Bacterial Toxins for Insect Control

COST 862 Workshop
(programme, abstracts)

15-17 September 2005
Nitra, Slovakia

Local coordinator: Ľudovít Cagáň

Scientific Organizing Committee:
Ľudovít Cagáň, Neil Crickmore, David Ellar, Ruud de Maagd, Juan Ferré, Bjarne Munk Hansen, Ralf-Udo Ehlers
## Wednesday, September 14

Collection of participants at Vienna and Bratislava airports.

## Thursday, September 15

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**Bacterial Toxins for Insect Control, COST 862 Workshop (programme, abstracts)**
15-17 September 2005, Nitra, Slovakia

### Thursday, September 15

17.00-17.15
Possibilities of the use of *Bacillus thuringiensis* and entomopathogenic fungi in the control of sawflies (Hymenoptera, Symphyta)  
A. Sierpinska

17.15-17.30
*Bacillus thuringiensis* pathovar israelensis, a potential biological control agent against leatherjackets *Tipula paludosa* and *T. oleracea* (Diptera: Nematocera)  
O. Strauch, J. Oestergaard, C. Belau, R. U. Ehlers

17.30-18.00
Discussion

19.00
Dinner at Hotel Olympia

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### Friday, September 16

#### Session 4: Safety and Ecology  
**Chair: Bjarne Mark Hansen**

9.00 – 9.15
Overview on the studies conducted in Italy on the effects of Bt GM plants on non-target organisms  
Barbara Manachini

9.15-9.30
The cereulide genetic determinants of emetic *Bacillus cereus* are plasmid-borne  
Florence Hoton

9.30-9.45
The clonal structure of environmental *Bacillus thuringiensis* strains from north-east Poland correlates with their enterotoxin gene content but not their cry gene diversity.  
Izabela Swiecicka

9.45-10.00
Fate and effect of *B. thuringiensis* based insecticides in the human gut  
Andrea Wilcks

10.00-10.30
Discussion

10.30-11.00
Coffee Break

#### Session 5: Commercialization  
**Chair: Ralf-Udo Ehlers**

11.00-11.15
Global overview on Bt insecticides: Industrial perspectives  
Prem Warrior

11.15-11.30
Quality control of Bt products  
Jesko Oestergaard

11.30-11.45
EU Registration of Bt products  
Sergio Franceschini

11.45-12.00
REBECA - a project to review regulation of biological control agents  
Ralf-Udo Ehlers

12.00-12.30
Discussion

12.30-14.30
Lunch at Hotel Olympia

14.30-15.30
**General Discussion**  
**Chair: Neil Crickmore**

15.30-16.30
**Poster Discussion**

16.30-17.00
Coffee break

17.00-18.30
**Management Committee Meeting**

20.00
Dinner in local restaurant

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### Saturday, September 17

**Excursion**

8.00-10.00
Travel to southern Slovakia. Visiting maize fields attacked by Western corn root borer (*Diabrotica virgifera virgifera*).

10.00-12.00
Travel to Gabčíkovo and visiting dam on the Danube river.

12.00-13.00
Travel to Bratislava.

13.00-15.00
Free programme in Bratislava.

15.00-16.00
Short visit of Bratislava cathedral and castle.

16.00-18.00
Travel back to Nitra. Stop at maize fields attacked by the European corn borer (*Ostrinia nubilalis*).

18.00-19.00
Visit of Nitra castle.

20.00
Dinner at hotel Olympia.
Sunday, 18 July

8.00
Departure from Nitra (bus from Nitra to Bratislava and Vienna airport will be arranged).
Susceptibility of Greek populations of *Sesamia nonagrioides* (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) to a *Bacillus thuringiensis* endotoxin

P.G. Milonas¹, S.S. Andreadis², M. Savopoulou-Soultani², G. Mironidis² and N. Kagelaris²

¹Benaki Phytopathological Institute, ²Aristotle University of Thessaloniki

Baseline susceptibility to CryIAb toxin from Bacillus thuringiensis was determined for 3 populations of neonate Mediterranean corn borer, *Sesamia nonagrioides* Lefèbre (Serres, Thessaloniki, Larissa) and 1 of European corn borer, *Ostrinia nubilalis* (Hübner) (Serres) from Greece. *S. nonagrioides* and *O. nubilalis* neonate larvae were exposed to artificial diet treated on the surface of the diet with increasing Bt-toxin concentrations (ng/cm²). Larvae were kept in dark conditions at 25°C and mortality was evaluated after 7 days. The LC50 values for *S. nonagrioides* were calculated and they were 26.99, 28.02 and 27.44 ng/cm² for Serres, Thessaloniki, Larissa populations respectively. For *O. nubilalis* the LC50 value was determined as 29.32 (ng/cm²). A baseline susceptibility experiment was also performed for the population of *O. nubilalis* using artificial diets where the toxin was incorporated in the diet. The respective LD50 value was found as 20.64ng/gr. This study shows that both species seem to be equalled susceptible to *B. thuringiensis* CryIAb protein. No differences were detected among the *S. nonagrioides* populations and between the two species as well. For *S. nonagrioides* populations from Serres and Thessaloniki an F2 screen test was also performed to search for resistance alleles to *B. thuringiensis* toxin using Bt-corn seedlings. In total 41 isofemale lines were used and in none of them did we detect any allele conferring resistance to Bt-maize producing the CryIAb toxin.
Use of *Bacillus thuringiensis var. kurstaki* in crop and forest environments

Angela Niccoli  
*C.R.A. Consiglio per la Ricerca e la Sperimentazione in Agricoltura*  
*Istituto Sperimentale per la Zoologia Agraria*

Field and laboratory experiments have been carried out since 1980 to evaluate the effectiveness of Btk preparations in control of phytophagous insects in the order Lepidoptera, e.g. the European Grapevine Moth, the Olive Moth, the Gypsy Moth, the Brown Tail Moth, the Pine and Oak Processionary Caterpillars. In these investigations the mortality rates and/or the percentages of infestation related to the different species after the treatments were considered and the persistence of action in time after the applications was also evaluated. The infestation levels of the European Grapevine Moth and the Olive Moth, were satisfactorily reduced after treatments, even if this efficacy was not maintained in the subsequent generation. The effectiveness of Btk products in controlling the infestations of the two lymantriids was more evident, as the high levels of larval mortality after the treatments showed. The spore vitality of various Btk products on leaves of treated plants was also verified in different environments until 6 weeks after treatments. In the past few years my activity has turned to the evaluation of the effectiveness of different Btk formulations in the control of infestation of the Green Oak Leaf Roller Moth in mixed forests of deciduous oaks in Central Italy. The percentages of infestation of the vegetative apices after the treatment were significantly lower in the treated trees than in the control. Laboratory tests were carried out, in addition to field trials, to check the survival of Btk cells and Btk persistence of action. In consideration of the widespread distribution and of the recurrent damages of the Green Oak Leaf Roller Moth in the oak forests of Italy, deeper knowledge of the effects of the treatments in connection with larval development on different oak species is necessary. Planning further experiments on these issues in the near future, it seems to me, might be related to the topic discussed in the WG3 Working Group - Integrated Pest Management.
Insects, which cause serious damages in natural environments

Riziero Tiberi
Dipartimento di Biotecnologie agrarie
Università degli Studi di Firenze

In the field of forestry protection, my studies have focused mainly on the control of defoliating insects, which cause serious damages in natural environments. For this purpose for the past two decades in Central Italy several experiments have been carried out to test the efficacy of Btk preparations against lepidoptera, as Pine and Oak Processionary Caterpillars and the lymantriids, Lymantria dispar and Euproctis chrysorrhoea.

Commercial Btk products were used in the various studies, some of them in both field and laboratory trials, and the efficacy of the different products against the same species was compared. Effectiveness of the Btk products was also evaluated in relation to the larval age of the different defoliating species and to the dose and timing of application; persistence of action of the products was also taken into account.

Microbial preparations were employed against I and II instar larvae of Lymantria dispar, both in the field and in the laboratory, at two different doses. The larval mortality levels obtained were high enough to ensure the control of the infestations, independently of the dose employed. In the laboratory the survival of the spores and the persistence of the action of the Btk preparations were verified until 3 weeks after the treatment.

Experiments against the other two defoliators on broadleaved trees were conducted only in confined environment, where I and II instar larvae of Thaumetopoea processionea and III and IV instar larvae of Euproctis chrysorrhoea were treated. In the latter case the doses employed were higher because of the more advanced larval age. Anyway, the results obtained in both experiments were very satisfactory and the mortality percentages were similar to those of Lymantria dispar.

In consideration of the damages caused by the two lymantriids in Central Italy during their recurrent infestations and of the socio-sanitary problems connected with the urticant hairs of the Euproctis chrysorrhoea larvae, further tests are necessary about the use of Btk formulations in the control of these insects on different broadleaf species in forests with high environmental value and in environments frequented by man. Particular attention will be given to the study of other phytophagous insect species present in the environment where the control experiments will be carried out and to the investigation of the possible repercussions on populations of natural enemies.
Use of *Bacillus thuringiensis* var. *kurstaki* formulations for the control of harmful defoliators in the order Lepidoptera

Tiziana Panzavolta  
*Dipartimento di Biotecnologie agrarie Università degli Studi di Firenze*

During my studies in forest trees protection from insect pests, my attention has focused on the use of *Bacillus thuringiensis* var. *kurstaki* formulations for the control of harmful defoliators in the order Lepidoptera. My activity has been oriented particularly on the protection of pine forests from Pine Processionary Caterpillar infestations. To this regard various field and laboratory experiments on the use of Btk formulations were carried out, employing different doses against I and II instar larvae and also against IV and V instar larvae. The results of these tests showed high efficacy of the Btk products against early instar larvae, independently of the dose employed. On the contrary the same products, employed at higher doses against IV and V instar larvae, always caused similar or slightly higher larval mortality to that recorded in the field. The same trials were repeated, in the same periods, also in confined environment and their results confirmed those obtained in the field. In the laboratory the survival of the spores and the efficacy in time after the treatment of different Btk formulations were investigated.

The Pine Processionary Caterpillar is one of the most important phytophagous insects for the ecological and selvicultural damages caused to the pine forests and it is also one of the most feared species in urbanized environments because of the urticant hairs of larvae starting from the III instar and their effects on humans and other homeothermic animals. For these reasons it is my intention to carry on with the experiments on the use of Btk preparations mainly on III and IV instar larvae in the winter, when the nests are easily recognizable and nontarget species are no longer active. Since this approach has minimal impact on the natural environment, it would be recommended for use in areas where a certain amount of damage is tolerated. On the contrary its use in an urban environment, where the treatment threshold is very low because of the socio-sanitary problems connected with the urticant hairs, is still not sufficiently evaluated.
C-terminal extension effects on crystal formation and insecticidal properties of Colorado potato beetle-active *Bacillus thuringiensis* ä-endotoxins

Samir Naimov, Elena Martens-Uzunova, Mieke Weemen-Hendriks, Stefan Dukandjiev, Ivan Minkov, and Ruud A. de Maagd

Plant Research International Wageningen

Many Bacillus thuringiensis crystal proteins, particularly those active against lepidopteran insects, have C-terminal extensions that mediate bipyramidal crystal formation. These crystals are only soluble at high (>10) pH in reducing conditions such as generally found in the lepidopteran midgut. Most of the Colorado potato beetle-active toxins lack such an extension, yet some toxins with a C-terminal extension have cryptic activity against this insect, revealed only after in vitro solubilization. Crystal formation, morphology, protein content and activity against CPB were compared for two sets of proteins, the Cry1-hybrid SN19 and Cry3Aa, both with and without a C-terminal extension. Cry3Aa, with or without extension, formed flat square or rectangular crystals. SN19 (with extension) and its derivative without extension formed irregular inclusion bodies. All Cry3Aa and SN19 crystals and inclusion bodies were almost equally active before and after in vitro pre-solubilization and could be solubilized in diluted CPB midgut extract. In contrast, bipyramidal crystals of Cry1Ba were insoluble under these conditions. Our results suggest that bipyramidal crystal formation typical for proteins with a C-terminal extension may preclude activity against CPB, but that interfering with this crystal formation can increase the activity.
Laboratory evaluation of *Bacillus thuringiensis* compounds on the grape berry moth *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera, Tortricidae) and the mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae)

D.C. Kontodimas, O. Anastasopoulou, S. Chaleplidi and M. Anagnou - Veroniki Benaki Phytopathological Institute, Laboratory of Insect Microbiology and Pathology, Kifissia, Greece

The efficacy of some *Bacillus thuringiensis* compounds on the grape berry moth *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera, Tortricidae) and on the mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) was investigated. The products that have been tested were: Agree WP (*Bacillus thuringiensis* subsp. kurstaki / subsp. aizawai, *Thuricide* WP (B.t. subsp. kurstaki), Xentari WG (B.t. subsp. aizawai) and BMP 123 WP (B.t. encapsulated d-entotoxin). Three trials (and six repetitions in each trial) of each product were carried out in laboratory conditions (temperature: 26±1°C, relative humidity: 60±2% and photoperiod: 16 hours light / 8 hours dark). The products were mixed in three doses (recommended, ½ of the recommended and ¼ of the recommended) with the diet of the pests. Three days after the treatment, the mortalities of *L. botrana* larvae in the recommended doses for Agree, *Thuricide*, Xentari and BMP, were 82, 92, 93 and 54% respectively whereas the mortalities of *E. kuehniella* larvae were 95, 65, 18 and 25% respectively. Seven days after, the mortalities of *L. botrana* were 95, 93, 100 and 88% respectively and the mortalities of *E. kuehniella* were 100, 98, 25 and 43% respectively. In the lowest doses, seven days after the treatment, the mortalities were >57% for *L. botrana* and >17% for *E. kuehniella*. 
EU-project: “Protecting the Benefits of Bt-toxins from Insect Resistance Development by Monitoring and Management”

Christiane Saeglitz  
Institute of Environmental Research, Aachen University, (RWTH)

In a three-year EU-funded project, coordinated by Aachen University (RWTH), 11 partners from different countries cooperate to obtain detailed knowledge on Bt resistance development in the European corn borer and the Mediterranean corn borer. The aim of this project is to establish baseline data on pest behaviour, genetics, biochemistry, frequency of resistance alleles and molecular biology while trying to anticipate future evolutionary changes on all these levels. One core component, investigated by the RWTH partner, is to characterize the genetic diversity of ECB populations across Europe. Data from allozyme analysis and AFLP showed very small genetic differences among ECB populations across Europe. Further studies on the establishment of the baseline susceptibility of different European ECB populations to Cry1Ab were performed. There were no significant differences in the susceptibility of populations from different regions of Germany. One major problem arising in such tests is the origin, the efficacy and the longevity of toxins. Quality standards of toxins and test methods must be established for future monitoring to guarantee consistency of the results. The research activities of all different partners will be combined to deduce an overall resistance management plan for Bt-maize in Europe.
Biosafety Research of Transgenic Diabrotica-resistant Bt-maize: Development and Evaluation of Monitoring Methods

Ingolf Schuphan
Institute of Environmental Research (Biology V), Chair of Ecology, Ecotoxicology, Ecochemistry, Aachen University (RWTH),

Biosafety Research of Transgenic Diabrotica-resistant Bt-maize: Development and Evaluation of Monitoring Methods
A trial release of Bt-maize Mon88017 has been approved for 3 years in the context of a national joined research project funded by the Federal Ministry of Education and Research (BMBF, RWTH co-ordination. The possible effects of Bt-maize on non-target organisms will be investigated in the next 3 years in co-operation with 10 partner institutions. On an experimental field of 6 ha, Diabrotica-resistant Bt-maize Mon88017, a close isogenic line, and two conventional maize lines are cultivated in a plot design with 8 replicates each. The non-target studies include organisms of the edaphon, epigaeon and hypergäon. Additional work addresses the development and application of analytical methods, the activation and degradation behaviour of the Cry3Bb1 toxin, potential alternative host plants for Diabrotica, biotests, and the development of appropriate biometric methods for statistical data analyses.
It is of interest to discuss with/find partners for a future wide-ranging project focussing on resistance management which will include molecular approaches.
The cereulide genetic determinants of emetic Bacillus cereus are plasmid-borne

Florence Hoton  
Laboratory of Food and Environmental Microbiology, Croix du Sud, Belgium

The cereulide genetic determinants of emetic Bacillus cereus are plasmid-borne. Generally, food-borne illness caused by Bacillus cereus are relatively mild and do not last more than 24 h. However, the emetic syndrome can have a fatal and fulminant outcome. It was the case in Kinrooi (Belgium), in 2003, when a seven years old girl died after pasta consumption contaminated by B. cereus. Cereulide (a small cyclic dodecadepsipeptide [(D-O-Leu-D-Ala-L-O-Val-L-Val)3]) is thought to be responsible for the child’s death. This study shows that the genomic location of genetic determinants involved in the cereulide production is extrachromosomal, on a plasmid named pCERE01. Hoton, F. M., Andrup, L., Swiecicka, I. & Mahillon, J. (2005) The cereulide genetic determinants of emetic Bacillus cereus are plasmid-borne. Microbiology 151, 2121-2124.
Genetic exchanges among members of the Bacillus cereus group: conjugation and mobilization of small and large plasmids between B. cereus and Bacillus thuringiensis.

Jacques Mahillon
Laboratory of Food and Environmental Microbiology, Croix du Sud, Belgium

This communication will focus on 1) the lateral gene exchanges driven by two conjugative plasmids, pAW63 and pXO16 originating from B. thuringiensis and 2) the detailed functional map of pAW63 and its relationship with the virulence plasmid pXO2 from Bacillus anthracis.
Large-scale production of Cry1Ab toxin from E.coli - its activity and stability after long time storage

Nguyen Thu Hang, Johannes A. Jehle
Labor für biotechnologischen Pflanzenschutz, Abt. Phytomedizin, Breitenweg 71, 67435 Neustadt/Wstr., Germany

The aim of the project is to produce standardised Cry1Ab, which can be used for the resistance monitoring of the European Corn Borer and/or other Cry1Ab target insects. Commercial cultivation of transgenic Bt plants will require appropriate case-specific resistance monitoring and general surveillance of non-target effects. For that reason, the production and supply of standardised Cry1Ab is a pre-requisite for monitoring studies. Our major objective is to validate monitoring methods and to produce Cry1Ab standards that can be used for mentoring and risk assessment studies. Production of the standardised Cry1Ab toxin as well as formulation of standardised methods will be recorded. We are also conducting bioassays using neonate larvae of Ostrinia nubilalis and the biological activity of each Cry1Ab batch will be compared. In order to investigate their stability and activity, these toxin charges will be stored under different temperature regimes and biochemical and biological analyses will be carried out. This project is funded from 2005 until 2008 by the German Federal Ministry of Science and Education.
Risk assessment of the development of resistance traits of the invasive western corn rootworm (Diabrotica virgifera virgifera) against Bt-maize due to the use of alternative host plants

Joachim Moeser & Stefan Vidal, Institute of Plant Pathology and Plant Protection, Georg-August University Goettingen, Germany

The use of Bt-maize is regarded as a valuable management tool against the invasive maize pest, Diabrotica virgifera virgifera (Coleoptera, Chrysomelidae) which was introduced into Europe in the 1990s. Besides environmental concerns, one of the major problems regarding the use of this technology is the possible development of resistant insect strains. The use of transgenic maize in integrated pest management approaches requires resistance management to avoid a possible rapid selection of resistant insects. Therefore we are studying possible mechanisms of selection of resistant D. v. virgifera under semi-field conditions. The Bt-maize developed to counter D. v. virgifera larval damage expresses the Cry 3Bb1 toxin in relatively low doses. The use of alternative host plants (grassy weeds or monocot crops) during part of their lifecycle could therefore lead to larvae which have been exposed to only limited amounts of toxin. Especially the first larval stage could feed on alternate host plants and move to maize once this food source becomes depleted. As older larvae seem to be more tolerant to low doses of the expressed Cry3Bb toxin this could lead to partially resistant individuals. These could build up a resistant population if no resistance management plan is being implemented. Furthermore the impact of possible refuge strategies is being evaluated (seed mix vs distinct blocks of Bt and refuge maize). Larval behavior and performance will be studied on these interfaces of transgenic and conventional maize with regard to resistance management strategies.
The potential use of Bt toxins in Italian Natural Reserves

Filippo Castiglia
Azienda Regionale Foreste Demaniali, Palermo

In the Natural Reserve “Bosco della Ficuzza, Bosco del Cappelliere, Rocca Busambra, Gorgo del Drago” one of the most interesting example of Mediterranean forest wide 3.600 hectares dominated by Evergreen oak (Quercus ilex) and Downy oak (Quercus pubescens s.l.). In the last years Downy oak suffered for increasing attacks of the lepidopteranspecies Tortrix viridana L. and Lymantria dispar L.. Those Lepidoptera in the stadium of larva is able to cause severe damage to the single tree, eating the most part of leaves. Occasional attacks are quite normal and never ask for treatment, but in the last three years the population explosion is repeating year after year. In many cases the pests attack the same tree or group of tree, so it’s going to lose the capacity of reacting to defoliation. The effects of their activity include reduction in tree growth, crown dieback and tree mortality. In extreme situations of outbreaks, nearly 100% tree mortality may occur over large areas Moreover some people also have allergies to the urticacious hairs on L. dispar larvae, further compromising their recreational experience.

The high level of biodiversity and the site importance classified as SIC according to Dir CEE 92/43 and ZPS (Dir CEE 79/409) need great attention to the different causes of this phenomena (climatic, lack of wood cure, decreasing of predation). Anyway a direct control of population density of those lepidopteran using bacterial toxins could be the on of the most auspicable control method in a natural reserve. Bt toxins should be used for their specific activity and low environmental impact.

So we are planning to monitor the population of T. viridana and L. dispar using traps and pheromones. However data regarding the biosafety of the Bt toxins are essential in the case of natural reserves. For this reason, according to the fly period, the products could be applied only those trees with an high numbers of larvae, near to the roads. Moreover infestation of the two lepidoptera should be monitored by GPS to verify the progression of the attacks and the success of the treatments. In this way we aim to reduce population in the most important places for diffusion of these pests and try to preserve an important natural areas

At the moment we are moving the first steps to control those lepidopterans, there is not experience of using BT in our forests. So everything from susceptibility of target and no target insects, to rate and period of treatment, needs to be studied before apply treatment. Other potential microbial control as the naturally-occurring bacteria Streptococcus faecalis and Pseudomonas spp. occasionally cause high levels of mortality (up to 60%) under outbreak conditions (Podgwaite 1981). Investigations in our forests of the natural pathogens present of these Lepidoptera could also help to control them in a more environmental friendly way.

Another interesting aspect to be considered in the natural areas is the similarity among the natural strains of Bt, present in the Reserve, and the strains commercially available. This could be interesting in the view of preserving natural biodiversity. Podgwaite, J.D. 1981. Environmental persistence of gypsy moth NPV. pp. 479-487. In Doane, C.C., and M.L. McManus, Eds. The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C.

Podgwaite, J.D. 1981. Environmental persistence of gypsy moth NPV. pp. 479-487. In Doane, C.C., and M.L. McManus, Eds. The gypsy moth: Research toward integrated pest management. Technical Bulletin 1584. USDA, Washington, D.C. comments: Azienda Regionale Foreste Demaniali, in charge of manage the Natural Reserve “Bosco della Ficuzza, Bosco del Cappelliere, Rocca Busambra, Gorgo del Drago”, is planning the activities to control this emergency and will appreciate the participation to following workshops of his representative (Dr. Filippo Castiglia, Dr Agatino Sidoti) in the IPM working group.
Could *Bacillus thuringiensis* endo-toxins mutants overcome resistance to *Bacillus thuringiensis*

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Collection of entomopathogenic bacteria and their toxicity against fly pests

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A precious collection of entomopathogenic soil-occurring bacteria have been built in Sardinia (Italy) during the last few years as the result of a sampling activity in different areas of Europe, Asia, South America, Africa. Most of the isolates have been identified as Bacillus thuringiensis by morphological and genetic observations. A preliminary characterization of the collection have been based on the detection of some cry genes with general and specific primers, and the analysis of the protein profile by SDS PAGE.

Laboratory bioassays have been carried out to test the toxicity of these bacterial isolates against three important fly pests: 1) the poliphagous Medfly (Ceratitis capitata Wied.), one of the world’s most destructive fruit pest, 2) the Olive Fruit Fly (Bactrocera oleae Gmel.) which represents the most important olive pest, and 3) the Housefly (Musca domestica L.), a very common and world-wide species of medical and veterinary importance.

Among about 300 different bacterial isolates, 4 were significantly toxic to Housefly adults, 1 to Housefly larvae, 11 to adult of Olive Fruit Fly, 20 to Olive Fruit Fly larvae, 6 to adult of Medfly and 26 to Medfly larvae.

Toxicity assays were also performed on the Hymenoptera Opius concolor Szepl. and Muscidifurax raptor Girault and Sanders (Olive Fruit Fly and Housefly pupal parasidoids, respectively), to evaluate the ecological impact of these bacteria on the natural demographic regulators of these pests. No significant toxicity was detected against these useful insects.

The isolates which showed a significant toxicity are actually in course of characterization.

Experimental treatments in olive crops against Olive Fruit Fly adults and in livestock against both Housefly adults and larvae were carried out using new formulations based on the most toxic bacterial strains. Sampling methods for the monitoring of fly populations and strategies for the distribution of the bacterial formulations were studied. Encouraging results have been obtained comparing to fly densities of untreated areas.

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From the Bacillus thuringiensis discovery, in 1977, the interest in the search for other suitable biological control agents has constantly increased. Microorganisms are currently under intensive study for use as biopesticides and several fungal species including Metarhizium anisopliae, Verticillium lecanii and Beauveria bassiana are being used as biocontrol agents for a number of crop, livestock and human nuisance pests.

Of course the availability of satisfactory tools for pathogenic strains’ identification and monitoring has been a central theme in using microorganisms for biological control. This theme is furthermore supported by a persistent paradigm in insect pathology, which suggests that the host insect is the predominant influence on the population genetics of insect-pathogenic microorganisms.

Recent findings led to a significant theory shift: habitat selection, not host insect selection, drives the population structure of insect-pathogenic microorganisms.

In other words, habitat preferences should be considered as a primary feature for selecting microbial strains to be used in insect biocontrol efforts, particularly if microorganisms cycling and survival are key considerations. Undoubtedly insects, invertebrates in general terms, host numerous bacteria, with interactions ranging from pathogenesis to symbiosis. While certain symbiotic relationships have been well studied little is known about the dynamics of pathogenic bacterial communities.

Actually, since fungal and bacterial diseases in insects are more common and widespread than we would expect, our first concern should be an accurate evaluation of microbial biodiversity associated to pests, in order to obtain useful information about novel species of potential interest for pest control in conventional and organic agriculture in Europe.

In this regard techniques, such as 16S rDNA-DGGE, able to examine in situ the microbial ecology associated to specific habitat appear to be the simplest way to proceed.

The Denaturing Gradient Gel Electrophoresis (DGGE) represents an innovative approach that allows investigating on microbiota associated to particular habitats, encompassing microorganism’s cultivation and providing thus a more realistic view of microbial diversity in the ecosystems.

DGGE analysis of 16S rRNA gene fragments generated by Polymerase Chain Reaction (PCR) by using general eubacterial primers could allow to eubacterial DNA separation from the total insects DNA: appropriately chosen DNA region amplification and sequencing could provide a believable picture of microbial associates to insects, with particularly regard to the culturable portion.

Simultaneously to screening programs for new strains, it is necessary to delineate molecular markers to facilitate strains identification and able to correlate strains with particular pathogenic biotypes. With this aim, PCR-based techniques could be presumptively employed to further characterize at molecular level bacterial strains with regards to their pathogenic potential.
Use of *Bacillus thuringiensis* against the lepidopterous defoliators in oak forests

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Mediterranean oak forests are exposed periodically to infestations of gypsy moth (Lymantria dispar L.), commons lackey moth (Malacosoma neustria L.), and green oak leaf roller moth (Tortrix viridana L.). Outbreaks of these species can cause the total defoliation of thousands of hectares, thus worsening oak health conditions, limiting their productivity, and threatening the plants’ survival.

In order to control gypsy moth infestation, from 1990, aerial spray trials using several preparations of *Bacillus thuringiensis* Berl. subsp. *kurstaki* (Btk) were performed in Sardinian cork oak forests.

We determined the product and modality of application most suitable to a Mediterranean environment. Btk did not have any direct effect on gypsy moth parasites and predators activity, whose incidence in treated and untreated areas seemed to be dependent on host densities. Moreover, the effect of a high dose of Btk on richness and abundance of non target lepidopterous fauna was similar to that of completely defoliated cork oak forests heavily infested by *L. dispar*.

Based on these preliminary trials, a microbiological control program to limit the damage caused by *L. dispar* and *M. neustria* was implemented in approximately 60,000 hectares of Sardinian cork oak forests from 2001 to 2005. In all areas, 4 litres per hectare (42.4 BIU/ha) of Foray 48B, a Btk-based insecticide, were sprayed at ultra low volume with a helicopter having 4 micronairs. The control program was effective, causing an adequate protection of spring budding of cork oaks, with an indirect protection of cork production.

The treatments with Foray 48B demonstrated good efficacy also in pubescent oak forests infested by *Tortrix viridana*, even though the larvae of this species live and feed inside shelters they make by rolling up the leaf edge, which protect them from the insecticide.

In the COST program we are very interested in: 1) test in laboratory the toxicity of Bt strains against the oak lepidopterous defoliators; 2) carry out field test with Bt products of the European companies in order to determine the efficacy against oak pests and to evaluate the effects on non target arthropods.
Overview on the studies conducted in Italy on the effects of Bt GM plants on non target organisms

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Laboratory tests and field experiments were conducted to determine the effects of corn (Zea mays) expressing Cry1Ab protein derived from Bacillus thuringiensis Berliner subsp. kurstaki, on non-target insects and on soil fauna. Chrysopeola carnea Stephen was feed whit the aphid Rhopalosiphum padi L. reared in laboratory on Bt and on traditional. No detrimental effects of transgenic Bt corn on preimaginal development and mortality of C. carnea were observed. The abundance of aphids was not affect by the presence transgenic corn crops. The statistical analyses revealed no significant differences in the abundance of this sucking insect in isogenic corn and Bt corn crops.

Moreover investigation was done on different parasitoids of ECB eg. Lydella thompsoni Hert and Trichogramma brassicae Bedz. In field Coleoptera Carabidae and Nematoda were studied, as typical soil fauna, comparing community structures and degree of specific biodiversity in transgenic and isogenic corn crops. The ground beetle assemblages and the nematofauna were quite similar, and any differences appeared to have no correlation with the presence of Bt maize. The biodiversity of the soil communities was quite low for both types of crops and, on comparing the two types of hybrid corn crops, no significant differences were found in any of the indices analyzed. However some differences were recorded in the case of fungal feeders nematodes when the BT GM plant was growth in silty soil. The effect of Bt plants on soil fauna was also investigated in the case of Bt Brassica napus (Cry IAc), Bt Solanum melongena (CryIIIB), Bt Solanum tuberosum (CryIAb). Discussion for differences or lack of them are discussed.

2. Monitoring of European corn Borer (Ostrinia nubilalis Hb.) resistance to Bt toxins in Italy

Field corn, Zea mays L., is one of the main crop plant engineered genetically to express the Cry 1Ab protein derived from Bacillus thuringiensis Berliner var. kurstaki (Koziel et al. 1993) with an insecticidal activity against Ostrinia nubilalis Hub. (European corn borer).

The widespread use of maize expressing Bt á-endotoxin might lead to development of resistance in insect pest to Bt toxins, such concept is based largely on predictions from population genetic and computer models (Gould, 1998). Moreover the potential for adaptation of several insect to conventional insecticides and in some cases to transgenic crops has already been documented. Commercial products based on the bacterium B. thuringiensis have been routinely used for insect control for about 40 years. The European corn borer has never developed resistance to the insecticidal proteins contained in such products. However, in some cases, other lepidopteran insects belonging to the same family of European corn borer or to families phylogenic closed, have developed resistance to B. thuringiensis microbial insecticides (Bailey et al., 1998) and to several proteins insecticide derived from Bt. Many different approaches are requested to study and forecast the phenomena. For example baseline susceptibility of European corn borer larvae (Ostrinia nubilalis Hb.) to a commercial formulation of B. thuringiensis var. kurstaki was determined for different strains in 7 geographic locations in the corn area of North Italy (Po Valley). For the bioassay 7 different doses, raised on a meredic diet, were used and the data was analyzed with the Probit analysis. Differences in the susceptibility among the northern Italian populations of O. nubilalis were recorded, which can be attributed to natural variability. The ECB collected near Como and Turin was the most susceptible, respectively with LCS0 of 0.23 and 0.57 mg/ml. In addition data are available for the susceptibility only to the Cry1Ab toxin alone. Moreover F2 screen and discriminate dose used to select resistant strains are in progress. Discussion on the research done in Italy and on the problems connected to the different methods will be provide.
The 140 kDa δ-endotoxin of the Bacillus thuringiensis strain 1.1

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The 140 kDa δ-endotoxin of the Bacillus thuringiensis strain 1.1, which exhibits β-glucosidase enzymatic activity, is involved in the process of spore germination based on the following data: (i) the 140 kDa δ-endotoxin of the Bt 1.1 is on the spore coat and in this location displays enzymatic activity, (ii) substrates of β-glucosidase lead to germination of spores of the Bt strain 1.1 but not to the acrystalliferous strain 4.1, in which the 140 kDa δ-endotoxin was not detected in the spores. Additionally the spores of both strains can be germinated by glucose. (iii) reduction of enzymatic activity of δ-endotoxin, by incubation of spores far from the β-glucosidase’s pH optimum or by antibodies (directed against the 140 kDa δ-endotoxin or against β-glucosidase) results also in reduction of germination.
Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of Bacillus thuringiensis ssp. israelensis

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Classical biological control is the most successful and promising way to replace chemical pesticides. The sub-species israelensis of Bacillus thuringiensis (Bti) is a safe and efficient agent to control mosquito larvae and hence mosquito-borne diseases. One approach to overcome the low efficacy and short half-life in nature of current formulations of Bti is by expressing the toxin genes in recombinant cyanobacteria as a delivery system. Attempts to express Bti toxin in cyanobacteria have been carried out during the last ten years. Toxicities of the transgenic strains were however very low, even under regulation of strong promoters, too low to be effective in vivo. We have derived and patented a series of transgenic Anabaena PCC 7120 (a nitrogen-fixing, filamentous cyanobacterium) expressing combinations of 3 genes coding for two ?-endotoxins and an accessory protein from Bti. The toxins operate in different modes of action thus synergistically. The genes and their products are found in nature, and the transformed organism (Anabaena sp.) is ubiquitous in water bodies around the world, hence safe for use from environmental viewpoint. The transgenic Anabaena strains are long lasting alternatives to Bti for field delivery with exceedingly high mosquito larvicidal activities: they serve as food source for the larvae and proliferate in their breeding habitats. Releasing such recombinant strains to open environment is still a major obstacle in exploiting this biotechnology.
Fate and effect of *B. thuringiensis* based insecticides in the human gut

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Animal studies with germfree rats harbouring a human faecal flora were performed with one *B. cereus* and two *Bt* strains. The survival and persistence of the strains was studied, as also in vivo enterotoxin production. Furthermore an effect on the surrounding flora was studied by bacteriological counting and DGGE.

2) *Bacillus cereus* like organisms isolated from ready-to-eat food, including fruit and vegetables, and their relationship to commercial strains.

Nearly 50,000 food samples were analysed, 0.5% had counts of Bc like bacteria above 10-4 cfu/g. High counts were beside starchy, cooked products, also found in fresh cucumbers, tomatoes and peppers. In these vegetables, the majority of isolated strains could not be distinguished by RAPD and plasmid profiling from commercial strains used in biocontrol. This indicates that residues of commercial biopesticides based on Bt can be found on vegetables.
Assessing the Environmental Effects of Transgenic Crops: a Quantitative Approach

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The knowledge about the effects of genetically modified plants on non-target organisms is increasing, nevertheless results from experimental studies are not univocal. In a recent review of laboratory studies (Lovei and Arpaia, Ent.Appl.Exp.,114: 1-14, 2005) it has been highlighted that globally 18 predator species and 14 parasitoid species were tested; most in a few or even a single experiment. Whole orders (e.g. Diptera) were almost neglected in biosafety studies, moreover very often the ecological realism of such studies is not sufficient to mimic natural conditions. An aggregate evaluation indicates that a fair number of cases (30 % for predators, 39,8 % for parasitoids) singled out a significant negative effect on individual species. This evaluation points out the need for experimental data gathered in more natural conditions (greenhouse and field studies) to adequately assess any impact even in the pre-release phase of development.

As it comes to field studies, a large and diverse array of different methods and techniques has been adopted, not always in the most appropriate way.

In the framework of the research project Bt-BioNoTa, funded by the EU, it has been developed a quantitative approach for monitoring. It can be assumed that in many cases taxonomic knowledge is only partial and, in such situations the use of organismal taxonomic units (OTU) can be turn out acceptable and preferable to lumping species into larger units (orders, families). Moreover, field sampling will often be performed by technicians or parataxonomists that are able to recognise distinct taxonomic units, but not to allocate in different higher units.

In our experimental fields the presence and abundance of herbivores and predators were monitored weekly by visual samplings in three different crops (canola, potato, eggplant) for three years (2001-2003). Specimen were then pooled in OTUs (6-15, according to the crop). Several multivariate methods were utilised and data analyses were performed for a total of 8 field experiments.

Based on our analyses, we suggest that profile analysis (e.g. by using additive correspondence analysis) should be a first logical step. Comparisons between differently treated (or affected) areas with a diversity indexes (sensu Southwood, Ecological Methods, Chapman and Hall, 1978) can be done by applying multivariate methods, based on distance measures and permutations.

The possible identification of taxa significantly associated with a particular treatment (e.g. a new GM crop or trait) is a very important objective. The selection of indicator species (if they exist) in this case, would only be done after the data collection step and would be numerically supported by statistical analysis. For this goal, an interesting new method is the Indicator Species Analysis.

The general outcome of field studies indicate a general similarity of species assemblage between transgenic crops and their near isogenic control. Nevertheless, some taxa may need further studies, for example flea beetles (Coleoptera: Chrysomelidae, Alticinae) in eggplants showed a different degree of aggregation between treatments. Moreover the dynamics of some flower dwelling species (e.g., Thripidae, Staphilinidae) may deserve more studies in order to clarify some aspects of their interactions with their host plants.
The clonal structure of environmental Bacillus thuringiensis strains from north-east Poland correlates with their enterotoxins gene content but not with cry gene diversity

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B. thuringiensis, thanks to its high delta-endotoxin specificity against target insects, is the most commonly used microbial pesticide throughout the world. In spite of a wide variety of B. thuringiensis strains, the search for novel insecticidal activities is still relevant. New isolates of this bacterium can display previously unknown activity against a number of pests, which are not controlled with the available delta-endotoxins, and provide novel alternatives for coping with the problem of resistance. Except for the accumulation of entomopathogenic inclusions encoded by cry genes located on large plasmids, this bacterium appears to be indistinguishable from Bacillus cereus, an opportunistic human pathogen ubiquitous in soil, water and foods. Many strains of B. cereus cause diarrhoea, which is attributed to the combinatorial action of various enterotoxins, such as the haemolytic (HBL) and non-haemolytic (NHE) enterotoxins. Although B. thuringiensis is generally regarded as a beneficial microorganism, there are some indications of enterotoxins synthesis by this bacterium. Yet, little is known about B. cereus-like enterotoxins produced by environmental B. thuringiensis strains. Considering the taxonomic similarity between B. thuringiensis and B. cereus and the extensive introduction of B. thuringiensis into human and animal food during its usage as insecticide, studies on the B. thuringiensis virulence factors presence in environmental strains are of major importance.

In the present study, the abundance of the delta-endotoxin, and the haemolytic and non-haemolytic enterotoxins genes was investigated in over one hundred B. thuringiensis isolates recovered from the intestines of small wild mammals from NE Poland on the basis of PCR amplification. To confirm the outcomes for enterotoxin genes testing, Southern blot hybridisation was also applied to all of the strains under study. The genetic relationship between the strains was determined on the basis of pulsed-field gel electrophoresis (PFGE).

The percentage of B. thuringiensis strains harbouring genes coding for toxins active against Lepidoptera (cry1, cry2, cry9) was very high (64%), compared to that of Diptera specific strains (cry4, 14%). No strain with cry genes coding for proteins directed against coleopteran larvae and nematodes was found. Among genes of the HBL enterotoxin, hblA was the least abundant, present in 15% of B. thuringiensis strains only, whereas hblB and hblC were noted in over 50% of the isolates. The genes coding for the Nhe toxin were found in higher number of the isolates (95, 75, and 85% for nheA, nheC, and nheB, respectively). Following digestion with NotI and Ascl, only eight distinct PFGE pulsotypes were found among the isolates, indicating a clonal structure for the B. thuringiensis population from NE Poland. Interestingly, no correlation was observed between the DNA pulsotype strains and their crystal gene content and diversity. However the presence of enterotoxins genes was highly correlated to DNA type of the isolate. The results presented here help to explain the ecological significance of B. thuringiensis.
Insecticidal properties and mode of action of Cry15Aa endotoxin from *Bacillus thuringiensis* serovar. *thompsoni* HD542

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The majority of the insecticidal proteins produced by *Bacillus thuringiensis* (Bt), also known as delta-endotoxins or Cry proteins, are are probably organized in a conserved three domain-structure. However, other types of insecticidal Bt proteins with a 3D structure likely to be different from most Cry proteins have been found. The co-crystallizing Cry 15Aa and 40 kDa proteins are examples of members of a smaller non-three domain homology group of Bt toxins.

In order to investigate the insecticidal properties and mode of action of Cry15Aa/40kDa protein we have cloned both genes individually as well as the entire Cry15Aa operon and expressed them in E.coli XL-Blue. The basic biochemical properties of both proteins such as solubilization and protease stability were assessed. Toxicity of individual pure proteins, co-expressed or in vitro mixed proteins, as well as effects of solubilization and trypsin-pretreatment were tested for *Manduca sexta*, *Pieris rapae*, *Cydia pomonella*, *Spodoptera exigua*, and *Helicoverpa armigera*. We show for the first time that these proteins can be solubilized under insect-midgut like conditions and that solubilization and/or trypsin “activation” can increase the potency in an insect species-specific manner.
Contribution of Intrachem Production Srl to COST 862 Action

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In COST Action- Gruppo 3. On literature many laboratory screening on different Bt target are available. Sometime field performance are difficult to extrapolate from laboratory. Efficacy of different product depends on toxins expressed by the Bt strain and contained on the product.

In order to predict performance of different product Intrachem would be interested on screening different strains on such target on a field trial/small plot trials.

Potential new targets for BT in Italian situation (including even targets where a better efficacy is required)

Application on orchards (northern Italy – Po Valley):
- Anarsia lineatella (peach tree borer)
- Cydia funebrana (plum moth)

Application on Vegetables & strawberry (Southern Italy _ Open fields & Protected crops)
- Noctuids moths
- Spodoptera littoralis (Egyptian cootn leafworm)
- Spodoptera exigua (Beet armyworm)
- Agrotis segetum e Agrotis ypsilon (Cutworms)
- Chrysodeixis chalcites
- Autographa gamma

The topics of the COST Action – Group 5 would be to coordinate the development of standard QC protocols, EU proposal for new project and to establish agreements on QC – protocols. It would be fundamental for the process development, quality control and regulatory issues of the BT products.

Another topic where the COST Action would find some important synergy would be on the EC registration procedures of BT. The COST Action - Working Group 5, could have an active contribution to the discussion, offering its interpretation of the possible data requirements to advise the E.U. regulatory authorities and the EC notifiers. It could be used as a Forum for Companies, scientists and extension services to discuss regulatory issues.

comments: Intrachem has been involved since the beginning in the preparation of the proposal and it is very much interested on the outcoming of the Cost action.
Collection of Bt strains from Silesia region in Poland

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Research concerning Bacillus thuringiensis carried out since 80. in Institute of Genetics and Microbiology (University of Wrocław, Poland) resulted in the collection of 98 Bt strains originating from Wrocław, Karkonosze National Park and Osola area (Lower Silesia region, Poland) as well as from the Upper Silesia region. Majority of strains (62) was isolated from soil. The remaining ones (36) originated from tree leaves (mainly from beech and oak). According the biochemical tests 83 Wrocław isolates were grouped into several types. The most abundant (48 isolates) being type I consist of subspecies japonensis, jinghongiensis, yoso. Type II containing subspecies kurstaki, kenya, aizawai, sumiyosehiensis, fukuokaensis, higo includes 15 strains; type III (subsp. finitimus) includes five isolates; type IV (subsp. tochigiensis) – one strain; type V (subsp. alesi) – three isolates; type VI (subsp. thompsoni, coreanensis, medellin) – two strains; type VII (subsp. darmstadiensis) – two isolates; type VIII (subsp. tohokunensis) – two isolates; type IX (subsp. galleriae) – two strains; type X (subsp. neoleoensis) – one strain; type XI (subsp. ostrinae) – one isolate. Fifteen isolates were classified based on H-antigen agglutination. Thirteen of them were recognized as B. th. kurstaki. Two environmental isolates were designated as the strains of the new serotype – B. th. wratislaviensis (H-47).

All Bt isolated were screened with regard to their plasmid content. All of them carried extra-chromosomal elements and all displayed a complex plasmid pattern including molecules ranging in molecular weight from several MDa to more than 120 MDa.

The results of bioassays (measured by the corrected larvae mortality) showed differential toxicity (0-87%) against some dipteran and coleopteran larvae: Musca domestica, Aedes aegypti, Culex pipiens, Drosophila melanogaster, Tenebrio molitor, Alphilobius diaperinus, Blatta orientalis, Blattella germanica, Periplaneta americana.
Ecological impact of transgenic plants expressing B. thuringiensis genes

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The ecological impact of transgenic plants expressing B. thuringiensis genes, is poorly known. Concerning beneficial insects, several contradictory reports have been published; some authors reported negative effect of Bt on some predators (Chrysoperla carnea) whereas others did not find Bt-related mortality. The well-known work on the effects of transgenic pollen in the Monarch butterfly, is actually considered as inconsistent. The effect on natural enemies (parasitoids and predators) of Bt plants needs further studies Our research team aims to determine the effect on both pests and natural enemies of Bt-based transgenic plants. We are currently analysing the sensibility of several pests versus different bacterial toxins and we aim to determine the effect of supplying B. thuringiensis-fed preys to predators and parasitoids. The insects that will be studied are important in pest control since they are key pest of corn, potato or tobacco. Among these insects, Ostrinia nubilalis and the aphids are particularly interesting. The former because of being a pest of corn, the first transgenic culture grown in Spain; and the latter because of the role aphids play in the ecosystem, since many predators feed on them. Regarding natural enemies, the effect of Bt and Bt-plants will be studied for Harmonia viridis, Chrisoperla carnea and coccinellidae (predators), as well as for parasitoids of the Cotesia genus and Aphidiidae. The variation in the toxin expression level, both in time and tissue, has been poorly studied and has not been, up to date, related to the upper trophic level: insects rearing on or parasitizing target pests. We also plan to study the expression pattern of the bacterial genes in the transgenic culture: when, how much and where are they produced. The information on toxin expression and results from bioassaying natural enemies with “Bt-pests” will improve our knowledge on transgenics ecological effects and may be very useful to assess transgenics sustainability.
**Bacillus thuringiensis pathovar israelensis**, a potential biological control agent against leatherjackets *Tipula paludosa* and *T. oleracea* (Diptera: Nematocera)

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Tipula paludosa is the major pest in grassland and turf, also causing damage in horticulture and ornamental crops. The damaged area exceeds 300,000 ha of grassland in Germany. In the past the organophosphate Parathion was used to control *T. paludosa*. Since this insecticide is no longer available, no other chemical compounds have been registered. Biological alternatives have been investigated over the last 2 decades of which Bti is the most promising. The results on the toxicity of Bti against the different larval stages of tipulids and field trials will be reviewed. *B. thuringiensis israelensis* is the predominant serovar present on the grass foliage and may therefore be considered as a naturally occurring antagonist of tipulids. However, the toxicity of the Bti toxins against tipulids is low compared to that recorded for mosquitoes. It was reported, that tipulid larvae are susceptible to Cry11A and specific receptor proteins in the insect gut has been detected. Nevertheless, Anopheles larvae are 10,000 fold more susceptible to Cry11A then leatherjackets. There is some evidence that the Cry4 toxins are of more importance against leatherjackets. Tipulids have 4 larval stages. In laboratory trials the LD90 of Bti increased up to 10 times during the larval development (L2: 0.03 mg, L3: 0.31 mg, L4: 1.7 mg/larva). Until now, a successful control in the field could be obtained only for L1-L3 stages. Trials with sprayed Bti against *T. paludosa* (20 kg/ha, 3000 ITUs/mg) and *T. oleracea* (45 kg/ha, 1200 ITUs/mg) and with Bti baits against *T. oleracea* (150 kg/ha, 900 ITUs/mg) were carried out. The sprayed formulation reached an efficacy of almost 80%, whereas 61% were recorded with the bait. The related costs for such applications would range between 300 and 800 € per ha, which is probably too much for a widespread practical use. New Bt strains or optimized formulations may improve this situation in the future.
Isolation of vegetative isolates of Bacillus thuringiensis (Bt) from the phylloplane

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Two media were developed that were able selectively to recover Bt from leaves while in the vegetative as opposed to the spore form. These and non-selective media were used to follow the appearance of Bt in both spore and vegetative forms on the leaves of clover (Trifolium hybridum) in the field over two growing seasons. Vegetative forms were present for two, restricted periods while spores were recoverable for a longer period but were also subject to rapid rises and declines in numbers. The major, competitor spore former was found to be Bacillus cereus but the appearance of this organism did not markedly coincide with that of Bt.

The isolates of Bt found growing in vegetative form were characterised genetically and seemed to represent a stable, indigenous population which cycled between the vegetative and spore forms. The initial origin of these strains was shown, predominantly at least, to be the soil. The most common delta-endotoxin genes possessed by the vegetative isolates was shown by PCR to be cry1 types.
Research interests of Department of Genetics, Faculty of Biological Sciences in Valencia

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Our research interest is focused on the knowledge of the molecular bases of Bacillus thuringiensis mode of action and specificity, to be able to develop rational strategies to solve problems related to insecticidal spectrum and resistance, which are inherent to any insecticidal molecule to be used long lasting for pest control.

We are currently working with the Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say), an exceptionally destructive insect pest for potato, tomato, pepper and other solanaceous crops in Europe, North and Central America, responsible of important economic losses in the agricultural sector.

Traditionally this pest control has been performed with synthetic chemical insecticides that besides the environmental problems their use carries on have caused the development of resistance in this insect to a wide variety of them. Therefore, the need to implement alternative strategies for effective pest control has become nowadays an imperative to be faced.

Cry3 toxins are active against coleopteran insects, order to which Leptinotarsa decemlineata belongs. However, one of the biggest impediments to our understanding of the toxic action of coleopteran-specific Cry3 Bacillus thuringiensis toxins is the almost complete lack of information about the nature of the insect target receptors with which Cry3 toxins interact on the midgut of coleopteran larvae and the biochemical mechanism by means of which these toxins cause midgut cells to die.

The main goal of our research is the identification, purification and characterization of membrane proteins able to bind Cry3 in CPB brush border midgut membrane vesicles (BBMV) in order to assess their role in in vivo insecticidal activity and their ability for in vitro pore formation. The results of this research will broaden our knowledge on Cry3 toxins mode of action and this will allow a more efficient control of this devastating insect pest.
Management of insect resistance to Bt toxins

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Continuous exposure to Bt toxins will, sooner or later, select for resistance in exposed insect populations. The particular resistance management strategies to apply will depend on the genetic and biochemical characteristics of resistance. The past and current cases of insect resistance to Bt are reviewed with the aim to draw general conclusions to guide the strategies to be implemented in the European scenario.
Cellular mode of action of the Bacillus sphaericus binary toxin

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Bacillus sphaericus binary toxin (Bin) is one of the few available biopesticide to control populations of Anopheles and Culex mosquitoes, the main vectors of Malaria and West Nile Virus, respectively. The use of this biopesticide is threatened by the emergence of Culex resistant strains to Bin. The identification of Cpm1 (Culex pipiens maltase 1) as the receptor of Bin in the midgut epithelial cells of sensitive mosquitoes larvae has allowed to elucidate some mechanisms that render Culex mosquitoes resistant to Bin. Several resistant strains possess altered Cpm1 but other display intact Bin receptor. The occurrence of such strains lead us to focus on the cellular steps of the mode of action of Bin which happen after the binding of the toxin to its receptor. In previous studies we have expressed a Cpm1 construct in the mammalian epithelial MDCK cell line. We have shown that the characteristics of Cpm1 are fully conserved when Cpm1 is expressed in this cellular model: targeting to the apical side of polarized MDCK, anchoring by glycosylphosphatidylinositol, α-glucosidase activity. Bin is able to bind on MDCK cells that express Cpm1 and induces several cellular effects that have been observed in vivo among which the appearance of intracytoplasmic vacuoles. Our recent works on this cellular model show that Bin specifically induces the vacuolisation of compartments decorated with Rab7-GFP, a marker of late endosome and Lamp1-GFP, a lysosomal marker. Interestingly, BinB and BinA conjugated with Alexa546 and Alexa488 fluorescent dyes, respectively are also able to bind on the cell membrane of MDCK expressing Cpm1 and are internalized within few minutes at 37°C. After 24 hours of incubation, both BinB-A546 and BinA-A488 are observed inside the cytoplasm and are colocalized. They also induce the vacuolisation of compartments of late endosome and lysosome marker but neither BinB-A546 nor BinA-A488 are observed on the membranes of these vacuolating compartments. On the other hand they appear partially colocalized with the early endosomal marker Rab5. Preliminary observations of BinB-A546 and BinA-A488 internalization in vivo by MDCK expressing Cpm1 also suggest that they are not totally colocalized during the first minutes after incubation.
Possibilities of the use of Bacillus thuringiensis in the control of forest pests in Poland

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In 1994-2004 approximately 1.63 mln ha of coniferous forests was sprayed in Poland to control defoliating Lepidoptera and 0.25 mln ha to control defoliating Hymenoptera. It is a result of several Lepidoptera and Hymenoptera species outbreaks that develop cyclically in even-aged coniferous monocultures growing on poor site conditions. While there are 5 Bt insecticides registered to the use in forestry against defoliating Lepidoptera, there is no microbial insecticide, that could be used against defoliating Hymenoptera.

In Department of Forest Protection, Forest Research Institute in Warsaw one project concerning Bt is running: “Possibilities of the use of Bacillus thuringiensis and entomopathogenic fungi in the control of sawflies (Hymenoptera, Symphyta)”. The most important in the project remains an exploration for and evaluation of new Bt isolates for a control of sawflies. The insecticidal activity of new Bt isolates from sick and dead sawflies larvae is checked against healthy sawflies larvae (i.e. Diprion similis, D. pini, Macrodiprion nemoralis).

Bt from 38 forest Hymenoptera larvae were isolated. So far the insecticidal activity of 16 isolates was checked. The most promising Bt isolate caused 90 % mortality of D. pini larvae after 11 days (experiment run in 1998). The isolate lost its insecticidal activity after 7 years of the storing (in 2005 experiment the mortality of treated larvae was close to the mortality of control larvae). The screening programme will be continued for the nearest 3 years.
Use of Bacillus thuringiensis in vineyards and organic potatoes in Cyprus

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In Cyprus, the European Grapevine Moth, Lobesia botrana (Dennis & Sciff.) (Lepidoptera, Tortricidae) and the Potato Tuber Moth, Phthorimaea operculella (Zeller) (Lepidoptera, Gelechiidae) are the most important pests in vines and potatoes, respectively. The hazards of the conventional, broad spectrum acting insecticides to humans and environment are well documented. The Agricultural Research Institute (ARI) carries out experiments in order to find and recommend to the farmers alternative insecticides and methods against these pests. Experiments are carried out on the table grapes (var. Sultana), where Bacillus thuringiensis (Bt) and other "friendly" insecticides are used to control the 2nd and 3rd generations of the pest. At the same time, Bt is used with the Mating Disruption Technique of the pest. Here, Bt is used only in the periphery of the vineyard (3-4 peripheral lines), where the invasion of the mature females is more intensive and damage sometimes is significant.

The potato Tuber Moth is the most serious potato insect pest, both in the field and store. In the conventional potato cultivation, toxic chemical preparations have been used for many years. The ARI carries out experiments, concerning the organic farming and the potato pest control, including Potato Tuber Moth. To control and reduce the pest’s populations and infestation in the field, Bacillus thuringiensis, different bioinsecticides and combinations between them are used. In both occasions, Bt is treated immediately before egg hatch of the pests. Trials are being repeated in this year and will be continue in 2006.
Quantification of Insecticidal Crystal Proteins of *Bacillus thuringiensis* subsp. *israelensis*

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*Bacillus thuringiensis* subsp. *israelensis* (Bti) is commercially used for biological control of mosquitos and blackflies. The toxicity of Bti is caused by the insecticidal crystal proteins (ICPs) Cyt 1Aa, Cry 11Aa, Cry 4Aa and Cry 4Ba. The quality of Bti based products is determined by comparing the activity with an international standard in a bioassay using *Aedes aegyptii* and indicated as international toxic units (ITUs). This method is variable and costly. For optimisation of the production process, testing different media and formulation techniques and for quality control a faster and more reliable method was developed. ICPs are quantified by using monoclonal antibodies (MABs) in ELISA assays. Two different MABs, which bind specifically to different epitopes of each toxin, are necessary for the quantification. Nine MABs specific to Cyt 1Aa and 17 specific to Cry 11Aa have been produced and tested in different ELISA tests. The specificity of the MABs has been checked in western blots and in ELISA tests. Three MABs were produced, which detect the two Cry 4 proteins, but can not specifically distinguish between these highly homologous toxins. For the production of the MABs mice spleen cells were fused with Ag 8 cancer cells. The hybridoma cells were cultured and the MABs were purified by using an Amicon Press and protein G. Cyt 1Aa, Cry 11Aa and the two Cry 4 proteins can now be quantified with MABs in sandwich ELISA assays. The method can be used for quality control, but also for studies on the mode of action and receptor binding.
Isolation and characterisation of *Bacillus thuringiensis* from noctious insects of eastern black sea region of Turkey

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Various hazardous insects attack the forests and hazelnut of Eastern Black Sea region. Most of them are belong to the orders of Lepidoptera and Coleoptera. The serious damagers of forests are Dendroctonus micans, Ips sexdentatus, Ips typographus, Amphimallon solstitiale, and Curculio elephas, and the damagers of hazelnut are Gypsonema dealbana, Lymantria dispar, Hyphantria cunea, Curculio nucum, Melolontha melolontha, Xyleborus dispar, Agelastica alni, Obera linearis, Anoplus roboris and Malacosoma neustria. Rrecent concern on the hazardous effects of chemical pesticides in the environment and human made us consider finding more effective and safer control agent against these pests. In order to discover a significant control agent, we have isolated Bacillus thuringiensis from M. melolontha, M. neustria, X. dispar, D. micans and H. cunea. These isolates have been characterised based on morphological, biochemical, physiological, molecular and immunological tests, and their insecticidal effects on these pests were determined. The cry gene content and insecticidal activities indicate that especially B. thuringiensis isolates of M. melolontha, M. neustria, and X. nucum are very promising biological control agents against pests of forest and hazelnut of Eastern Black Sea region of Turkey.

Keywords: Biological control, Forest pests, Hazelnut pests, Bacillus thuringiensis
Characterization and Insecticidal Activities of *Bacillus thuringiensis* (Berliner) Strain Isolated from *Curculio nucum* L.

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Bacillus thuringiensis producing insecticidal crystal proteins have been used as the microbial insecticides. These proteins had high toxicity against insects of Lepidoptera, Coleoptera and Diptera orders. In this study, Bacillus thuringiensis strain isolated from insect, *Curculio nucum* L. (Coleoptera, Curculionidae), damaging in hazelnut was compared to the reference strains, by electron microscopy, SDS-PAGE analysis, plasmid pattern, cry genes content, H-serotyping and insecticidal activity. Isolate CnBt produced bipyramidal and cuboidal shaped parasporal inclusions. SDS-PAGE analyses have shown that CnBt isolate produced two proteins with molecular masses of about 65 and 130 kDa. After trypsin digestion of solubilized crystals, CnBt isolate produced a trypsin-resistant peptide of 60 kDa. PCR analyses have shown that CnBt have cry1 and cry2 genes. The only noticeable difference was the lack of two large plasmids, which compared to reference *B. thuringiensis* subsp. kurstaki. Toxicity tests were performed against 9 insect species of Lepidoptera, Coleoptera and Diptera orders. Toxicity tests have shown that CnBt against *Malacosoma neustria* and *Lymantria dispar* larvae has highly (90%) insecticidal activity.

Key words: Bacillus thuringiensis, cry gene, insecticidal activity, H-serotype
Proteolytical processing of B.t.-toxins in the midgut of target insects as reference systems for the identification of resistance mechanisms

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The application of Bacillus thuringiensis (B.t.) products as well as the cultivation of B.t.-plants can result in the development of resistant pest populations to the respective B.t.-toxins. Due to the toxic pathway of B.t.-toxins, the identification of resistance mechanisms in potential available resistant pest populations should be based on reference systems for physiological changes in the midgut of susceptible target insects. In different pest-B.t.-toxin-systems, two main mechanisms of resistance to B.t.-toxins - one proteinase-mediated and the other receptor-mediated - have been identified. Thus, reference systems on proteinase activities, proteolytical processing and receptor binding should be established with the susceptible pest populations in question. Exemplary, studies for the pest-B.t.-toxin-system European Corn Borer (ECB, Ostrinia nubilalis) – B.t.-toxin Cry1Ab are demonstrated. Studies on proteinase activities with midgut sap of 5th instar ECB larvae identified the digestive proteinases trypsin, chymotrypsin, elastase, and aminopeptidase. Besides, proteolytical processing of the B.t.-toxin Cry1Ab as present in transgenic corn as well as the respective protoxin were digested by all types of proteinases proved in the midgut sap of ECB, except aminopeptidase.
Extended PCR Screening for Novel Insecticidal cry and cyt Genes from Field-Collected Bacillus thuringiensis Strains

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Enhanced strategy using PCR will enrich the existing arsenal of Bacillus thuringiensis (Bt) insecticidal strains, identify novel genes or new combinations of known genes and predict their toxicities. We developed expanded sets of universal and specific primers for extended multiplex rapid PCR screening of Bt strains that harbor high variety of cry groups. Universal primers (Uns) were constructed from a highly conserved sequence in 54 cry’s, -1, -2, -3, -4, -5, -7, -8, -9 and -11, cyt1 and cyt2, encoding delta-endotoxins against lepidopteran, coleopteran and dipteran larvae and nematodes (Appl. Environ. Microbiol. 63: 4883-4890, 1997; Appl. Environ. Microbiol. 65: 3714-3716, 1999). DNA of each strain that reacted with at least one pair of Uns was identified with specific primers (selected from highly variable regions) for most of these genes, each producing a unique-size amplicon.

An alternative PCR analysis to screen for cry7 (promising for effective control and resistance management of agronomically important coleopteran pests) is proposed, based on the five conserved blocks of amino acids of Bt toxins and their encoding DNA sequences (Curr. Microbiol. 42: 96-99, 2000). The four direct primers were designed to amplify four distinct amplicons with the single reverse primer and create a fingerprint specific to cry7 genes. Modified profiles can identify new cry genes. This newly designed set of primers complements the existing PCR methodology for most currently known cry genes.

Screening Bt field-isolates with these Uns and a specific set of primers for dipteran cry’s and cyt’s discovered 11 strains that harbor gene(s) resembling cry11Aa of Bt subsp. israelensis. The profile of one of them revealed three major genes encoding Cry4B-, Cry11B- and Cyt2B- like proteins. The first shows a trans-membrane domain characteristic of the toxins, and the second is 98% homologous to Cry11Bb of Bt subsp. medellin. The genes were separately cloned into an expression vector of Escherichia coli and toxicity assays revealed that the new Cry11B-like toxin was more active against 3rd instar larvae of Aedes aegypti than Cry11Aa. While each, Cry4B-like and Cyt2B-like toxins displayed marginal activities, together they were highly synergistic. In addition, it was found that Cyt1Aa of Bt subsp. israelensis synergized both Cry4B-like and Cry11B-like delta-endotoxins against A. aegypti larvae.
Transgenic bacteria to raise efficacies of Bacillus thuringiensis

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Our attempts are distributed in three lines: isolation of new strains of Bacillus thuringiensis (Bti) with larvicidal activities higher than those of known strains, identification of novel insecticidal cry/cyt genes, and construction of transgenic organisms that will avoid selection of resistant pests.

Mosquitocidal transgenic bacteria: (i) The genes cry4Aa, cry11Aa, cyt1Aa and p20 from Bt subsp. israelensis (Bti) were cloned for expression in Escherichia coli in all 15 possible combinations. The two producing Cry4Aa, Cyt1Aa and P20, with or without Cry11Aa, display the highest toxicity against Aedes aegypti larvae ever reached in transgenic bacteria. The clones were bioassayed against 5 colonies of Culex quinquefasciatus, 4 of which had been selected for resistance against various combinations of the toxins. The wild-type, susceptible strain is over 5-fold more sensitive to the combination of all 4 genes than without Cy1Aa. This combination is only 2.5-3-fold less toxic to larvae of the resistant colonies, while the clone not producing Cyt1Aa is thousands-fold less toxic, except the colony selected with Cyt1Aa. The results confirm our notion that releasing recombinant bacteria expressing all Bti’s mosquito larvicidal toxins would be as effective as releasing Bti itself, but with additional advantages for mosquito control.

(ii) Several combinations of the four genes were cloned in the nitrogen-fixing, filamentous cyanobacterium Anabaena PCC 7120. The transgenic clone that express them all displays highest larvicidal activity, and may replace Bti for field delivery. Being of a laboratory origin, it will eventually disappear from the natural water body due to competition with endogeneous cyanobacteria, thus furnish temporal refugia between applications. It is cheaply mass-produced and its activity persists longer than Bti thus anticipated to be highly cost-effective.

Field-isolated Bt strains have been screened for new genes encoding Dipteran toxins by PCR. Profile of one strain revealed three major genes encoding Cry4-, Cry11B- and Cyt2- like proteins. The first shows a trans-membrane domain, characteristic to the Cry toxins. The Cry11B-like toxin is 98% homologous to Cry11Bb of Bt subsp. medellin. Both of the Cry proteins are toxic to A. aegypti larvae, and are synergized by Cyt1Aa.

Lepidoptera larvicidal activity for safe biological control: The genes cry1Ac and cry1Ca were isolated from Bt subsp. kurstaki HD-73 and aizawai 4J4, respectively and introduced into previously constructed E. coli expressing cyt1Aa and p20. Several clones with different combinations of the 4 genes were obtained and found to express the genes included. Toxicity of the clone expressing cry1Ac against susceptible larvae of Cotton Bollworm (Helicoverpa armigera) and Pink Bollworm (Pectinophora gossypella) was higher than of the clone that expressed cry1Ac, cyt1Aa and p20.

comments: I request that Dr. Eitan Ben-Dov (email address: etn@bgu.ac.il)) will be provided with support to also attend this important meeting. -Dr. Ben-Dov has no additional source of support so would not be able to participate unless funded. Thank you for consideration.
Photorhabdus toxins: novel alternatives to Bt toxins

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We will give a current overview of work on the Toxin complexes (Tc's) of Photorhabdus which have now been engineered into transgenic crops to give insect resistance. Full toxicity in recombinant E.coli requires three subunits A, B and C. The likely role of each of these subunits will be discussed and their interaction to give full recombinant toxicity will be described. These toxins now represent viable alternatives to those from Bt for conferring insect resistance in transgenic crops.
Resistance of Ostrinia nubilalis strains to Bt toxins

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Transgenic maize hybrids expressing toxins from Bacillus thuringiensis have been available in the U.S. for managing European corn borer (Ostrinia nubilalis Hubner) populations since 1996. However, there is concern that this technology will result in widespread resistance to Bt toxins. The high dose-refuge strategy is the currently accepted method for managing resistance in O. nubilalis. However, this strategy is based on a number of assumptions that are difficult to validate until resistance is identified and characterized. Resistant strains resulting from laboratory selections provide a potential tool to assess these assumptions in the absence of field derived resistant populations. However, such laboratory experiments are limited by their inability to reflect the selection intensity imposed on field populations exposed to transgenic plants. Our laboratory has been selecting O. nubilalis in the laboratory by exposure to Bt toxins incorporated into an artificial diet since 1997. A number of different colonies have been developed and are being used to document cross resistance, resistance mechanisms, and inheritance. Their performance on transgenic plants that express Bt toxins is also being evaluated.
Possible targets of Bt application in maize fields in Slovakia

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Until 2001, the most important pest of maize fields in Slovakia was the European corn borer (Ostrinia nubilalis) and cotton bollworm (Helicoverpa armigera) in some years. They are lepidopterans and it means that one strains of Bacillus thuringiensis could be effective against both pests. The application of Bt formulations against O. nubilalis and H. armigera also can be realised in the same time.

From 2001, new pest of maize was found in Slovakia. It was Western corn rootworm (Diabrotica virgifera virgifera). The methods of its control are different from those used against lepidopterans. The insect belongs to the order Coleoptera and Bt subspecies applicable against it is different. Also the method and time of application is different. Larvae are damaging maize roots during spring, adults can be found at maize fields during July, August and September.
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(Bacillus thuringiensis and Bacillus sphaericus. New toxin discovery, particularly discovery of new toxin types.
Toxin improvement, through mutagenesis and high-throughput screening techniques. Strain improvement for enhanced
environmental performance and through toxin combinations, especially through non-recombinant methods. Novel methods
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immunity as well as genetically inherited.)

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(B. thuringiensis in Integral Pest Management in Macedonia.)

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(Natural distribution of Bt in soil and other ecosystems, and in the search for new Bt strains active towards a wider range of insect orders. Finally, I think about interaction between Bt and other entomopathogens (mainly entomopathogenic fungi) for integrated control of insect pests.)

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(Selection of bacterial strains pathogenic to xylophagous insects in the forest (especially from Coleoptera; genus:Scolytus, Ips, Orthotomicus etc.), isolation and identification of toxins produced by by these strains. Methods of bacterial inoculum (toxins) application under the bark. Use of Bt in integrated pest management. Combined use of Bt with entomopathogenic fungi against Colorado Potato Beetle and some pest from Lepidoptera in the orchards.)

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