1911 Uber die Schalffsucht der Mehlmottentaupe. *Z. Gesamte Getreidewes.* 3· 63-70

Bernhard, K. 1986 Studies on the delta-endotoxin of *Bacillus thuringiensis* var *tenebrionis. FEMS Microbiol. Lett.* **33**: 261-266.

Boivin, G. 1985a Evaluation of monitoring techniques for the carrot weevil, *Listronotos oregonensis* (Coleoptera: Curculionidae). *Can. Entomol* **117**: 927-933

Boivin, G. 1985b *Anaphes sordidatus* (Girault) (Hymenoptera: Mymaridae), an egg parasite of the carrot weevil, *Listronotus oregonensis* (Le Conte). *Can. Entomol.* **118**: 393-394.

Boivin, G. and G. Bélair. 1989. Infectivity of two strains of *Steinernema feltiae* (Rhabditida: Steinernematidae) in relation to temperature, age and sex of carrot weevil (Coleoptera: Curculionidae) adults. *J. Econ. Entomol.* **82:** 762-765.

Boivin, G. and P. Sauriol 1984. Reseau de lutte integree: programme de dépistage. *Prod. Agric.* 7: 20-21.

Boyce, A. M. 1929. A study of the biology of the parsley stalk weevil. *J. Econ. Entomol.* 20: 814.

Brodeur, L. 1985. Eliminons le charançon de la carotte. *L'Horticulture.* 1:15.

Buchanan, L. L. 1932. The parsley and carrot weevil. *Bull. Brooklyn Entomol. Soc.* **27**: 7.

Carroll, J., J. Li and D. J. Ellar. 1989. Proteolytic processing of a coleopteran specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* **261**: 99-106.

Chandler, S. C. 1926. The economic importance of the carrot weevil in Illinois. *J. Econ. Entomol* **19:** 490 494.

Collins, R. D. and E Grafius 1986a Biology and life cycle of *Anaphes sordidatus* (Hymenoptera: Mymatidae), an egg parasitoid of the carrot weevil (Coleoptera: Curculionidae) *Environ. Entomol.* **15**: 100 105.

Collins, R. D. and E. Grafius. 1986b. Courtship and mating behaviour of *Anaphes sordidatus* (Hymenoptera. Mymatidae), a parasitoid of the carrot weevil (Coleoptera· Curculionidae) *Ann. Entomol Soc. Am* **79:** 31-33.

Collins, R. D. and E. Grafius 1986c. Impact of the egg parasitoid, *Anaphes sordidatus* (Hymenoptera Mymatidae), on the carrot weevil (Coleoptera Curculionidae) *Environ Entomol.* **15**: 469 475.

CPVQ (Conseil de producteurs vegetal du Québec) 1987. Légumes. Protection. Ministere de l'alimentation, des pêches et de l'agriculture du Québec. Agdex 250/605. Gouvernement du Québec, Québec.

de Barjac, H. and A. Bonnefoi. 1962. Essai de classification biochimique et sérologique de 24 souches de *Bacillus* du type *B. thuringiensis*. *Entomophaga.* 7. 5-31.

de Barjac, H. 1978. Une nouvelle variété de *Bacillus thuringiensis* tres

toxique pour les moustiques: *Bacillus thuringiensis* var. *israelensis*, sérotype 14. *C. R. Acad. Sci. (Paris) Ser. D* 286: 797-800.

de Barjac, H. 1981. Identification of H-serotype of *Bacillus thuringiensis*. In: Microbial Control of Pest and Plant Diseases, 1970-1980, ed. H. W. Burges pp. 35-43. London: Academic Press.

de Barjac, H and A. Bonnefoi 1968 A classification of strains of *Bacillus thuringiensis* Berliner with a key to their differentiation. *J. Invertebr. Pathol.* 11 335-347

de Barjac, H. and A Bonnefoi 1973 Mise au point sur la classification des *Bacillus thuringiensis*. *Entomophaga*. 18: 5-17.

Dulmage, H T 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti*. *J. Invertebr. Pathol.* 15: 232-239.

Ellar, D. J. 1990 Pathogenicity determinants of entomopathogenic bacteria. In: Proc. 15th Int Colloqium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, 20-24 August, 1990. pp. 298-302. Adelaide, Australia Society of Invertebrate Pathology.

Farkas, J., K Sebesta, K Horska, Z. Samek, L. Dolejs and F. Sorm. 1969. The structure of exotoxin of *Bacillus thuringiensis* var. *gelechiae*. *Coll Czech. Chem Commun.* 34 1118 1120.

Faust, R M. and L A. Bulla, Jr. 1982 Bacteria and their toxins as insecticides. In Microbial and Viral Pesticides, ed. E. Kurstak. pp. 75-208 New York. Marcel Dekker, Inc.

Ferro, D. N. and W D Gelernter 1989 Toxicity of a new strain of *Bacillus thuringiensis* to Colorado potato beetle (Coleoptera: Chrysomelidae) *J Econ. Entomol.* 82: 750-755.

Gill, S. S., E A Cowles and P V Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins *Ann. Rev. Entomol.* 37: 615-636

Gingrich, R. E., M Hauffer and N A. Allan. 1992a. Bioassay for ß-exotoxin and other compounds of *Bacillus thuringiensis* with larvae of the horn fly, *Haematobia irritans*. In: Spectrum of Activities of Varieties of *Bacillus thuringiensis*, eds. L. C. Lewis and H. T. Burges. Florida, Boca Raton. CRC Press.

Gingrich, R. E., M. Hauffer and N. A. Allan. 1992b. Bioassay of HD formulations of *Bacillus thuringiensis* with adults of the hairy goat louse, *Bovicola crassipes*. In: Spectrum of Activities of Varieties of *Bacillus thuringiensis*, eds. L. C. Lewis and H. T. Burges Florida. Boca Raton: CRC Press.

Goldberg, L. J. and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univeritattus*, *Aedes aegypti* and *Culex pipiensis*. *Mosq. News* **37**: 355-358.

Grafius, E. and R. D. Collins. 1986. Overwintering sites and survival of the carrot weevil, *Listronotus oregonensis* (Coleoptera Curculionidae). *Environ. Entomol.* **15**: 113-117.

Grafius, E., M. Otto and B. Collins. 1983. Detection and control of carrot weevil. *Mich. St. Univ. Ext. Bull.* E-890, 2pp.

Hannay, C. L. 1953. Crystalline inclusions in aerobic sporeforming bacteria. *Nature* **172**: 1004.

Harcourt, D. G. 1959. Insects of the season 1958 in the Ottawa area. *Can. Ins. Pest Rev* **37**. 80-84, 156, 179, 196.

Harcourt, D. G. 1963. Vegetable insects. *Can. Ins. Pest Rev.* **41**: 98

Harris, H. M. 1926. A new carrot pest, with notes on its life history. *J Econ. Entomol* **19**: 494-497

Heimpel, A. M. 1967. A critical review of *Bacillus thuringiensis* Berliner and other crystalliferous bacteria. *Ann Rev Entomol.* **12**: 287-322

Heimpel, A. M. and T. A. Angus. 1958. The taxonomy of insect pathogens related to *Bacillus cereus* Frankland and Frankland. *Can J Microbiol.* **4**: 531-541

Heimpel, A. M. and T. A. Angus. 1960. On the taxonomy of certain entomogenous crystalliferous bacteria. *J. Insect Pathol* **2**: 311-319

Henderson, L. S. 1939. A revision of the genus *Listronotus*: I (Curculionidae: Coleoptera). *Kan Univ Sci. Bull* **26**: 215-337

Herrnstadt, C., G. G. Soares, E. R. Wilcox and D. I. Edwards. 1986. A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. *Bio/Technology* **4**: 305-308

Herrnstadt, C., T. E. Gilory, D. A. Sobleski, B. D. Bennett and F. H. Gaertner. 1987. Nucleotide sequences and deduced amino acid sequence

of a coleopteran-active delta-endotoxin gene from *Bacillus thruingiensis* ssp. *san diago Gene (Amst)*. **57**: 37-46.

Hofmann, C., P. Luthy, R. Hutter and V. Pliska. 1988. Binding of the delta-endotoxin from *Bacillus thruingiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *Eur. J. Biochem.* **173**. 85-91.

Hofte, H. and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**: 242-255.

Holmes, K. C. and R. E. Monroe. 1965. Studies on the structure of parasporal inclusions from *Bacillus thuringiensis*. *J. Mol. Biol.* **14**: 572-581.

Ishiwata, S. 1901. On a kind of severe flacherie (Sotto disease). *Dainihon Sanshi Kaiho* **114**: 1-5

Ishiwata, S. 1905 About "Sotto Kin", a bacillus of a disease of the silkworm. *Dainihon Sanshi Kaiho* **160**: 1-8.

Ivinskiene, V 1978. Nonidentity of lecithinase and α-toxin from *Bacillus thuringiensis* Berliner *Tezisy Konf. Molodykh Uch. Inst. Zool. Parazithol Akad Lit. USSR* **2**: 13-15.

Jahn, N., W Schnetter and K Geider. 1987. Cloning of an insecticidal toxin gene of *Bacillus thuringiensis* ssp. *tenebrionis* and its expression in *Escherichia coli* cells. *FEMS Microbiol. Lett.* **48**: 311-316.

Jaquet, F., R Hutter and P. Luthy. 1987. Specificity of *Bacillus thuringiensis* delta-endotoxin. *Appl. Environ. Microbiol.* **53**: 500-504.

Jones, D R., V. Karunakaran and H. D. Burges. 1983. Phages naturally associated with the *aizawai* variety of insect pathogen *Bacillus thuringiensis* and their relevance to strain identification. *J. Appl. Bacteriol* **54** 373 377.

Knowles, B H and D. J Ellar. 1986. Characterization and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran specific δ-endotoxin. *J. Cell Sci* **83**. 89-101

Krieg, A. 1971a. Concerning α-exotoxin produced by vegetative cells of *Bacillus thuringiensis* and *Bacillus cereus*. *J. Invertebr. Pathol.*

17: 134-135.

Krieg, A. 1971b. Is the potential pathogenicity of bacilli for insects related to production of α-exotoxin. *J. Invertebr. Pathol.* 18: 425 426.

Krieg, A. 1986. *Bacillus thuringiensis*, ein mikrobielles Insektizid, Grundlagen und Anwendung. *Acta Phytomed* 10: 1-191.

Krieg, A. and O Lysenko. 1979. Toxins and enzymes of several species of *Bacillus*, especially of the *Bacillus cereus thuringiensis* group *Zbl Bakb. II. Abstr.* 134. 70 88

Krieg, A., A. M. Huger and W. Schnetter 1987a. *Bacillus thuringiensis* var. *san diego* strain M-7 is identical to the formerly in Germany isolated strain BI 256 82 *B. t.* ssp *tenebrionis* which is pathogenic to coleopteran insects. *J Appl. Entomol* 104 417 424

Krieg, A., A. M Huger, G A Langenbruch and W Schnetter 1983 *Bacillus thuringiensis* var *tenebrionis*· ein neuer, gegenuber Larven von Coleopteren wirksamer Pathotyp. *Z angew Ent* 96 500 508

Krieg, A., W. Schnetter, A M Huger and G. A Langenbruch 1987b. *Bacillus thuringiensis* ssp *tenebrionis* strain BI 256 82 third pathotype within the H-serotype 8A88. *Syst Appl. Microbiol* 9: 138 141.

Krywienczyk, J. and T. A. Angus. 1960. A serological comparison of the parasporal bodies of three insect pathogens. *J. Insect Pathol.* 2: 411-417.

Krywienczyk, J., H. T. Dulmage and P. G. fast. 1978. Occurrence of two serological distinct groups within *Bacillus thuringiensis* serotype 3a, b var. *kurstaki*. *J. Invertebr. Pathol* 31. 372 375

Kurstak, E. 1962 Donnees sur l'epizootie bacterienne naturelle provoquee par un *Bacillus* du type *Bacillus thuringiensis* sur *Ephystia kuhniella* Zeller. *Entomophaga Mem. Hors Ser.* 2· 245-247

Lambert, B. and M. Peferoen 1992 Insecticidal promise of *Bacillus thuringiensis* *Bioscience* 42 112 122

Levinson, B L., K L. Kasyan, S S Chiu, T. C Currier and J M. Gonzales, Jr 1990. Identification of β-exotoxin production, plasmids encoding β-exotoxin, and a new exotoxin in *Bacillus thuringiensis* by using high-performance liquid chromatography. *J.*

Bacteriol. **172**: 3172-3179.

Li, J., J. Carroll and D. J. Ellar. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature* **353**: 815-821.

Li, J., C. Henderson, J. Carroll and D. Ellar. 1988. X-ray analysis of the crystalline parasporal inclusion on *Bacillus thuringiensis* var. *tenebrionis*. *J. Mol. Biol.* **199**: 543-544.

Luthy, P., J. L. Cordier and H. M. Fischer. 1982. *Bacillus thuringiensis* as a bacterial insecticide: basic considerations and application. In: Microbial and Viral Pesticides, ed. E. Kurstak. pp. 35-74. New York: Marcel Dekker, Inc.

Martel, P., C. R. Harris and H. J. Svec. 1976. The life history of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae). *Can. Entomol.* **107**: 471-475.

Martel, P., H. J. Svec and C. R. Harris. 1975. Mass rearing of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae) under controlled environmental conditions. *Can. Entomol.* **107**: 95-98.

McConnel, E. and A. G. Richards. 1959. The production by *Bacillus thuringiensis* Berliner of a heat-stable substance toxic for insects. *Can. J. Microbiol.* **5**: 161-168.

McPherson, S. A., F. J. Perlak, R. L. Fuchs, P. G. Marrone, P. B. Lavtik and D. A. Fischoff. 1988. Characterization of the coleopteran-specific protein gene of *Bacillus thuringiensis* var. *tenebrionis*. *Bio/Technology*. **6**: 61-66.

Milne, R., A. Ge, D. Rivers and D. H. Dean. 1990. Specificity of insecticidal crystal proteins. In: Analytical Chemistry of *Bacillus thuringiensis*, eds. L. A. Hickle and W. L. Fitch. pp. 22-35. Washington D. C.: American Chemical Society.

Mohd-Saleh, M. B., C. C. Beegle and L. C. Lewis. 1980. Fermentation media and production of exotoxin by three varieties of *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **35**: 75-83.

Nickerson, K. W. 1980. Structure and function of the *Bacillus thuringiensis* protein crystal. *Biotechnol. Bioeng.* **12**: 1305-1335.

Norris, J. R. 1964. The classification of *Bacillus thuringiensis*. *J. Appl. Bacteriol.* **27**: 439-447.

Pepper, B. B. 1942. The carrot weevil, *Listronotus latiusculus* (Bohe.), in New Jersey and its control. *N. J. Agric. Exp. Stat. Bull.* **693** 1-20.

Pepper, B. B. and L. E. Hagmann. 1938 The carrot weevil, *Listronotus latiusculus* (Boh.), a new pest on celery. *J. Econ. Entomol* **31** 262 266.

Perron, J. P. 1971. Insect pests of carrots in organic soils of southwestern Quebec with special reference to the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae). *Can. Entomol.* **103**: 1441-1448.

Poinar, G. O., Jr. 1989. Examination of neoaplectanid species *feltiae* Filipjev, *carpocapsae* Weiser and *bibionis* Bovien (Nematoda: Rhabditida). *Rev. Nematol.* **12**: 375.

Poinar, G. O., Jr. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. In Entomopathogenic Nematodes in Biological Control, eds. R Gaugler and H. K. Kaya. pp. 23 61. Boston: CRC Press.

Ribier, J. and M. M. Le Cadet. 1973. Etude ultrastructurale et cinetique de la sporulation de *Bacillus thuringiensis* var. *berliner* 1715. Remarques sur la formulation de l'inclusion parasporale *Ann. Microbiol.* **17**: 373-375.

Rietmuller, U. and G. A Langenbruch 1989. Two bioassay methods to test the efficacy of *Bacillus thuringiensis* ssp. *tenebrionis* against the larvae of the Colorado potatoe beetle (*Leptinotarsa decemlineata*) *Entomophaga* **34**: 237-246

Searle, T. and W. N Yule 1988. Fungal control of the carrot weevil. *Proc. XVIII Int. Congress Entomol. Vancouver, B. C* p. 262

Sebesta, K and K. Horska. 1970. Mechanisms of inhibition of DNA dependent RNA polymerase by exotoxin of *Bacillus thuringiensis* *Biochem. Biophys. Acta* **209**: 357-367.

Sekar, V. 1988 The insecticidal crystal protein gene is expressed in vegetative cells of *Bacillus thuringiensis* var. *tenebrionis*. *Curr. Microbiol.* **17**: 347-350.

Simonet, D. E. and B. L Davenport 1981. Temperature requirements for development and oviposition of the carrot weevil. *Ann. Entomol. Soc. Am.* **74**: 312-315

Smith, R. A. 1987. Use of crystal serology to differentiate among varieties of *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **50**: 1-8.

St. Julian, G., L. A. Bulla and C. W. Hesseltine. 1971. Germination and outgrowth of *Bacillus thuringiensis* and *Bacillus alvei* spores viewed by scanning electron and phase-contrast microscopy. *Can. J. Microbiol* **17** 373-375

Stevenson, A B. 1976. Seasonal history of the carrot weevil, *Listronotus oregonensis* (Coleoptera Curculionidae) in the Holland Marsh, Ontario. *Proc Entomol Soc. Ont.* **107**: 71-78.

Stevenson, A. B. 1981. Carrot insects. Ontario Ministry of Agriculture and Food Fact Sheet No 81-007.

Stevenson, A B. 1985 Early warning system for the carrot weevil (Coleoptera Curculionidae) and its evaluation in commercial carrots in Ontario. *J Econ Entomol.* **78**. 704-708.

Toumanoff, C. 1954 L'action de *Bacillus cereus* var. *alesti* Toum. et Vago sur les chenilles de *Galleria melonella* L. and *Hyponomeuta eognatella*. *Ann Inst. Pasteur Paris.* **86**: 570-597.

Whitcomb, W D. 1965 The carrot weevil in Massachussetts: biology and control *Mass Agric. Exp. Stat. Bull* **550**: 1-30.

Widner, W R and H R Whiteley 1989 Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* ssp. *kurstaki* possess different host range specificities *J. Bacteriol.* **171**: 965-974.

Wu, D. and F. N Chang. 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* ssp. *israelensis* crystal *FEBS Lett* **190**: 232-236

Yamamoto, T. and R E McLaughlin. 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. *Biochem. Biophys. Res. Commun.* **103**: 414-421

Zehnder, G W. and W D Gelernter. 1989. Activity of the M-ONE formulation of a new strain of *Bacillus thuringiensis* against the Colorado potato beetle (Coleoptera: Chrysomelidae): relationship between susceptibility and insect life stage. *J. Econ. Entomol.* **82**: 750-761

CHAPTER 2

Response of the carrot weevil, *Listronotus oregonensis* (Coleoptera:Curculionidae), to strains of *Bacillus thuringiensis*.

ABSTRACT

Mortality and frass bioassays were done to investigate the toxicity of seven strains of *Bacillus thuringiensis* against the adult carrot weevils, *Listronotus oregonensis*. A semi-artificial diet of carrot foliage with 4% agar was selected to maximize feeding by the insects . *Bacillus thuringiensis* subsp. *tenebrionis* (BTT), and two unidentified *B. thuringiensis* strains, A30 and A429, gave the lowest LD_{50} values. The frass bioassay supported the conclusions of the mortality assay. Mortality of adults continued after their removal from the insecticidal medium, with the highest mortality being caused by strains A429 and BTT. Survivors from the frass bioassay, initially exposed to strains A30, A429, and BTT, did not resume normal levels of feeding after their removal from the insecticidal medium Attempts to reisolate the *B. thuringiensis* microorganisms from the experimental insects revealed that the carrot weevil harbours *B. thuringiensis*-like organisms in its gut and on other structures

INTRODUCTION

The carrot weevil, *Listronotus oregonensis* (Le Conte), is a major pest of Umbelliferous plants in the northeastern sectors of the United States of America (Chandler, 1926; Pepper, 1942; Whitcomb, 1965, Grafius and Otto, 1979) and Canada (Perron, 1971; Martel *et al.*, 1975; Stevenson, 1976). It attacks celery (Pepper and Hagmann, 1938) and parsley (Boyce, 1927), but its preferred host is the carrot with weed species such as wild carrots (*Daucus carota* L.), plantain (*Plantago* sp.), and dock (*Rumex* sp.) also serving as hosts (Pepper, 1942, Ryser, 1975)

The adult female lays eggs in the petiole and crown of the carrot. The larvae, upon emergence, tunnel into the root where they feed and develop until they exit into the soil and pupate (Perron, 1971) The carrot weevils overwinter in the soil as adults

Since 1967, carrot root injury caused by the carrot weevil ranged from 2-22% of the total crop production in the Quebec regions of Sherrington, St -Michel, and Ste Clotilde (Perron, 1971) with damage of up to 40% of the carrot crop when left uncontrolled (Boivin, 1985a). The packing and canning industries tolerate no more than 5% root injury (Perron, 1971) which makes efficient control measures necessary

The only control method available for the carrot weevil is the use of foliar insecticides against overwintered adults before they oviposit (Boivin, 1985a). However, the development of resistance to chemical insecticides by many pest insects, and the general concern for environment damage have resulted in the development of an integrated pest management program with emphasis on alternative control methods. There is little information on alternative control strategies in the literature. To date, an egg parasitoid, *Anaphes sordidatus* (Boivin, 1985b) and entomogenous nematodes, *Steinernema carpocapsae* [=*S. feltiae* (Poinar, 1989)], *S. feltiae* [=*S. bibionis* (Poinar, 1989)], and *Heterorhabditis bacteriophora* [=*H. heliothidis* (Poinar, 1990)] (Belair and Boivin, 1985, Boivin and Belair, 1989) have been examined

The entomopathogenic bacterium, *Bacillus thuringiensis*, occupies an important position in the field of biological control. *B. thuringiensis* is a Gram-positive, endospore-forming, soil-dwelling bacterium which

produces an insecticidal crystalline protein (ICP, also known as δ-endotoxin) during sporulation (Hofte and Whiteley, 1989). Once ingested by susceptible insects, the ICP dissolves in the midgut and is proteolytically activated to a smaller molecular weight toxin (Lilly *et al.*, 1980) These toxins bind to receptors on the brush border membrane of the midgut epithelial cells (Hofmann *et al.*, 1988a and b; Knowles and Ellar, 1986; Hendrickx *et al.*, 1989), causing cell lysis, feeding inhibition, and eventually death (Fast, 1981; Krieg *et al.*, 1983). Previously described strains have demonstrated activity against lepidopteran and dipteran species (Kurstak, 1962; Goldberg and Margalit, 1977). More recently, *B. thuringiensis* strains toxic to economically important pests of the order Coleoptera have been discovered (Krieg *et al.*, 1983; Herrnstadt *et al.*, 1986).

B. thuringiensis subsp *tenebrionis* [synonymous to *B. thuringiensis* subsp *san diego* (Krieg *et al.*, 1987)], a strain pathogenic to Coleoptera, together with six unidentified *B. thuringiensis* strains were used in the current study. The undefined strains, isolated from Canadian soils, were shown to be active against various coleopteran insect species (Moser, 1990).

The present study was done to evaluate the toxicity of the aforementioned strains on the carrot weevil and to determine survival of the insects after intoxication.

MATERIALS AND METHODS

Chemicals

Monobasic and dibasic potassium phosphate (KH_2PO_4 and K_2HPO_4), dithiothreitol, phenylmethylsulfonylfluoride, Ampicillin, Polymyxin B, and sodium carbonate (Na_2CO_3) were purchased from Sigma Chemical Company (Missouri,USA). Nutrient agar was purchased from BBL (Maryland,USA) and the Bio-Rad protein assay kit was from Bio-Rad (Ontario,Canada).

Bacterial cultures and spore-crystal suspensions

Bacillus thuringiensis subsp. *tenebrionis*, and the six unidentified strains of *B. thuringiensis* coded A30, A299, A311, A409, A410, and A429, were supplied by Imperial Chemical Industries Biological Products, North American Technical Centre (Ontario, Canada) The *Bacillus thuringiensis* strains were grown on nutrient agar buffered (pH 7 0) with equimolar concentrations (0.05M) of KH_2PO_4 and K_2HPO_4. After 5 days incubation at 30 °C, the bacterial lawn, based upon microscopic examination, consisted of vegetative cells, sporangia, spores and crystals. These stages were harvested by scraping, lyophilized, and stored at -20 °C.

Spore-crystal suspensions of each strain were used in all the toxicity assays To ensure that toxicity measurements would not reflect crystals containing different amounts of proteins, the concentration of crystal protein for each bacterial strain was determined. Spore crystal mixtures were suspended in 0 01% Triton X 100 (v/v) solution and washed three times by centrifugation (11,750 xg, 22 °C, 3 min) The final pellet was resuspended in a crystal solubilizing buffer [Na_2CO_3, 4 3 g/l; phenylmethylsulfonylfluoride (a protease inhibitor to prevent ICP degradation), 87 mg/l; dithiothreitol (to reduce disulfide bonds), 15.4 mg/L; pH 10.0] and solubilized at 42 °C by incubating for 2 h, vortexing every 30 min The suspension was centrifuged (11,750 xg, 22 °C, 3 min) and the supernatant assayed for total protein according to the Bradford method (1976) using a Bio Rad protein assay kit (Bio Rad, Ontario, Canada). The protein assay is based on a colour change of the dye, the optical density of the resulting solution increasing with increasing concentrations of protein. Bovine serum albumin was used as a standard.

Colony and rearing conditions

Carrot weevils available in laboratory culture were periodically supplemented with weevils from Agriculture Canada Research station (St. Jean-sur-Richelieu) to maintain hybrid vigor The colony was reared in an incubator with a photoperiod of 16 h (Martel *et al*, 1975) and a temperature regime of 24:16 °C (Day:Night). The adults were placed in

jars containing carrots, the carrots serving as both food and oviposition sites. After the insects oviposited in the carrots, the carrots were transferred to a plastic box having a screen on the bottom and this box was then placed inside another which had a solid bottom Once the larvae emerged they dropped to the lower box from whence they were collected and placed in moist sterile soil for pupation and adult emergence. These adults were used to both maintain the colony and for experimental purposes.

Selection of diet maximizing insect feeding

To select a diet that would be available year round, and act as a gustatory stimulant ensuring uptake of the ICP placed on it, six diets were fed to the adult insects:(1) carrot root, (2) softened (steamed) carrot root with 2% (w/v) agar, (3) fresh foliage, (4) foliage extract containing 4% (w/v) agar, (5) foliage homogenate in 4% (w/v) agar, and (6) 4% (w/v) agar Diets 4 and 5 were prepared by suspending 22 g of chopped foliage in 70 ml of water Foliage homogenates consisted of the fibrous and soluble portions of the suspension whereas the extract was rendered low in fiber by gravity sedimentation. Equal volumes of autoclaved agar were added to the diets to produce the final concentrations of agar. Abundant food was placed in plastic Petri dishes (15x100mm diameter) with moist filter paper to which 10 adults were added The plates were placed in the incubators and the number of frass pellets counted daily for three days The filter paper was changed daily for fresh counts. There were three replicates for each treatment.

Insecticidal Bioassays

Two different bioassays were done to measure the toxicity of the strains against the carrot weevil: one based upon adult mortality and the other a modification of the frass production assay of van Frankenhuyzen and Gringorten (1991) regarded as a rapid method to determine the specificity of numerous *B. thuringiensis* toxins against Lepidoptera.

Diet 5, the optimum medium for insect feeding, was offered to the

insects as food cylinders (6x6mm diameter; 10 cylinders/plate) cut from foliage homogenate supplemented with agar (Chapter 2, "Selection of diet maximizing insect feeding"). The cylinders were coated with test spore crystal suspensions by dipping them into the different doses of rehydrated spore-crystal mixtures in distilled water, agitating the preparations for 5 sec and allowing them to air-dry. The ICP doses included 75, 150, 225, and 300 μg protein/ml Distilled water served as a control

Adults, collected 3-8 days after eclosion, were added to the diet in Petri dishes (15x100mm diameter) containing moistened filter paper. Insects were allowed to feed on Diet 5 for 8 days in the mortality bioassay, and for six days in the frass bioassay both at 25°C in darkness. Adults were treated in groups of 15 insects (8 ♂♂, 7 ♀♀) per plate with a total of 60 adults per dose

Mortality was monitored daily over the assay period and the median lethal dose (LD_{50}) for each test strain was calculated by probit analysis (Finney, 1971). The cumulative decrease in number of frass pellets per insect exposed to the same ICP doses over a period of 6 days was calculated. This approach deviated from that of van Frankenhuyzen and Gringorten (1991) in which the number of insects failing to produce frass pellets was recorded The concentration of protein required to inhibit frass production by 50% (FP_{50}) was determined

Insect survival and frass production after removal from the diet containing ICP

Lepidopteran larvae are known to survive intoxication by *B. thuringiensis* (Milne *et al.*, 1990). The consequence of limited intoxication for beetles is not known Because the survival of insects formerly exposed to *B thuringiensis* influences overall efficacy of the insecticide, it was necessary to determine the long term effect of *B thuringiensis* strains on adults previously exposed to the ICP at the LD_{50} values Survivors within a given treatment, at 8 days for mortality and 6 days for frass production, were consolidated on untreated food cylinders in Petri plates with moistened filter paper. The insects were incubated at a temperature regime of 25.16 °C (Day:Night) with a 16:8 h (Day Night)

photoperiod. Mortality was recorded over a period of seven days and the frass was counted for five days.

Detection of B. thuringiensis-*like bacteria*

Control adults exhibited mortality. Therefore, to confirm the validity of mortality of insects treated with the *B. thuringiensis* strains, Koch's postulate was initiated. *B. thuringiensis* or *B. thuringiensis* like organisms were detected in treated as well as control insects. Screening for possible contamination by *B. thuringiensis* of the control insects was done. Several untreated insect body parts were smeared on two media. NAP (Nutrient agar + 5μg/ml Polymyxin B sulfate + 4μg/ml Ampicillin) which favors competitive growth of *B. thruringiensis* relative to other microorganisms, and NA (Nutrient agar) as a control. Both media were subsequently incubated at 37 °C for 4 days. NAP was modified from NPP (Nutrient agar + 5μg/ml Polymyxin B sulfate + 4μg/ml Penicillin G) (Saleh *et al.*, 1969). The structures plated on the media included larval gut; gut from unfed adults, carrot fed adults, and carrot fed adults from another location (St. Jean-sur-Richelieu), haemolymph, and elytra. To check for the possible source of contamination, carrots and soil were washed with sterile distilled water from which an aliquot (40 μl) was spread on the media plates. *B. thuringiensis*-like organisms were identified by the Smirnoff differential stain to detect the vegetative cells, spores and crystals (Smirnoff, 1962).

Data analysis

The LD_{50} values (median lethal dose) were calculated using probit analysis (Finney, 1971). The FP_{50} values (dose inducing 50% decrease in frass production) were calculated by linear regression analysis of the frass bioassay data. Statistical analysis of paired data was performed using Student's t-test. One-way analysis of variance (ANOVA) in conjunction with Tukey's test was used to compare groups of means. Statistical significance was taken at $p<0.05$. Results are presented as means ± standard error of the mean.

RESULTS

Diet selection

To select for the diet conducive to food consumption, the effect of food formulae on adult defecation rate was determined. Carrots did not elicit frass production to the same extent as fresh foliage (Fig. 1). However, in both cases the addition of agar enhanced frass production. Homogenizing the foliage and adding agar strongly enhanced frass production with diet 5 (foliage homogenate with 4% agar) being optimum for frass production whereas agar alone was the least effective. Although not quantified, it was observed that the insects attacked diet 5 more avidly than the other formulations.

Determination of crystal protein content

To standardize the amount of insecticidal proteins of the different strains for use in the bioassays, the protein content of the crystals was determined (Fig. 2). The results varied with the isolates ranging from 2.11 ± 0.063 μg protein/ mg spore-crystal mixture for *B. thuringiensis* subsp. *tenebrionis* to 100.6 ± 11.07 μg protein/ mg spore crystal mixture for strain A410.

ICP effects on insect mortality and frass production

The effect of *B. thuringiensis* strains on adult weevil mortality established the lowest LD_{50} value for strain A429 and the highest value for strain A311 (Table 1). The rank order of toxicity for the different strains was A429 = BTT = A30 > A299 > A410 > A409 > A311. Figure 3 shows dose-response curves of the most toxic strains and a less toxic isolate for comparison purposes. The most toxic strains displayed relatively steep slopes with strain BTT showing the steepest slope value (1.80 ± 0.25).

To further assess the toxicity of the *B. thuringiensis* strains,

Figure 1. The effect of food composition on adult rate of defecation. 1, carrot root; 2, carrot root with 2% (w/v) agar; 3, fresh foliage; 4, foliage extracts containing 4% (w/v) agar; 5, foliage homogenate in 4% (w/v) agar; 6, 4% (w/v) agar. (N=3).

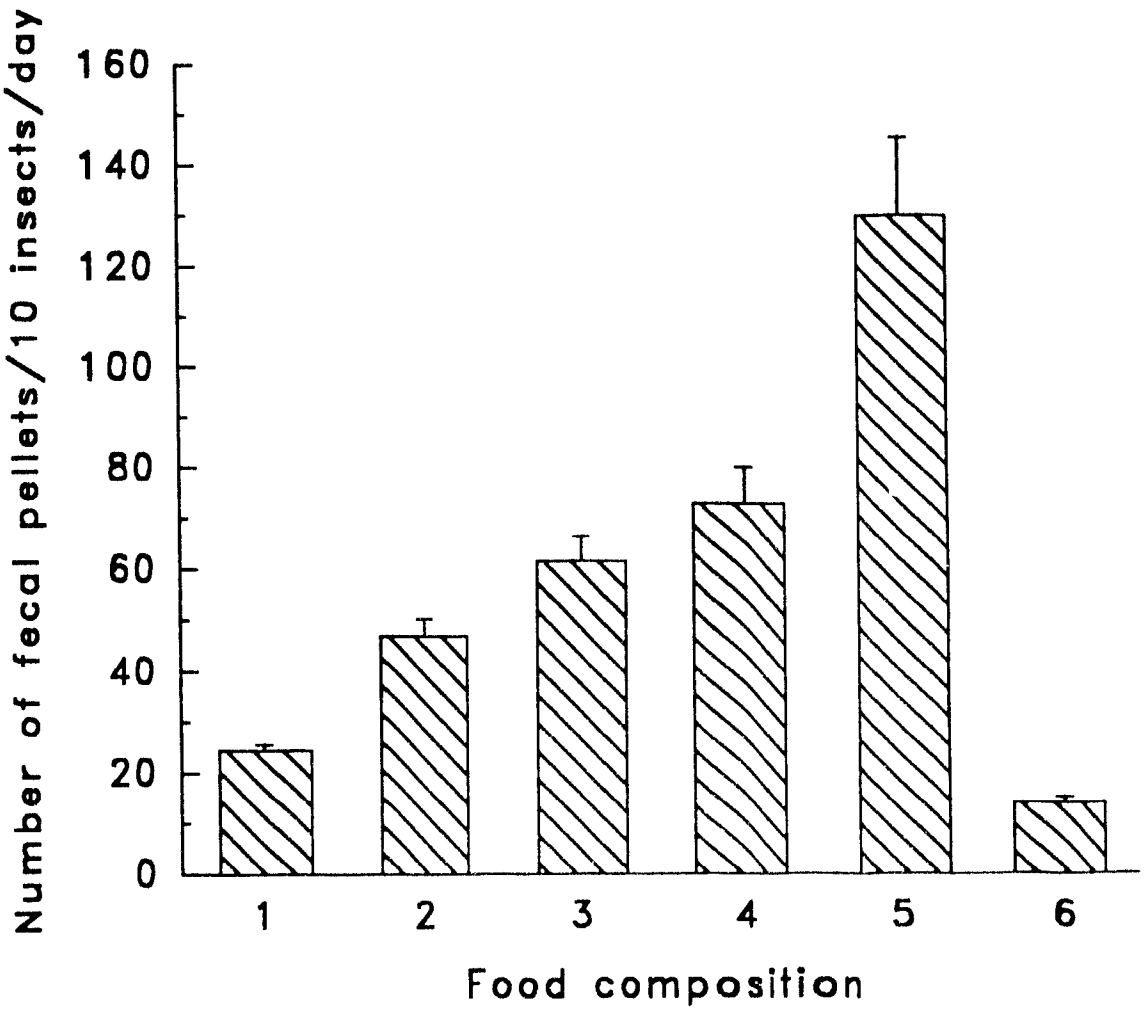

Number of fecal pellets/10 insects/day
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Food composition

Figure 2. Crystal-spore mixture protein content of the different bacterial strains. (N=5).

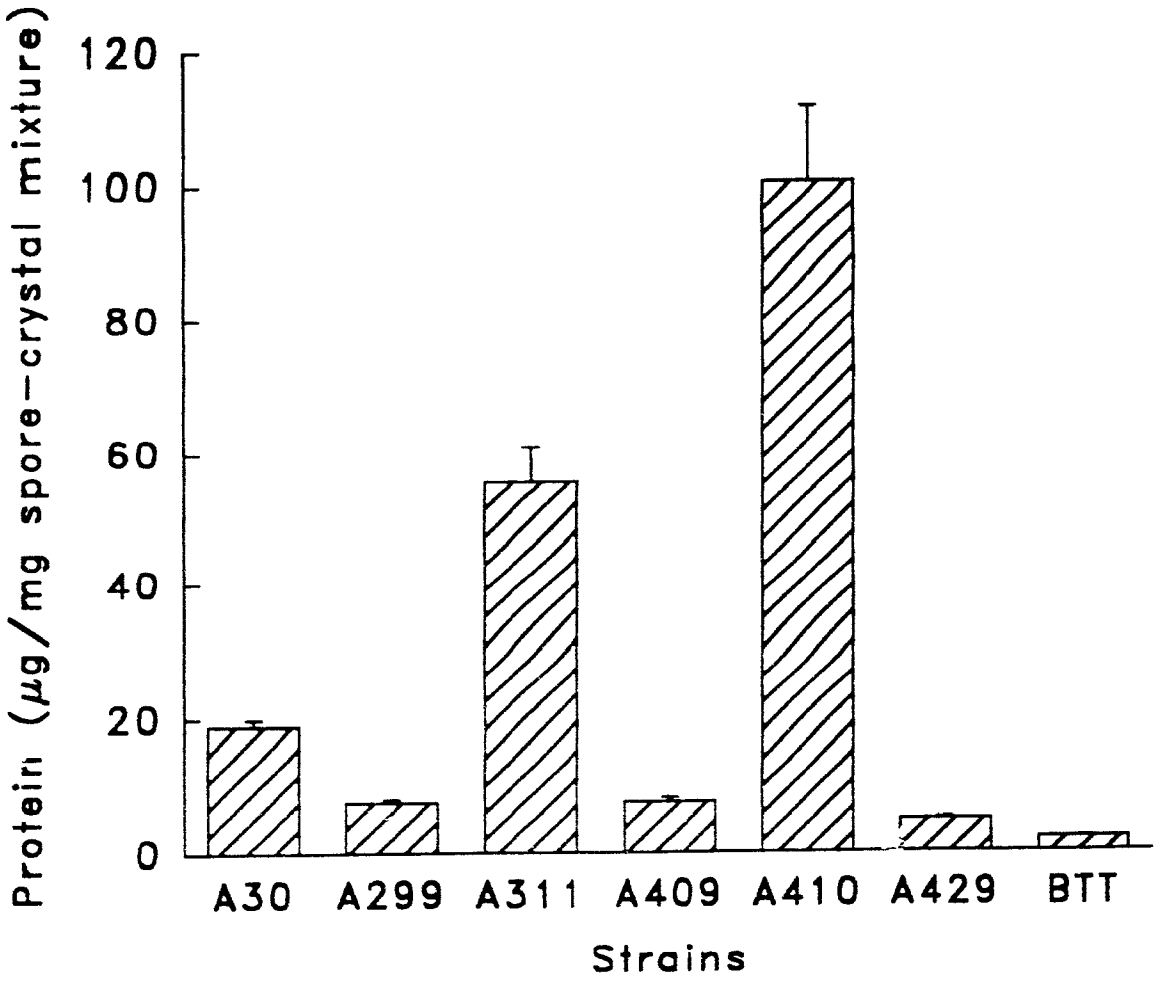

Protein (µg/mg spore-crystal mixture)
120
100
80
60
40
20
0
A30
A299
A311
A409
A410
A429
BTT
Strains

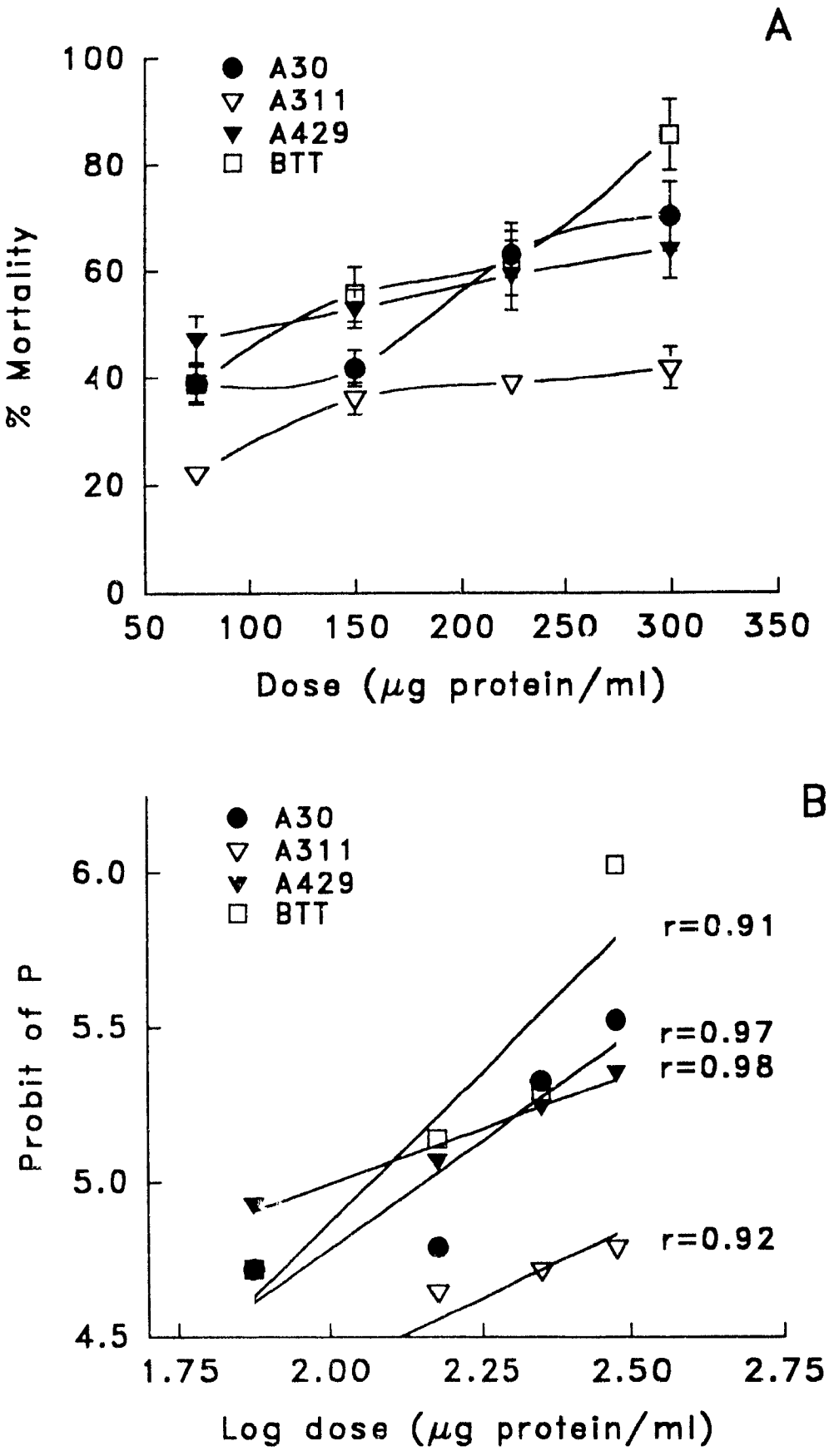
Table 1 The median lethal dose (LD_{50} µg protein/ml) for adults of the carrot weevil, *Listronotus oregonensis*, exposed to designated *Bacillus thuringiensis* strains for 8 days at 25°C

Strain	LD_{50}[a] (µg protein/ml)	95% fiducial limits (µg protein/ml)	Slope[b]
A429	102	52 - 200	0.71 ± 0.07
BTI	118	89 - 155	1.80 ± 0.25
A30	143	101 - 204	1.38 ± 0.11
A299	230	143 - 370	1.01 ± 0.05
A410	255	196 - 331	1.90 ± 0.08
A409	429	201 - 914	0.64 ± 0.02
A311	461	266 - 800	0.90 ± 0.03

a N=3

b Mean ± standard error of the mean

Figure 3. (A) Dose-response curves of the most virulent strains, A30, A429, and BTT, compared to strain A311 which has low virulence. (B) Corresponding probit regression lines. The data represents means of 3 mortality bioassays. P represents percent kill


A
100
80
60
40
20
0
% Mortality
A30
A311
A429
BTT
50
100
150
200
250
300
350
Dose (μg protein/ml)
B
6.0
5.5
5.0
4.5
Probit of P
r=0.91
r=0.97
r=0.98
r=0.92
1.75
2.00
2.25
2.50
2.75
Log dose (μg protein/ml)

their effect on feeding inhibition, as indicated by a decrease in frass production, was determined. Strains A429 (FP_{50}= 10.67 ± 1.70 μg protein/ml) and BTT (FP_{50} = 41.94 ± 4 20 μg protein/ml) caused the greatest decrease in frass pellet production followed by strain A30 (FP_{50}= 94.58 + 9.80 μg protein/ml) (Fig.4). Strain A311 (FP_{50}= 1234.37 ± 185.15 μg protein/ml), however, was not significantly different from the control groups There was a significant correlation between insect mortality and reduction in frass production (r= 0 99) (Fig. 5). At all dosages of ICP, strains A30, BTT, and A429 showed a significant decrease in the rate of frass pellet production per insect over a period of 6 days as compared to the less toxic strain A311 Figure 6 shows a representative result of the influence of selected strains of *B. thuringiensis* at one dosage (225 μg protein/ml) on frass production The number of frass pellets per insect exposed to strains A30, BTT, and A429 exhibited a 3-4 fold decrease after 6 days of intoxication. Conversely, the decrease in number of pellets produced per insect exposed to strain A311 was not significantly different from the control group.

Insect survival and frass production after removal from diet containing ICP

When adult weevils surviving ar LD_{50} dosage for a given ICP strain were placed onto untreated diet 5 for 7 days, mortality continued For strains A299, A311, A409, A30 and A410, mortality was not significantly different from the control (10-20%) (Fig. 7) However, mortality of survivors previously subjected to strains A429 and BTI ranged between 45 55%

Surviving adults previously subjected to LD_{50} dosages of ICP of strains A30. BTT, and A429 displayed a continuously low defecation rate (3-6 frass pellets/insect/day) as compared to the control insects (10 14 frass pellets/insect/day) during the 5 days of exposure to untreated diet (Fig. 8). Strain A311 elicited frass production comparable to the control groups (8-12 frass pellets/insect/day).

Figure 4. (**A**) Dose-response curves of *Bacillus thuringiensis* strains A30, A429, BTI, and A311 in terms of the cumulative production of frass pellets over a period of 6 days. (**B**) Corresponding regression lines.

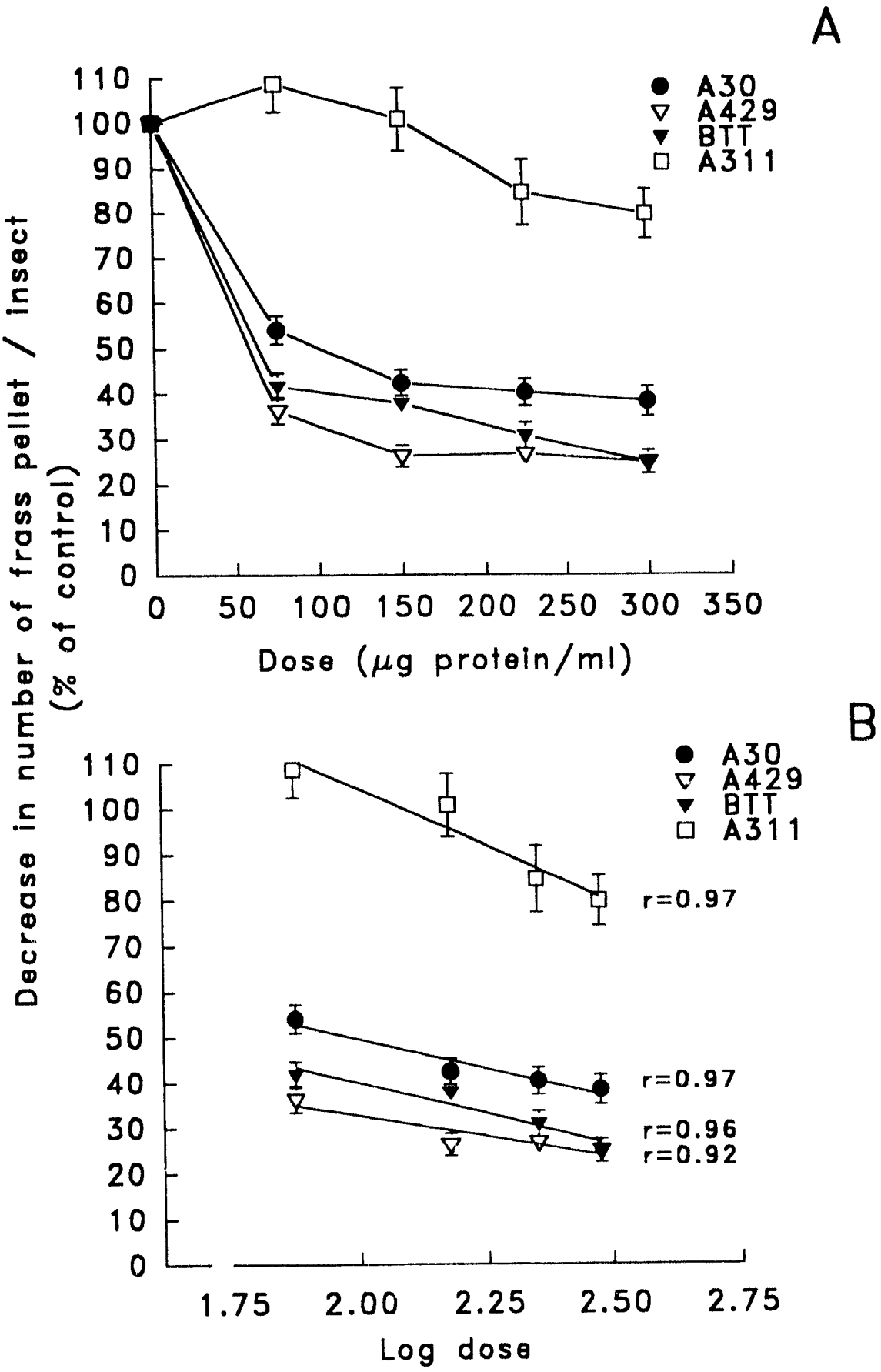

A
A30
A429
BTT
A311
Decrease in number of frass pellet / insect (% of control)
Dose (μg protein/ml)
B
r=0.97
r=0.97
r=0.96
r=0.92
Log dose

Figure 5. The relationship between FP_{50} values (dose inducing 50% decrease in frass production) of *Bacillus thuringiensis* strains A30, A429, BTI, and A311 and their corresponding LD_{50} values (median lethal dose).

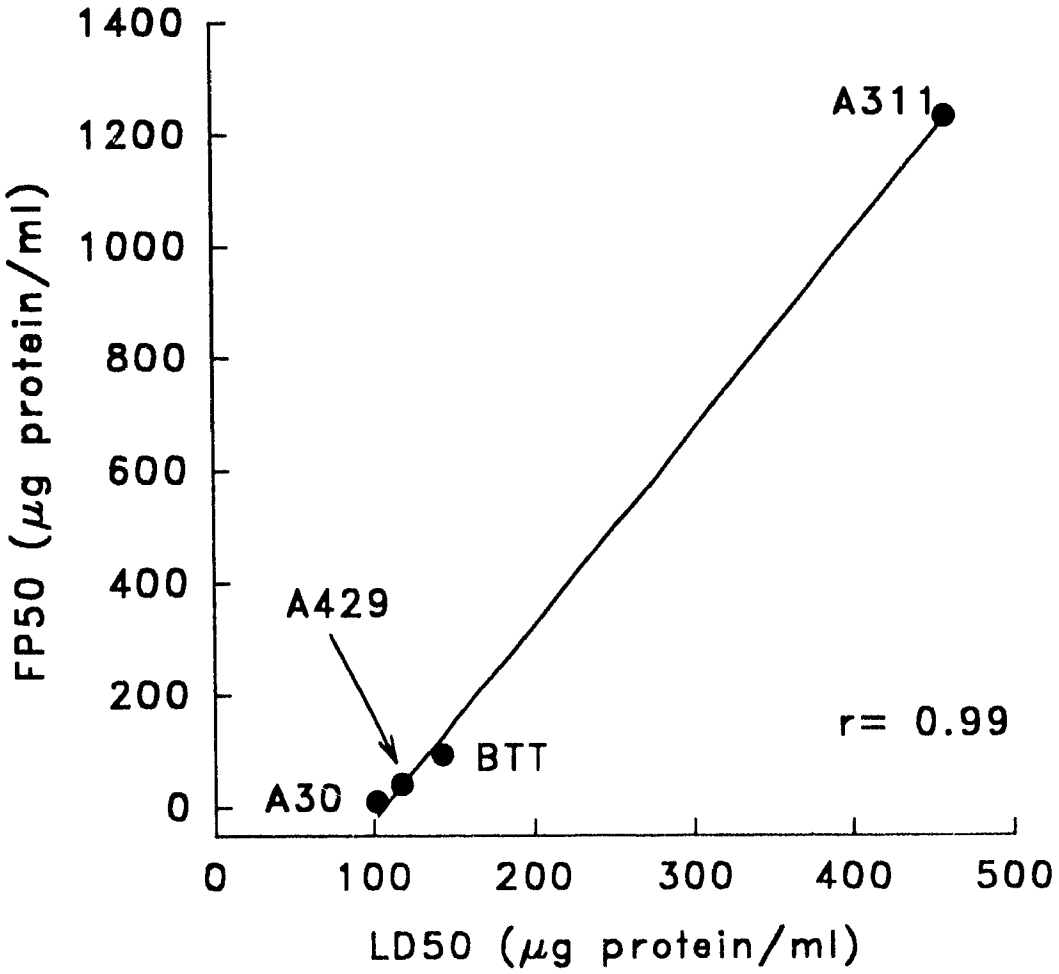

1400
1200
1000
800
600
400
200
0
FP50 (μg protein/ml)
A311
A429
BTT
A30
r= 0.99
0
100
200
300
400
500
LD50 (μg protein/ml)

Figure 6. The effect of strains A30, A429, BTT, and A311 of *B. thuringiensis* on the rate of defecation in a frass bioassay using a representative dosage of 225μg protein/ml. The data represents the mean of 3 bioassays containing 60 insects per treatment.

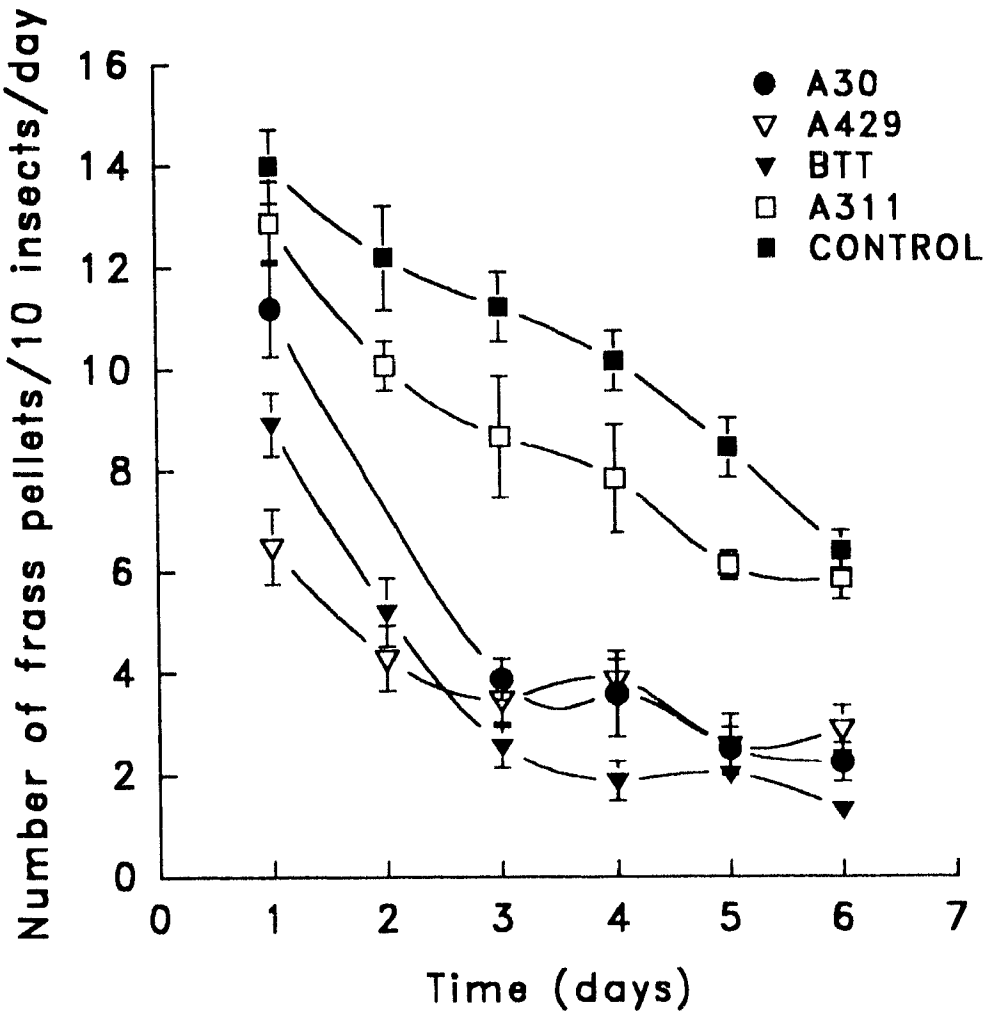

Number of frass pellets/10 insects/day
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
Time (days)
A30
A429
BTT
A311
CONTROL

Figure 7. Cumulative 7-day mortality of the adults of *Listronotus oregonensis* that had survived a prior 8-day exposure to the LD_{50} dosage insecticidal crystal proteins of different strains of *B. thuringiensis*.

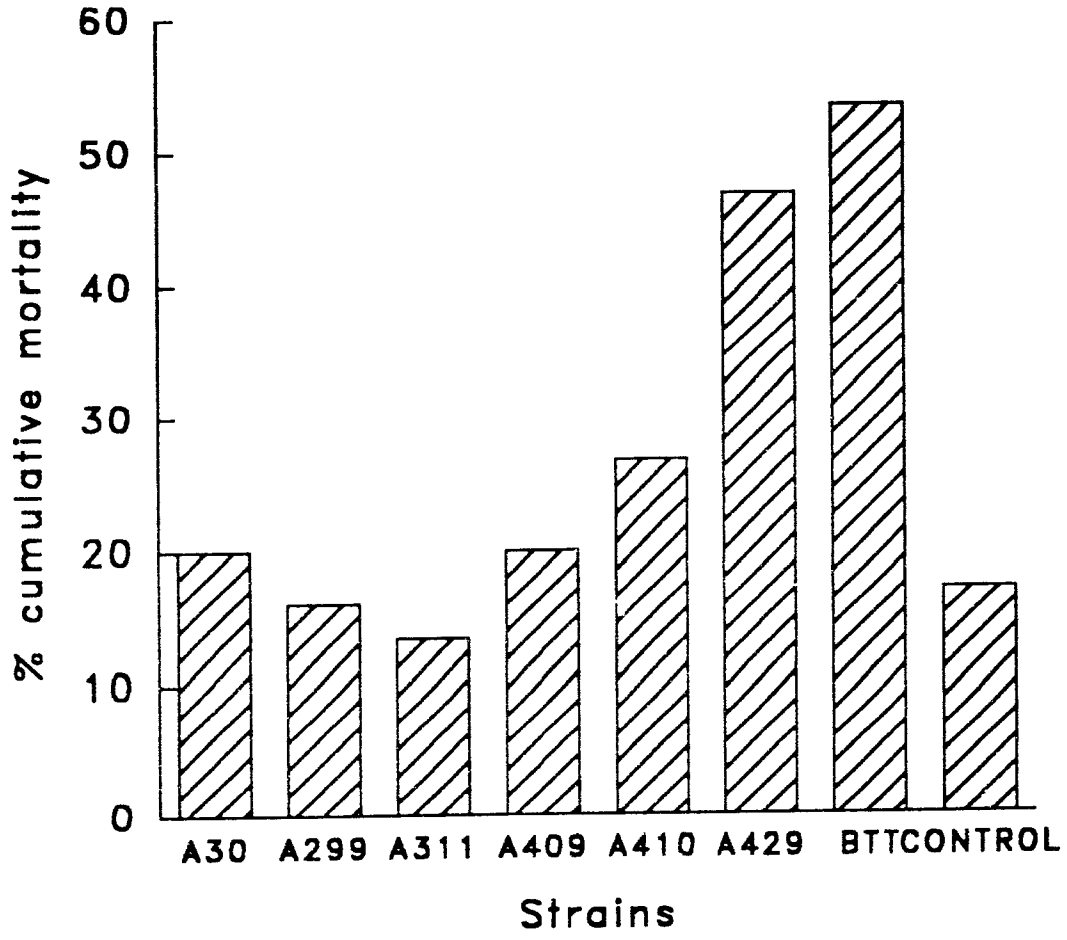
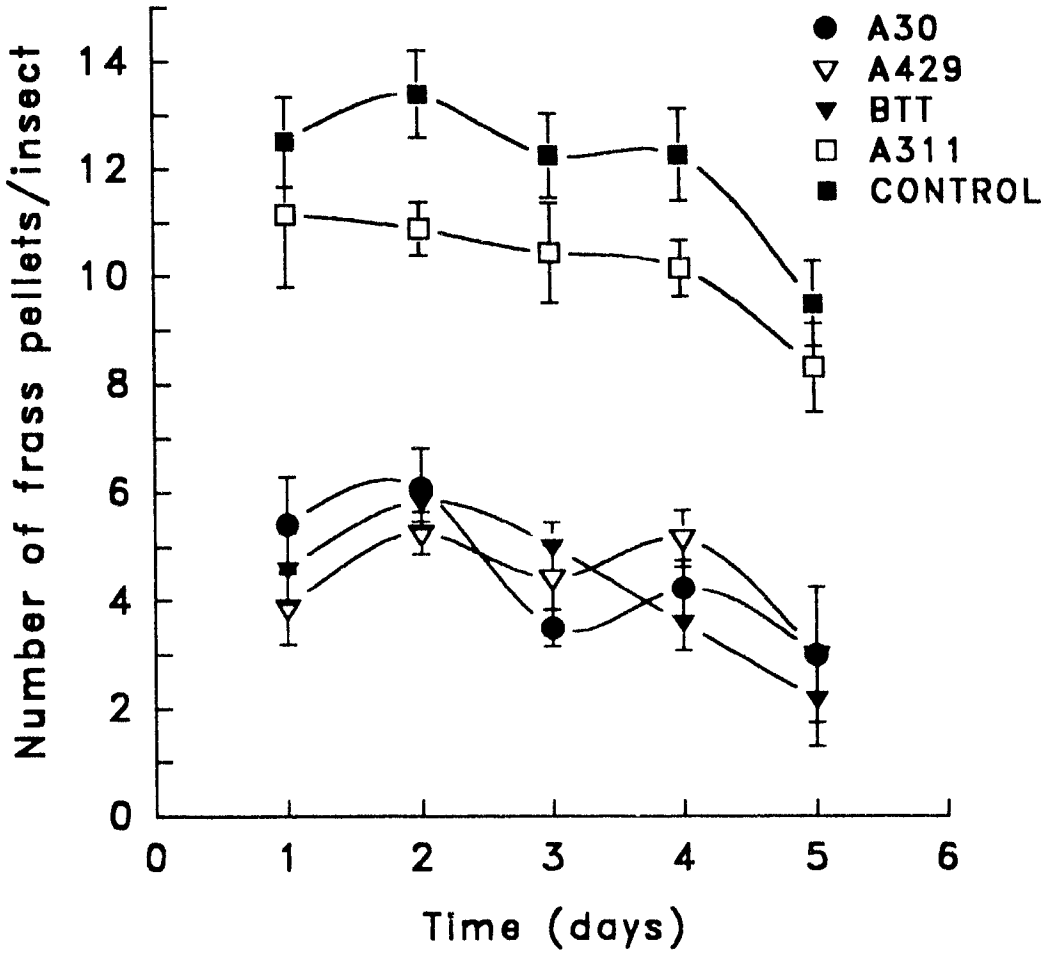

60
50
40
30
20
10
0
% cumulative mortality
A30
A299
A311
A409
A410
A429
BTTCONTROL
Strains

Figure 8. Rate of frass production of *Listronotus oregonensis* previously exposed to LD_{50} values of *Bacillus thuringiensis* strains A30, A429, B11, and A311


A30
A429
BTT
A311
CONTROL
Number of frass pellets/insect
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
Time (days)

Detection of B. thuringiensis-*like bacteria*

B.thuringiensis subsp. *tenebrionis* readily grew on NAP whereas no Gram negative bacteria grew confirming the validity of the medium for selecting for *B thuringiensis* (Table 2). *B. thuringiensis*-like organisms were detected in and on all structures except the haemolymph of adults, larval gut and on the carrot on NAP medium. In addition, there was no evidence of *B. thuringiensis*-like organisms in larval gut, haemolymph of larvae and adults, elytra, and carrots when streaked on NA.

DISCUSSION

The discovery of *B. thuringiensis* isolates with toxic activity against coleopterans (Krieg *et al.*, 1983; Herrnstadt *et al.*, 1986) has initiated an interest in evaluating ICP toxicity and identifying and explaining susceptibility in pest insects. Different species of coleopterans display variable degrees of sensitivity to *B. thuringiensis* isolates including the Colorado potato beetle, *Leptinotarsa decemlineata*, the elm leaf beetle, *Pyrrhalta luteola*, the boll weevil, *Anthomonus grandis grandis*, the yellow mealworm, *Tenebrio molitor*, and the black vine weevil, *Otiorhynchus sulcatus*, (Herrnstadt *et al.*, 1986). This study reports the relative toxicity of several strains of *B. thuringiensis* with known activity against coleopterous insects (Moser, 1990) to the adult stage of the carrot weevil, *Listronotus oregonensis*. The results show that three out of the seven strains tested are pathogenic to these insects.

The lack of availability of the natural diet (fresh foliage) of the adult weevils year round has lead to the search for a more practical alternative. The preferred food source in terms of fecal production (as a measure of consumption rate) was found to be the semi-artificial diet composed of chopped foliage with 4% agar. The foliage can be chopped and stored with water at 20°C until needed Members of the Umbelliferae family are aromatic due to the presence of essential oils and resins

Table 2. Detection of *Bacillus thuringiensis*-like bacteria in/on insect structures of untreated adult *Listronotus oregonensis* and in the insect environment.

	TREATMENT								
MEDIUM	L	ANF	AF	SJ	Haem	Elyt	Soil	Carr	BTT
NAP	+	++	+	+		+	+	_	+++
NA	_	++	++	++	_	_	+	_	+++

L, larval gut; ANF, nonfed adult gut, AF, fed adult gut; SJ, gut of fed adults from another location (St. Jean-sur-Richelieu); Haem, haemolymph form adults; Elyt, elytra; Carr, carrot; BTT, pure culture of *Bacillus thuringiensis* subsp. *tenebrionis* (a positive control); NAP, nutrient agar + Polymyxin B sulfate + Ampicillin; NA, nutrient agar, +, preserce of *B thuringiensis*-like bacteria; -, absence.

(Berenbaum, 1990). Chopping up the foliage may have released the chemicals causing a strong attraction stimulus to the insect relative to the intact fresh foliage thus creating a high consumption rate. The agar clearly does not play a nutritional role but may help preserve the texture of the foliage and minimize water loss from the diet which collectively would be conducive to feeding and/or frass production The diet also ensured maximum initial feeding of toxin-contaminated diet.

Although a method to standardize the potency (IU/mg) of the active ingredient of *B thuringiensis* strains toxic to coleopterans using the Colorado potato beetle as the test insect has been proposed (Ferro and Gelernter, 1989), a publicly available *B. thuringiensis*-coleopteran specific standard has not been developed to date (Tompkins *et al.*, 1990). Therefore, the active ingredient in the bioassays in this study was based on the protein content of the crystals per weight of spore-crystal mixture of each strain as determined by analytical methods.

The low LD_{50} values of strains BTI, A429, and A30 established these strains as the most toxic In addition, BTI has the lowest crystal protein content followed by A429. This suggests that BTI possesses the most potent toxin with A429 in second place Although the protein content (μg/mg spore-crystal mixture) of strains A409 and A299 are low compared with the toxic strain A30, their corresponding LD_{50} values are relatively high, implying that the crystals are less toxic. As judged by its LD_{50} value, strain A30 was found to be relatively toxic, although its crystal protein content was higher than that of BTT and A429 This suggests that the toxicity of the endotoxin of strain A30 is less than that of the latter two strains

The ranking order of toxicity of the three selected strains is a function of the percentage of insect kill being sought. The steepness of the slopes will dictate the rank of the strains, *e.g.*, the rank order of toxicity for a 50% kill is A429 > BTT > A30 while for a 70% kill it is BTI > A30 > A429 In all cases, the slopes were significantly greater than or equal to unity thus, insect demise was the more immediate result of toxicosis as opposed to septicemia (Bucher,1973).

While mortality of survivors previously exposed to ICP dosages at the LD_{50} values of most of the bacterial strains were not significanty

different from the controls, the percent mortality due to strains A429 and BTT were significantly different from the control groups, but not from each other. This further suggests that the latter strains are more toxic than the former and that the effect of strains A429 and BTT at that dose is irreversible. The long-term mortality effect of these strains at the LD_{50} dose might be of economic importance under field conditions since only a minimum number of sprays would be required to achieve effective control In contrast, a more repetitive spraying scheme might be in order for strain A30 to obtain similar results.

An alternative procedure for assessing ICP toxicity against beetles clearly established in this study is the frass production assay, an indication of feeding inhibition The dose-response curves for the effective strains regarding the decrease in number of frass pellets per insect correlated well with those of the mortality bioassay In general, the frass production curves trace a dosage dependent response with respect to reduced feeding. Mortality of insects caused by ICPs is generally slow being mediated by a series of events such as cessation of feeding, development of septicemia from germinating *B. thuringiensis* spores or other opportunistic microorganisms all of which complicated by starvational stress and an irreparable damaged gut which eventually cause insect mortality (Hall and Dunn, 1958; Heimpel and Angus, 1959, Somerville *et al.*, 1970, van Frankenhuyzen and Gringorten, 1991). Thus, this assay offers an expedient means of reliably determining bacterial isolate toxicity against beetles similar to the assay protocol of van Frankenhuyzen and Gringorten (1991)

At all test dosages of the highly toxic strains, feeding was significantly reduced by the third day of treatment. Beyond that period, feeding remained very low This may indicate that the gut was severely damaged by day three Following intoxication and exposure to untreated diet, frass production remained very low for insects exposed to the effective strains, which suggests that the gut had undergone irreversible paralysis. This is unlike the spruce budworm, *Choristoneura fumiferana*, in which sublethal levels of *B thuringiensis* did not inhibit midgut tissue regeneration (Milne *et al* , 1990)

The detection of *B thuringiensis*-like bacteria in the gut of

untreated insects from 2 distinct cultures made it impossible to fulfil Koch's postulate using the present method. The absence of these organisms in the haemolymph of the insects suggests that the insects were not diseased. These microorganisms might be part of the natural flora of the insect gut which was picked up from the soil. Additional tests are required to confirm the identity of *B. thuringiensis*-like bacteria.

In conclusion, 3 of the 7 strains are promising for use against the carrot weevil. For greatest efficacy, an optimal relationship between the insect and the toxin is required. Therefore, further research into the mode of action would be useful.

REFERENCES

Bélair, G. and G. Boivin. 1985. Susceptibility of the carrot weevil (Coleoptera: Curculionidae) to *Steinernema feltiae*., *S. bibionis* and *Heterorhabditis heliothidis*. *J. Nematol.* **17**: 363 366.

Berenbaum, M. R. 1990. Evolution of specialization in insect-umbellifer associations. *Ann Rev Entomol.* **35** 319 343

Boivin, G. 1985a. Evaluation of monitoring techniques for the carrot weevil, *Listronotus oregonensis* (Coleoptera; Curculionidae) *Can. Entomol*. **117**: 927-933.

Boivin, G. 1985b. *Anaphes sordidatus* (Girault) (Hymenoptera: Mymaridae), an egg parasite of the carrot weevil, *Listronotus oregonensis* (le Conte). *Can. Entomol* 118 393-394.

Boivin, G. and G. Bélair. 1989 Infectivity of two strains of *Steinernema feltiae* (Rhabditida, Steinernematidae) in relation to temperature age and sex of carrot weevil (Coleoptera; Curculionidae) adults. *J. Econ. Entomol*. 82 762-765.

Boyce, A. M. 1927. A study of the biology of the parsley stalk weevil. *J. Econ. Entomol*. 20(6): 814.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem* **72**: 248-254.

Bucher, G. A. 1973. Definition and identification of insect pathogens in regulation of insect populations by microorganisms. *Annals Acad. Sci.* **217**: 8-17.

Chandler, S. C. 1926. The economic importance of the carrot weevil in Illinois. *J. Econ. Entomol.* **19**: 490-494.

Fast, P. G. 1981. The crystal toxin of *Bacillus thuringiensis*. In: Microbial Control of Insects and Mites, eds H. D. Burges and N. W. Hussey. pp. 223-248 New York: Academic Press.

Ferro, D. N. and W D. Gelernter 1989. Toxicity of a new strain of *Bacillus thuringiensis* to Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* **82**: 750-755.

Finney, D. J. 1971. Probit analysis. Cambridge University Press, Cambridge, England.

Goldberg, I J. and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaemia unguiculata*, *Culex univeritattus*, *Aedes aegyptii* and *Culex pipiensis*. *Mosq. News* 37: 355-358.

Grafius, E. and M Otto 1979. Detection and control of the carrot weevil. *Michigan State University Ext. Bull.* E-890. 2p.

Hall, I M. and P H. Dunn. 1958. Susceptibility of some insect pests to infection by *Bacillus thuringiensis* Berliner in laboratory tests. *J. Econ. Entomol.* 51. 296-298

Heimpel, A. M. and T A. Augus. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. *J. Insect. Pathol.* 1: 152-170.

Hendrickx, K , A De Loof and H Van Mellaert. 1989. Binding of *Bacillus thuringiensis* delta-endotoxin to brush-border membranes of *Manduca sexta*. Receptor, membrane transport, Verlag Publications Nato Series. Berlin, eidelberg. H29, 344-351.

Herrnstadt, C., G. G Soares, E R. Wilcox and D. L. Edwards. 1986. A new strain of *Bacillus thuringiensis* with activity against coleoptera insects *Bio/Technology*. 4: 305-308.

Hofmann, C., P. Luthy, R. Huetter and V. Pliska. 1988a. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *Eur. J. Biochem* 173: 85-91.

Hofmann, C , H Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens and H. Van Mellaert. 1988b Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high affinity binding sites in the rush-border membrane of target insect midguts. *Proc. Natl Acad. Sci USA* 85. 7844-7848.

Hofte, H and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis* *Microbiol Rev* 53: 242-255.

Krieg, A , A. M. Huger and W Schnetter. 1987. *Bacillus thuringiensis* var. *tenebrionis* strain M-7 is identical to the formerly in Germany isolated strain BI 256-82 *B. t* ssp. *tenebrionis* which is pathogenic to coleopteran insects. *J. Appl. Entomol.* 104: 417-424.

Krieg, V A , A M Huger, G A. Langenbruch and W. Schnetter. 1983.

Bacillus thuringiensis var *tenebrionis*, a new pathotype effective against larvae of Coleoptera. *Z. Angew. Entomol.* 96: 500 508.

Kurstak, E. 1962. Donnees sur l'epizootie bacterienne naturelle provoquee par un *Bacillus* du type *Bacillus thuringiensis* sur Iphystia kuhniella Zeller. *Entomophaga Mem. Hors Ser.* 2: 245 247

Knowles, B. H. and D. J. Ellar. 1986. Characterization and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran specific δ endotoxin. *J. Cell Sci.* **83**: 89-101.

Lilly, M., R. Ruffel and H. Soumerville. 1980. Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis*. *J. Gen. Microbiol.* 118: 1-11.

Martel, P., H. J. Svec and C. R. Harris. 1975. Mass rearing of the carrot weevil, *Listronotus oregonensis* (Coleoptera; Curculionidae) under controlled environmental conditions. *Can. Entomol* 107. 95 98.

Milne, R., A. Z. Ge, D. Rivers and D. H Dean. 1990 Specificity of insecticidal crystal proteins. In. Analytical Chemistry of *Bacillus thuringiensis*, eds. L. A. Hickle and W. I Fitch. pp. 22-35. Washington D.C : American Chemical Society.

Moser, L. 1990. Imperial Chemical Industries Biological Products, North American Technical Centre, Mississauga, Ontario, Canada (see Appendix).

Pepper, B. B. 1942. The carrot weevil, *Listronotus oregonensis* (Bohe) in New Jersey and its control. *N. J. Agric. Exp. Str. Bull.* 693: 1 20.

Pepper, B. B. and L. E. Hagmann. 1938. The carrot weevil, *Listronotus oregonensis* (Boh.), a new pest on celery. *J.Econ. Entomol.* 31: 262 266.

Perron, J. P. 1971. Insect pests of carrots in organic soils of southwestern Québec with special reference to the carrot weevil, *Listronotus oregonensis* (Coleoptera; Curculionidae) *Can. Entomol.* **103**: 1441-1446.

Poinar, G. O., Jr. 1989. Examination of the neoaplectanid species *feltiae* Filipjev, *carpocapsae* Weiser and *bibionis* Bovien (Nematoda. Rhabditida). *Rev. Nematol.* 12 375.

Poinar, G. O., Jr. 1990. Taxonomy and biology of Steinernematidae and

Heterorhabditidae. In: Entomopthogenic Nematodes in Biological Controls, eds. R. Gaugler and H. K. Kaya. pp. 23-61. Boston: CRC Press.

Ryser, B. W. 1975. Investigations regarding the biology and control of the carrot weevil *Listronotus oregonensis* (Le Conte) in New Jersey. M.S. Thesis, Rutgers University. New Brunswick, New Jersey. 141 pp.

Saleh, S. M., R. T. Harris and O. N. Allen. 1969. Method for determining *Bacillus thuringiensis* var. *thuringiensis* Berliner in soil. *Can. J. Microbiol* **15** 1101-1104

Smirnoff, W. A. 1962. A staining method for differentiating spores, crystals, and cells of *Bacillus thuringiensis* (Berliner). *J. Insect. Pathol.* **4**. 384-386.

Somerville, H. J., Y. Tamada and E. M. Omi. 1970. Lethal effect of purified spore and crystalline endotoxin preparations of *Bacillus thuringiensis* on several lepidopteran insects. *J. Invertebr. Pathol.* **16**: 241-248.

Stevenson, A. B. 1976. Seasonal history of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae) in the Holland Marsh, Ontario. *Proc. Entomol. Soc. Ontario* **107**: 71-78.

Tompkins, G., R. Inglar, M. Mendelsohn and P. Hutton. 1990. Historical aspects of the quantification of the active ingredient percentage for *Bacillus thuringiensis* products. In: Analytical Chemistry of *Bacillus thuringiensis*, eds. L. A. Hickle and W. L. Fitch. American Chemical Society, Washington D.C. pp.9-13.

van Frankenhuyzen, K. and J. L. Gringorten. 1991. Frass failure and pupation failure as quantal measurements of *Bacillus thuringiensis* toxicity to lepidoptera. *J. Invertebr. Pathol.* **58**: 465-467.

Whitcomb, W. D. 1965. The carrot weevil in Massachusetts. Biology and control. *University Massachusetts Agric. Exp. Str. Bull.* 550: 1-30.

CONNECTING STATEMENT

In Chapter 2, seven strains of *Bacillus thuringiensis* active against Coleopiera were evaluated for their toxicity against the adult stage of the carrot weevil, *Listronotus oregonensis*. Three strains were selected with high toxicity and one strain with low toxicity to determine differences in modes of toxicity of the strains in Chapter 3. The solubility of the crystals of the three toxic strains compared with a weakly toxic strain and the proteolytic modification of the solubilized crystals in carrot weevil gut extract were studied.

Chapter 3

Solubilization and proteolytic processing of *Bacillus thuringiensis* δ-endotoxin in the midgut fluids of the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae), *in vitro*

ABSTRACT

Several strains of *B. thuringiensis* active against Coleoptera were selected for toxicity against the adult carrot weevil. Both, solubilization and proteolytic processing of the δ-endotoxins of the toxic strains (BTT, A30 and A429) and a weakly toxic strain (A311) were examined to determine their relationship to toxicity. Crystal solubility increased as the buffer pH increased and, with the exception of strain A311, was correlated with LD_{50} values implying that toxin availability may explain toxicity. Proteolytic activation of the crystals from the toxic bacterial isolates with carrot weevil gut extract yielded similar electrophoretic profiles. Unlike strain A311, strains BTI, A30 and A429 possessed a putative toxic protein band (66-67 kDa). These results suggest that the relative low toxicity of strain A311 crystal is due, in part, to the absence of the toxic moiety in the δ endotoxin.

INTRODUCTION

The carrot weevil, *Listronotus oregonensis* (Le Conte) (Coleoptera: Curculionidae) is a major pest of Umbelliferous plants in the northeastern states of the United States of America (Chandler, 1926; Pepper, 1942; Whitcomb, 1965; Grafius and Otto, 1979) and Canada (Perron, 1971). Its preferred host is carrots, and when the insect is left uncontrolled, up to 40% of the crop has been reported to be damaged in Quebec (Boivin, 1985). The adult female lays eggs in the petiole and crown of the carrot and, upon emergence, the larvae tunnel down into the soil and pupate (Perron, 1971). The carrot weevils overwinter in the soil as adults. Three highly virulent strains of *Bacillus thuringiensis* active against the adult stage of the carrot weevil were selected (Chapter 2).

Bacillus thuringiensis is a Gram-positive, spore-forming, soil-dwelling bacterium characterized by its ability to produce insecticidal crystalline proteins (ICPs) during sporulation. The ICPs, also designated as δ endotoxins, are toxic to many insects belonging to the orders Lepidoptera, Diptera and Coleoptera (Hofte and Whiteley, 1989). The crystalline protoxins that dissolve in the midgut have relative molecular masses (Mr) of 70 kDa-135 kDa depending on bacterial variety (Li, *et al.*,1991) Gut proteases release the active toxin which then binds to receptors on the midgut epithelium (Hofmann *et al.*, 1988a,b) and induces leakage channels in the midgut cell membranes (Crawford and Harvey, 1988). Death of the insect ensues due to gut and haemolymph ion contamination leading directly and indirectly to starvation and septicemia (Hofte and Whiteley, 1989)

The basis for the differential toxicity of the *B thuringiensis* strains for the carrot weevil may be attributed to one of the three stages associated with the mode of action of the δ-endotoxin for other insect species as proposed by Aronson *et al.*(1991). These stages include solubilization of ICPs, proteolytic activation of the protoxin and toxin binding to specific receptors The importance of solubility has been demonstrated for 14 serotype of *B thuringiensis* strains against three lepidopteran species known to respond differently to the δ-endotoxins (Jaquet *et al.*, 1987). Although proteolytic processing of the protoxin is

essential for the activation of the toxin, rapid deg adation of the toxin might be an additional factor explaining reduced toxicity. However, according to Slaney *et al.* (1992) using *B thuringiensis* subsp *morrisoni* strain EG2158 against the Colorado potato beetle, *Leptinotarsa decemlineata*, and the southern corn rootworm *Diabrotica undecimpunctata*, the rapid proteolytic degradation of the toxin in digestive fluid of the former insect did not hinder toxicity The toxicity and specificity of δ endotoxins have been shown to correlate with the presence of high affinity binding sites in the brush border membrane of target insect midgut epithelium (Hofmann *et al* , 1988a, b) Their study concentrated on *B thuringiensis* strains which differed in their insecticidal activity toward tobacco hornworm, *Manduca sexta*, and cabbage butterfly, *Pieris brassicae* Receptor binding studies by Van Rie *et al* (1990) have shown that resistance of the laboratory-selected Indianmeal moth, *Plodia interpunctella*, strain to *B thuringiensis* subsp. *berliner* ICP was correlated with a reduction in affinity of the membrane receptor for the toxic protein. However, different classes of ICP may bind to distinct receptor sites (Hofmann *et al* , 1988b) This may explain the sensitivity of *P. interpunctella* to a second type of insecticidal crystal protein that seems to recognize a different receptor (Van Rie *et al.* , 1990).

The examination of the three steps in the mode of action of *B. thuringiensis* δ-endotoxins should determine the factors responsible for the differential activity of the four strains of *B thuringiensis* against the carrot weevil. In this study, the two primary key steps, solubility and proteolytic processing, involved in the activation of the toxin were investigated.

MATERIALS AND METHODS

Chemicals

Monobasic and dibasic potassium phosphate (KH_2PO_4 and K_2HPO_4, respectively), 2-b-mercaptoethanol, boric acid (H_3BO_3), phosphoric acid (H_3PO_4), and acetic acid (CH_3COOH) were purchased from Sigma Chemical

Company (Missouri, USA). Glycerol and sodium dodecyl sulfate (SDS) were purchased from the United States Biochemical Corporation (USA) and Tris-HCl from Boehringer Mannheim (Quebec, Canada). Bromophenol blue was acquired from Bio-Rad (Ontario, Canada), diethylether from BDH (USA), and nutrient agar from BBL (Maryland, USA).

Insects

Carrot weevil available in laboratory culture and periodically supplemented with carrot weevils from Agriculture Canada Research Station (St Jean-sur Richelieu) were used to maintain hybrid vigor. The insects were reared on carrots at a temperature day:night cycle of 24:16 °C with a 16 h photoperiod (Martel *et al.*, 1975). For additional details consult Chapter 2

Bacterial strains

Bacillus thuringiensis subsp. *tenebrionis* and unidentified *B. thuringiensis* strains coded A30, A429, and A311 with activity against *Listronotus oregonensis* (supplied by Imperial Chemical Industries, Biological Products, Mississauga, Ontario) were grown on nutrient agar supplemented with equimolar concentrations (0.05M) of KH_2PO_4 and K_2HPO_4 (pH 7.0) After 5 days incubation at 30 °C the bacterial lawn which, based upon microscopic examination consisted of vegetative cells, sporangia, spores, and crystals was then scraped and lyophilized.

Midgut isolation and digestive fluid pH

The adult carrot weevils were anaesthetized for 10 min with diethylether in a jar Imagos with amputated legs were pinned at the pronotum and last abdominal segment onto a wax plate. The alimentary canal was exposed by removing the elytra, hind wings, abdominal tergites, metanotum, and pronotum (Calder, 1989) Since protease activity is located in the midgut, the pH of the anterior and posterior midgut was determined using a microelectrode (Beetrode™ pH microelectrode, World

Precision Instruments, Conn., USA) and a liquid junction reference electrode (World Precision Instruments, Conn., USA) connected to the pH meter. To determine whether the type of food influenced midgut pH, the adults were fed on either fresh foliage or chopped foliage with 4% agar (Chapter 2) for three days prior to exposing the gut. Twenty pH readings were taken for each treatment.

Solubilization of crystals at selected pH values

To study the effect of the pH on crystal solubilization, 1 mg of spore-crystal mixture of each strain was washed 3 times with 0.01% Triton X-100 (Chapter 2). The final pellet was suspended in 200μl of Universal buffer (2.7ml of concentrated H_3PO_4, 2.29ml concentrated glacial acetic acid, and 2.48g H_3BO_3 per 100ml final volume) at pH 6.0 to 11.0 with constant ionic strength (Koller *et al.*, 1992) in 1.5ml microcentrifuge tubes at 30 °C for 2 hrs. At 30 min intervals, designated 5μl aliquots of 0.5M NaOH were added to arrest proteolysis (Brussock and Currier, 1990). The samples were centrifuged at 11,750 xg for 3 min and the supernatant assayed for its protein content according to the Bradford method (1976) using a Bio Rad protein assay kit (Bio Rad, Ontario, Canada). The assay is based on the colour change of a dye in response to protein binding. Bovine serum albumin was used as a standard. The percent solubility was based on the amount of protein in the supernatant relative to the total solubility of the crystals dissolved in 200 μl of 3.3M NaBr (Bernhard, 1986).

To determine the amount of protein per crystal per strain, the pellets were resuspended in a known volume of Universal buffer at the corresponding pH, and the number of unsolubilized crystals counted using a haemocytometer. The absorbance of the dye-protein complex of the solubilized crystals at 595 nm was then subtracted from the total absorbance in 3.3 M NaBr solution. In the latter solution, the crystals were totally solubilized. The difference in absorbance was transformed to amount of protein corresponding to the unsolubilized crystals (pg/crystal/strain).

Residence time of food in the gut

To determine a time range for the midgut protease activation of the protoxins, it was necessary to ascertain an approximate range of time that the ICP would be in the gut. Adult weevils were primarily fed carrot roots (orange colour) for 3 days. They were then fed foliage with 4% agar (green colour). The time from the initiation of insect feeding on the green coloured food source to the first observation of green frass was determined to be 68-159 min. While recognizing that the time represented the total passage time as opposed to the duration of food in the midgut, the upper limit for incubating the crystals with midgut fluids was 2 h. This time is regarded valid due to the midgut occupying approximately 75% of the gut volume.

Electrophoresis of protease-digested crystals

The alimentary canal of adult weevils were excised as described previously ("Midgut isolation and digestive fluid pH"), homogenized in 50mM Tris buffer (pH 8.0) and then centrifuged at 11,750 xg for 5 min. Percent solubility of crystal proteins of the strains in Tris buffer at pH 8.0 was not significantly different from that in Universal buffer at the same pH. The crystals were solubilized as in "solubilization of crystals at selected pH values", but with 50mM Tris buffer (pH 8.0) as opposed to Universal buffer. Preliminary results of crystal solubilization established the same level of solubilization in 50 mM Tris buffer (pH 8.0) as in the Universal buffer at the same pH. The ICP solutions were incubated with gut juices (1:5 v/v gut juice:crystal protein solution) producing a final concentration of 3μg of crystal protein per 1 μl of final suspension. The samples were incubated at 30 °C for 5 min, 30 min, 1 h, and 2 h which span the time of food passaging in the gut. Control samples consisted of solubilized crystals.

Samples dissolved in solubilizing buffer (0.5M Tris HCl, pH 6.8; 10% glycerol, 10% w/v SDS, 5% 2-b-mercaptoethanol, 0.05% w/v bromophenol blue) (Laemmli, 1970) in a ratio of 1:3 were boiled for 5 min at 100 °C, loaded onto a sodium dodecyl sulfate-polyacrylamide gel (10%

w/v) and run for 45 min at 200 mV. The molecular weights of protein standards included myosin (200.0 kDa), ß-galactosidase (116.2 kDa), phosphorylase B (97 4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21 5 kDa) (Bio-Rad, Ontario, Canada). The gels were stained with 0.1% Coomassie blue (Bio Rad, Ontario, Canada) in fixative (40% methanol with 10% acetic acid) and destained in the fixative.

Data analysis

Statistical analysis of paired data was performed using Student's t-test. One way analysis of variance (ANOVA) complimented by Tukey's test was used to compare groups of means. Statistical significance was taken at $p<0.05$. Results are presented as means ± standard error of the mean.

RESULTS

pH measurement of midgut digestive fluids fed on selected diets

The anterior and posterior midgut pH readings of insects fed either diet were not significantly different (Table 1).

Effect of pH on crystal solubilization

The crystal solubility of the four isolates of ICPs increased as the pH increased from 6.0 to 11.0 (Fig. 1). Strain A30 had the greatest solubility for the pH range tested while strain A311 had the least. The percentage solubility at a given pH varied with the bacterial strain. At the pH of the midgut (8 0), the order of solubility for the strains was A30 (69%) > BTI (55%) > A429 (37%) > A311 (14%).

A correlation (r=0 98) was found between the solubility of the more toxic ICP isolates at pH 8.0 and their LD_{50} values (Fig. 2). LD_{50} values increased with increasing solubility. This was not the case for strain A311 which had a high LD_{50} value but a low percent solubility.

Table 1. pH measurements of the midgut of the carrot weevil, *Listronotus oregonensis*, fed selected diets.

FOOD	MIDGUT SECTION [a]	
	Anterior	Posterior
Fresh foliage	8.19 ± 0.17	8.27 ± 0.21
Foliage with 4% agar	8.05 ± 0.18	7.74 ± 0.09

a Mean ± standard error of the mean, N=20

Figure 1. The solubility of the crystals of different strains of *Bacillus thuringiensis* in Universal buffer for 2 h at selected pH values. Each point represents the mean ± standard error, N=3.

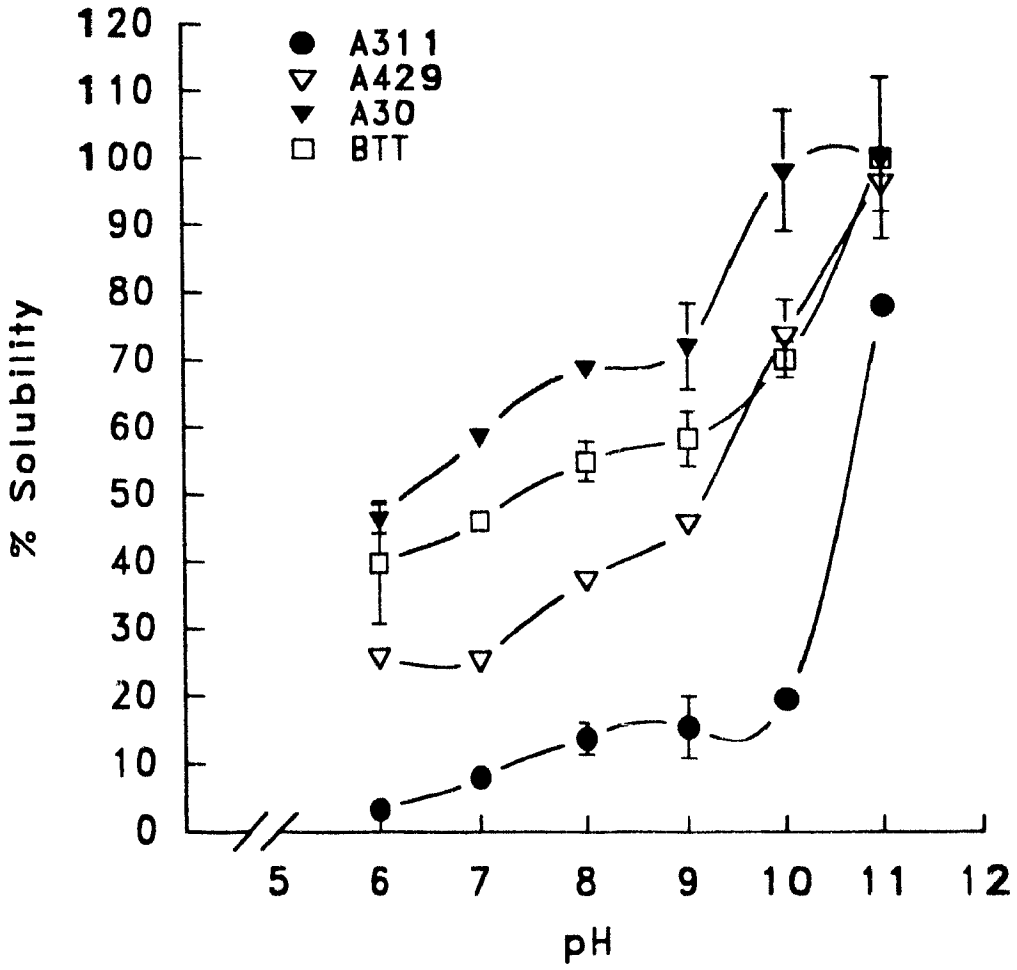
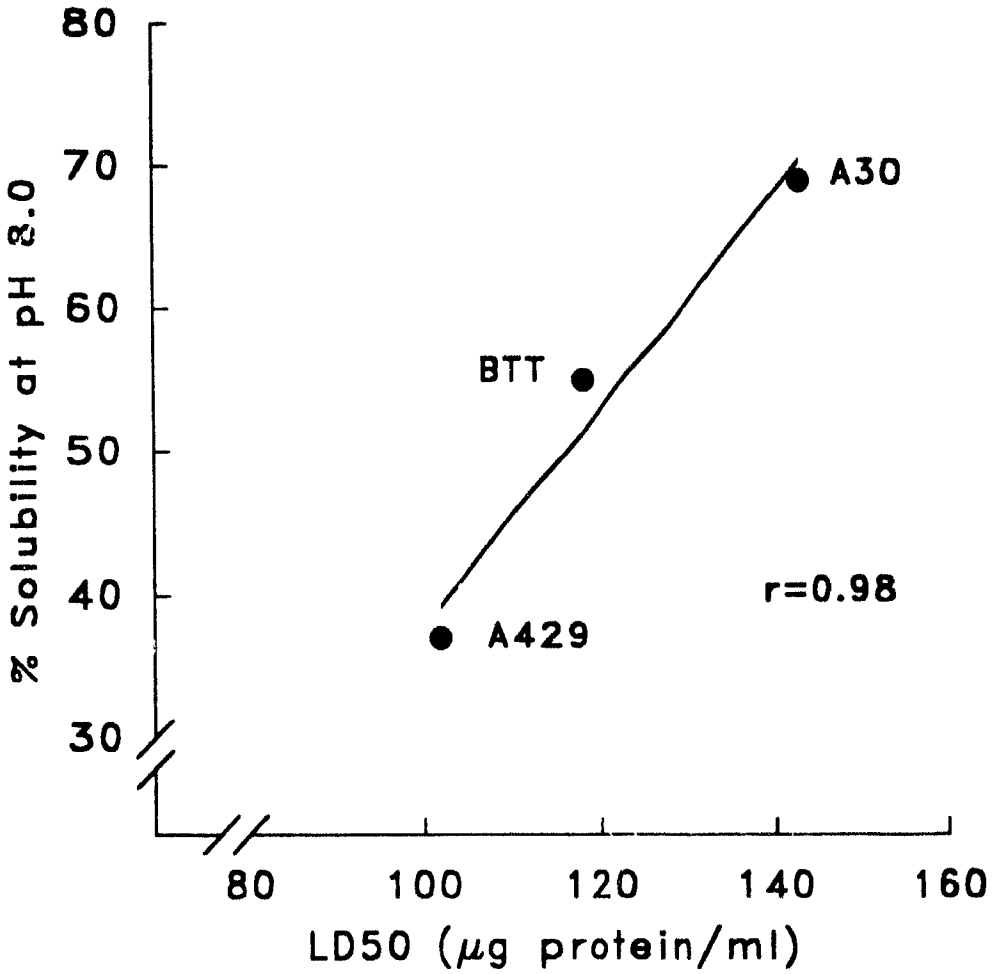

A311
A429
A30
BTT
% Solubility
120
110
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
12
pH

Figure 2. Relationship between the percent solubility of A429, BTT and A30 strains of *Bacillus thuringiensis* at pH 8.0 and their LD_{50} values against the adult carrot weevils (see Chapter 1 for LD_{50} values).


80
70
60
50
40
30
% Solubility at pH 8.0
A30
BTT
A429
r=0.98
80
100
120
140
160
LD50 (μg protein/ml)

The determination of the protein content per crystal per strain revealed that the least toxic strain, A311, had a significantly higher protein content than the more toxic ICPs of A429, A30, and BTT (Fig. 3).

Electrophoretic profile of protease-digested crystals in insect midgut fluids

The major proteins in the lanes with and without gut solubilized ICPs for strains A30, A429, and BTT consisted of polypeptides with apparent molecular weights of 70-71 kDa, 66 KDa and 33-36 kDa (Fig. 4). With strain BTT, these polypeptides remained stable after 2 h of proteolytic processing with weevil gut extracts. However, with strains A30 and A429, the band corresponding to the 71 kDa polypeptides diminished by 1 h postincubation as the 66 kDa band increased. These changes also occurred with strain A429 in addition to the appearance of a thin 58 kDa band as incubation continued. Smaller bands of 33-36 kDa also seemed to shift into smaller molecular weight polypeptides for the highly toxic isolates. The largest polypeptide that appeared in strain A311 was 35-36 kDa in weight when no protease had been added (Fig. 4). Proteolytic digestion for 2 h did not degrade this band.

DISCUSSION

Coleopterans including those susceptible to *B. thuringiensis* subsp. *tenebrionis* have been shown to have an acidic midgut environment (Murdock *et al*., 1987, Koller *et al*., 1992) essential to cysteine and aspartic proteases that predominate in the gut of phytophagous beetles (Thei and Houseman, 1990). However, the midgut pH of the adult carrot weevil proved to be slightly alkaline. Although this might imply that *B. thuringiensis* strains toxic to most Coleoptera with acidic midgut fluid would be ineffective against the carrot weevil, purified CRY III gene product, the crystal protein of *B. thuringiensis* subsp *tenebrionis*, with activity against *Chrysomela scripta* (midgut pH 5.0) is soluble under both moderately acidic and moderately alkaline conditions (Bernhard, 1986;

Figure 3. Protein content per crystal of the different strains of *Bacillus thuringiensis* in Universal buffer (pH 8.0). Each bar represents the mean ± standard error, N=3.

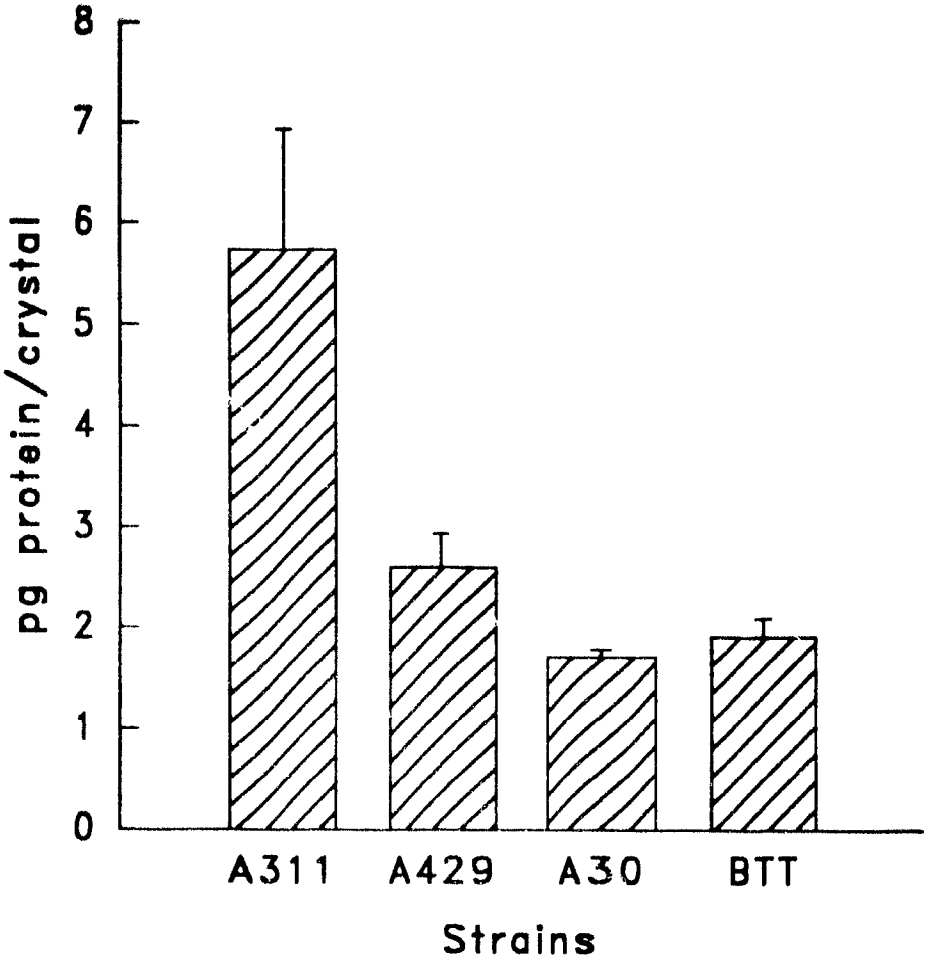
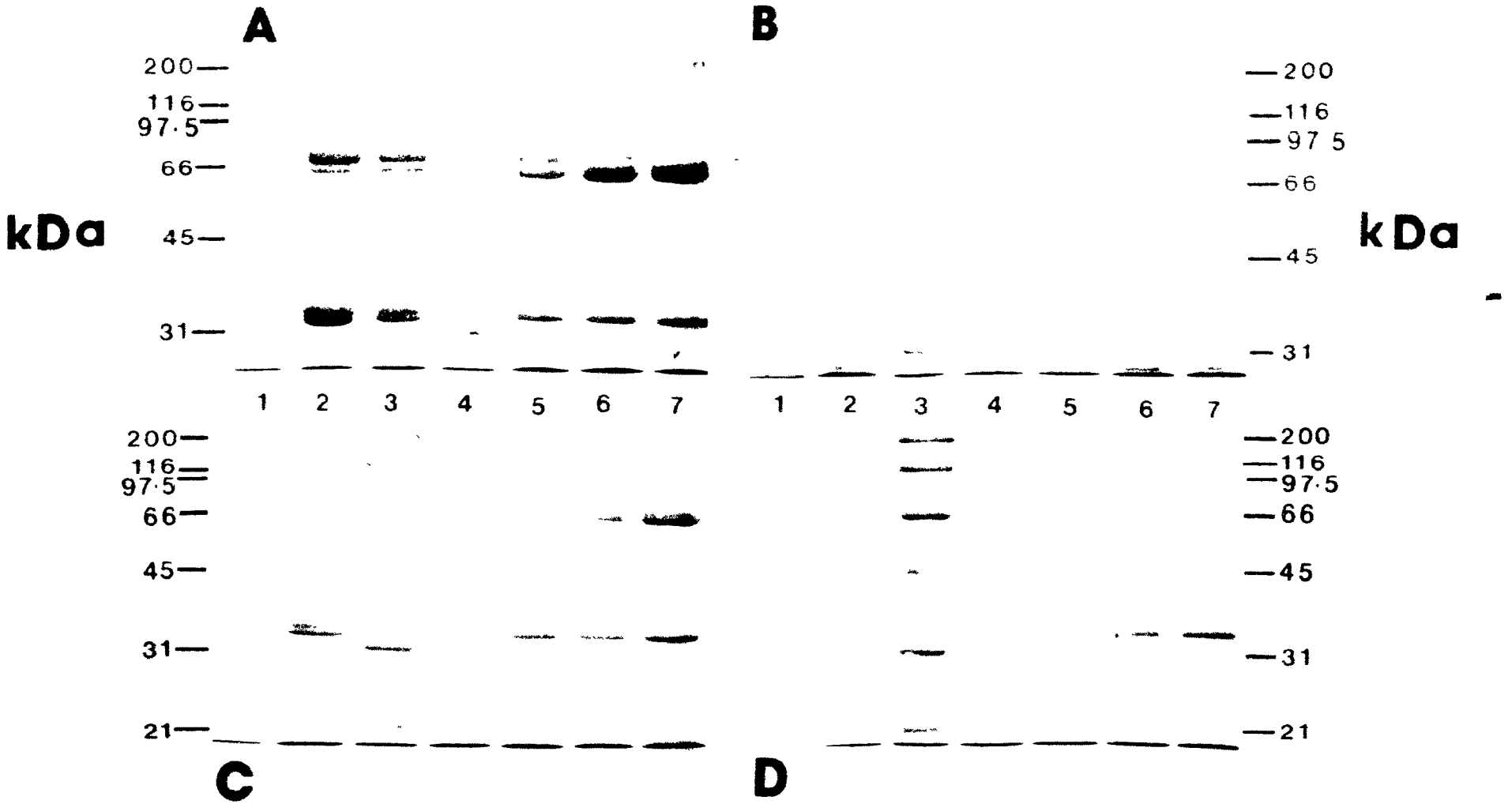

8
7
6
5
4
3
2
1
0
pg protein/crystal
A311
A429
A30
BTT
Strains

Figure 4. Proteolytic processing of the crystals of *Bacillus thuringiensis* strains A429 (A), BTT (B), A30 (C), and A311 (D) in the gut juice of the *Listronotus oregonensis*. For strains A311, A30, and BTT, lane 1 = gut extract, lane 2 = solubilized crystal, lane 3 = broad range molecular weight standards, lanes 4-7 = gut extract with crystals at increasing times of incubation (5 min, 30 min, 1 h, and 2 h). The difference with strain A429 is that lanes 3 and 4 are interchanged. Concentration of protein applied is 30 μg/10 μl.


A
B
kDa
200
116
97·5
66
45
31
1 2 3 4 5 6 7
C
D
21

Koller *et al*, 1992) making it potentially soluble with the midgut environment of a wide range of coleopteran hosts. In the present study, the test crystals from the different bacterial sources, including the *B. thuringiensis* subsp. *tenebrionis* control culture, appeared to increase in solubility throughout the pH range tested. This result differs from Koller *et al* (1992) in which purified crystals had limited solubility at pH 5.0 to 9.0. The difference may reflect bacterial isolate and or cultural conditions. However, the continuous increase in solubilized protein may represent leaching of spore proteins over the pH range of 6.0 to 9.0. At pH>10.0 the crystals may have solubilized explaining the sharp rise in the solubility observation. This being the case, spore crystal mixtures are not acceptable for crystal solubilization studies. That is not to say that spore crystal samples would be invalid for analyses of activity spectra since Jacquet *et al* (1987) reported that for susceptible insects toxin activity spectra was the same with both purified crystals and spore crystal mixtures. The results also pose the question as to whether the carrot weevil belongs to the type III group of insect, that require both spores and crystals for intoxication. Solubility of the toxin, while generally not thought to be a barrier to toxicity in susceptible insect, is an essential step in the expression of toxicity (Koller *et al*, 1992). The crystal solubility of strains A429, A30, and BII, which are relatively toxic to the adult carrot weevil (Chapter 2), at pH 8.0 is significantly more soluble than that of the less toxic strain A3II. This suggests that the midgut environment may be more conducive to solubilizing the toxins of the former strains than A3II ICP.

Since expression of toxicity is a function of solubility at the midgut pH (8.0), one would expect to associate high toxicity with high crystal solubility. Surprisingly, a relatively low solubility was observed for the most toxic strain (A429) with increasing solubility correlating with increasing LD$_{50}$ values. This inverse relationship between toxicity and crystal solubility might suggest that the active toxins of the more toxic strains bind to receptors with greater affinity and/or induce cellular damage more quickly than the less toxic isolates.

The amount of total protein per crystal was negatively correlated with the insecticidal activity of the strain since with the highly toxic

strains A429, A30, and BTT, having the lower total protein content per crystal than the least toxic strain A311. This also suggests that toxicity may be more dependent on the solubilization of specific protein components in the crystal rather than the quantity of protein in the crystals.

Unlike the lepidopteran-active crystal, CRY IA(c) which proteolytically breaks down from the 135 kDa protoxin to a stable 55-68 kDa toxin (Slaney *et al.*, 1992), none of the strains tested revealed a band larger than 71-72 kDa. The CRY IIIA toxin is synthesized as an unstable 70-73 kDa protein, which is cleaved to the stable 66-67 kDa protein in *L. decemlineata* (Carroll *et al.*, 1989, Slaney *et al.*, 1992). Strains A30, A429, and BTT possessed both polypeptides, and after 1-2 h of incubation in gut fluid, the 70-73 kDa band diminished as the 66-67 kDa band increased in intensity with prolonged incubation Not surprisingly, strain A311 appeared to lack the 70-73 kDa and 66-67 kDa bands in the absence of gut fluid, suggesting that the relatively weak toxicity of the strain may be due to the absence of a highly toxic moiety It is also possible that the 66-67 kDa may be present at physiologically significant levels but not detectable by electrophoresis. These observations argue in favour of either the total absence, or the presence of the toxic moiety in minute quantities in strain A311

The literature shows that proteolytic digestion is important for both activation and detoxification or degradation of the crystals (Nakamura *et al.*, 1992) Proteolysis of CRY IIIA in the digestive fluid of the Colorado Potato Beetle caused the complete degradation of the 67 kDa polypeptide after 16 min (Slaney *et al* , 1992) However, none of the strains with polypeptide 66-67 kDa showed any signs of detoxification after 2 h This suggests that the toxic moiety is stable (i e protease resistant) in the gut throughout the residence time of food The failure of the 66-67 kDa band of strains A30, A429 and BTT to be digested in the gut fluid of the carrot weevil may represent the absence of a proteolytic enzyme with the ability to digest that protein

Further research involving receptor binding studies should be undertaken to reveal the presence of a site of action (receptor binding site) of the toxins in the midgut epithelium brush-border membrane of the

carrot weevil. A comparative receptor binding study for the 4 strains would be an important step in further understanding the mode of action of the toxic strains, and in examining toxicity of strain A311.

REFERENCES

Aronson, A. I., E.- S. Han, W. McGaughey and D. Johnson. 1991. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects *App. Env. Microbiol.* 57(4) 981-986.

Bernhard, K. 1986 Studies on the delta-endotoxin of *Bacillus thuringiensis* var. *tenebrionis*. *FEMS Microbiol. Lett.* 33: 261-266.

Boivin, G. 1985. Evaluation of monitoring techniques for the carrot weevil, *Listronotus oregonensis* (Coleoptera; Curculionidae). *Can. Entomol* 117 927-933.

Bradford, M 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding *Anal. Biochem.* 72: 248-254.

Brussock, S. M and I C. Currier. 1990. Use of sodium dodecyl sulfate-polyacrilamide gel electrophoresis to quantify *Bacillus thuringiensis* δ-endotoxins. In: Analytical Chemistry of *Bacillus thuringiensis*, eds. L. A. Hickle and W. L. Fitch. pp. 78-87. Washington D.C American Chemical Society.

Calder, A A 1989 The alimentary canal and nervous system of Curculionoidae (Coleoptera). gross morphology and systematic significance *J. Natural History.* 23: 1205-1265

Carroll, J., J. Li and D. J. Ellar. 1989. Proteolytic processing of a coleopteran specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* 261: 99-106.

Chandler, S. C 1926. The economic importance of the carrot weevil in Illinois *J. Econ Entomol.* 19: 490-494.

Crawford, D N and W R Harvey. 1988 Barium and calcium block *Bacillus thuringiensis* subspecies *kurstaki* δ-endotoxin inhibition of potassium current across isolated midgut of larval *Manduca sexta*. *J. exp. Biol* 137: 277 286.

Grafius, E. and M. Otto. 1979. Detection and control of the carrot weevil. *Michigan State University. Ext. Bull.* E-890. 2p.

Hofmann, C., P. Luthy, R. Hutter and V. Pliska. 1988a. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush-border membrane

vesicles of the cabbage butterfly. (*Pieris brassicae*). *Eur. J. Biochem.* **173**: 85-91.

Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens and H. Van Mellaert. 1988b Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high affinity binding sites in the rush-border membrane of target insect midguts. *Proc. Natl. Acad. Sci USA* **85**: 7844-7848.

Hofte, H. and H R Whiteley 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**: 242-255.

Koller, C. N., L. S Bauer and R. M. Hollingworth. 1992 Characterization of the pH-mediated solubility of *Bacillus thuringiensis* var. *san diego* native δ-endotoxin crystals. *Biochem. Biophys. Res. Comm.* **184**: 692-699.

Jaquet, F., R. Hutter and P. Luthy. 1987. Specificty of *Bacillus thuringiensis* delta-endotoxin. *Appl. Environ. Microbiol* **53**: 500-504.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680 685.

Li, J., J. Carroll and D. J Ellar. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2 5 Å resolution. *Nature* **353** 815 821.

Martel, P., H. J. Svec and C R Harris. 1975 Mass rearing of the carrot weevil, *Listronotus oregonensis* (Coleoptera. Curculionidae) under controlled environmental conditions. *Can. Entomol.* **107** 95 98.

Murdock, L. L., G L. Brookhart, P. F. Dunn, D. E. Foard, S. Kelley, I Kitch, R. E Shade, R. H. Shukleand and J. L. Wolfson 1987. Cysteine digestive proteinases in Coleoptera. *Comp. Biochem. Physiol.* **87B**. 783-787

Nakamura, K., R. Murai-Nishioka, M. Shimizu, K. Oshie, K. Mikitami, K Oeda and H. Ohkawa. 1992. Insecticidal activity and processing in larval gut juices of genetically engineered 130 kDa protein of *Bacillus thuringiensis* subsp. *aizawai*. *Biosci. Biotech. Biochem.* **56(1)**: 1-7.

Pepper, B. B. 1942. The carrot weevil, *Listronotus oregonensis* (Bohe) in New Jersey and its control. *N. J. Agric. Exp. Str. Bull.* 693· 1-20.

Perron, J. P. 1971. Insect pests of carrots in organic soils of southwestern Québec with special reference to the carrot weevil, *Listronotus oregonensis* (Coleoptera: Curculionidae). *Can. Entomol.* 103: 1441-1446.

Slaney, A. C., H. L. Robins and L. English. 1992. Mode of action of *Bacilius thuringiensis* toxin Cry IIIA: An analysis of toxicity in *Leptinotarsa decemlineata* (Say) and *Diabrotica undecimpunctata howardi* Barber *Insect Biochem. Molec. Biol.* 22· 9-18.

Thei, N. M. and J. G. Houseman. 1990. Identification of cathepsin B, D and H in the larval midgut of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleptera· Chrysomelidae). *Insect Biochem.* 20: 313-318.

Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett and H. Van Mellaert. 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis. Science* 247: 72-74.

Whitcomb, W. D. 1965. The carrot weevil in Massachusetts. Biology and control. *University Massachusetts. Agric. Exp. Str. Bull.* 550: 1-30.

CONCLUSION AND FUTURE RESEARCH

Laboratory studies indicated that selected strains of *Bacillus thuringiensis* active against Coleoptera are potentially effective biological control agents of the adult carrot weevil. These strains could be incorporated into an integrated pest management (IPM) program.

Bacillus thuringiensis subsp. *tenebrionis* and strains A30 and A429 were selected as the most toxic among the seven strains that were tested against the carrot weevil adults. The fact that survivors of the insect having been exposed to these three strains do not resume normal levels of feeding suggests that irreversible gut paralysis has occurred which could translate into increased crop protection. At this point, experiments under field conditions would have to be carried out to test the strains as possible candidates for carrot crop protection. The formulation of these strains is also critical for field efficacy. It is necessary that the insect ingest a lethal dose before they stop feeding. The addition of baits and feeding stimulants could be advantageous as attractants. In addition, the protection of the bacterial mixture from sunlight would be essential since these are photosensitive. One factor that would be useful to examine is the effect of the strains on adult weevil fecundity of insects surviving the toxins. Although an attempt at that has been made, the study failed to yield any suitable results. The decrease in the rate of oviposition of insects subjected to the pathogen would be of great benefit in the control of this pest.

Studying the mode of action of the bacterium against this pest leads to a better understanding of the mechanisms involved in toxicity. The importance of solubility to toxicity should be tested by feeding adult weevils previously solubilized ICPs. It should also be determined, using purified ICP, if the carrot weevil requires both spores and crystals to be effectively managed. This would have substantial impact on the use of transgenic carrots containing only the δ-endotoxin. The 66-67 kDa protein band is known to be the toxic moiet of *B. thuringiensis* subsp. *tenebrionis* and should be tested for toxicity in strains A30 and A429.

The *Bacillus thuringiensis* strains hold promise as a control agent of the carrot weevil.

APPENDIX

Biological Products

Dr. W.N. Yule
Department of Entomology
Faculty of Agriculture
Macdonald College
McGill University
21,111 Lakeshore Road
Ste Anne de Bellevue, PQ
H9X 1C0

ICI Biological Products
North American
Technical Centre
2101 Hadwen Road
Sheridan Park
Mississauga, Ontario
Canada L5K 2L8
Telephone (416) 823 7160
Fax (416) 823 0044

Ref: DJG/File IA3-14-02

October 25, 1990

Dear Dr. Yule,

Please find enclosed six Bacillus thuringiensis strains (A30, A299, A311, A409, A410, A429) on Nutrient Agar slants. These strains have been isolated here at the ICI Biological Products, NATC, laboratory and are natural isolates from Canadian soil or grain samples. The six strains were shown to have activity against various insect species in insect bioassay screening.

Regarding your letter sent to Dr. Gannon dated October 18th; unfortunately, the strains which we are sending have not been identified as B. thuringiensis tenebrionis. These are natural isolates of B.t. which have been shown to be active against coleopteran insect species by bioassay. All of the strains sent have been chosen for this characteristic. I hope this is not a disappointment to you. If you really need an identified tenebrionis culture, we can access one for you through another division of ICI; so please let me know if this would help your research.

As a reminder, the details of our agreement with regard to these strains can be found in the Secrecy Agreement which is also enclosed.

If you have any further questions regarding these cultures, please feel free to contact either Dr. Roger Bernier or me. We look forward to further discussions.

Sincerely,

Lori Moser
Microbiologist

Encl.
HD-MISC/1090.252

c.c. R.L. Bernier

Biological Products

ICI Biological Products
North American
Technical Centre
2101 Hadwen Road
Sheridan Park
Mississauga, Ontario
Canada L5K 2L3
Telephone (416) 823 7160
Fax (416) 823 0044

Ms. Fabianne Saade
Dept. of Entomology
MacDonald College
21111 Lakeshore Rd.
Ste. Anne de Bellevue,
P.Q.
H9X 1C0

December 13, 1990

Dear Ms. Saade,

Enclosed in this package you will find 2 nutrient agar slants inoculated with our control strain of B.t. tenebrionis. Before sending the strain to you I have checked its purity, Gram stain and ability to produce crystals. The strain is characteristically a Gram positive rod that produces a large central spore and small crystals upon lysis. The isolated colony morphology is creamy white with irregular edges. In general, all B.t. strains grow best at 30°C and will enter lysis stage on day 3 or 4.

Good luck in your research and do not hesitate to call if you have any questions.

Guy Moser
Microbiologist