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SCREENING OF NEW B. THURINGIENSIS STRAINS FOR INTEGRATED PEST MANAGEMENT IN COSTA RICA

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EXECUTIVE SUMMARY

A collaborative program was initiated between Ecogen Israel Partnership (EIP - a biotechnology R&D subsidiary of Ecogen Inc., based in Jerusalem Israel) and a team of scientists at the University of Costa Rica (San-Jose). A four year program was devoted to the development of biological insecticides that are improved strains of *Bacillus thuringiensis*, a naturally occurring bacterium.

In a world that suffers from the ecological hazards due to overwhelming utilization of chemicals and particularly chemical pesticides, it has become a global decree to identify replacements and among whom were proposed a variety of biopesticides. It is due to that rationale that the two teams embarked on the research program with the impetus of developing a whole family of cost-effective Bt-based products. The teams focused their efforts on three major pests of vegetable crops and primarily on old-world bollworm (*Heliothis armigera*), Egyptian leafworm (*Spodoptera littoralis*) and diamondback moth (*Plutella xylostella*) which are devastating pests of cruciferous and solaneous vegetables.

It was clear to all parties involved in the R&D program already from the onset of the project, that in order to attain cost-effective pest control, it is possible to use naturally occurring Bt strains that are isolated from different niches in the environment. However, such isolates required significant improvements before they could turn into a commercial product. Within four years, the program resulted in the isolation of three highly potent Bt strains each against one of the above mentioned pests. This was followed by intense research which adopted sophisticated techniques in molecular biology that had a great impact on the quality of the genetically engineered constructs of the Bt strains. The naturally occurring strains were manipulated so as to enhance the expression of toxin plasmid genes as well as broadening of the pest spectrum.

The main accomplishments of the joint project were the identification of useful Bt-based biopesticides, their improvement by genetic manipulation and last but not least the targeting of those strains towards major pests that in the future will have to be controlled by biorational means. We believe that the close collaboration between the two teams, will create the foundation for a highly qualified research in Costa-Rica whose scientists are eager to make progress in such a prominent area.

RESEARCH OBJECTIVES

The objectives of the project were as follows:

1. Screening of highly effective Bt isolates against diamondback moth (*P. xylostella*) an old world bollworm (*H. armigera.*) and Egyptian leafworm (*S. littoralis*).
2. Characterization of stable strain out of these isolates.
3. Optimization of industrial fermentation process of the Bt isolates.
4. Development of "Crop-tailored" formulation which will be adjusted to each crop and every pest (i.e. *P. xylostella* and *S. littoralis* in cruciferous and *H. armigera* in solaneuos crops).
5. Genetic improvement of Bt-isolates by recombinant DNA-technology to form combined biopesticide for the control of *S. littoralis* and *P. xylostella* in cruciferous crops.
6. Development of greenhouse simulation experiments and extended field trials for evaluation of biopesticidal rate of control, mode of action and Bt incorporation into a regime of Integrated Pest Management (IPM).

The objectives were set to accomplish the goal of development of biologicical pesticides. The proposed antagonists have been novel isolates of Bacillus *thuringiensis* (Bt) which were selected on the basis of their effectiveness against the above mentioned pests of cruciferous and solaneous vegetables. The project was conducted with a scientific team from the University of Costa Rica since their country became a major producer and exporter of fresh vegetable to the South and North-American markets. The significance of the proposed project had to be evaluated in light of the urgent need to abandon the use of chemical pesticides which have undoubtedly proven to be ecologically hazardous. The need to replace chemical pesticides became particularly essential for vegetable crops since those are marketed as fresh commodities and no chemical residues are allowed to remain on them.

Bacillus thuringiensis based products have been used for the control of many different lepidopteran pests and the general impression was that with the improvement of the isolates and with better formulation, the end-use product could become competitive with the chemical pesticides based on cost-effectiveness. However, certain improvements were required primarily in the case of vegetable pest control mainly in respect to the residual effectiveness over time.

Our project addressed the major issues by searching for novel and better isolates and by genetic manipulations of the existing Bt strains. The project fits very well into the on-going research by other scientists around the world. In the last decade, many laboratories have devoted their expertise to the establishment of screening methods with higher resolution and to the use of sophisticated recombinant-DNA technology for improvement of the existing isolates. The status of the latter in the area of Bt related R&D is discussed in two review articles that were published by senior scientists from Ecogen Inc. (Appendix I).

The innovation in the project resides with the fact that through genetic manipulations we established the foundations for development of novel Bt strains with a broad spectrum of insecticidal activity capable to control cost-effectively the prominent pests of leafy vegetables.

The program was originally proposed as a joint project between the R&D company (its name was recently changed from FRM Agricultural Sciences Partnership to Ecogen Israel Partnership - EIP) and a team of scientists at the University of Mauritius. This cooperation never succeeded to take-off and following the advise of the A.I.D. authorities, a new arrangement was established between EIP and a team of scientists at the University of Costa Rica, San Jose. The team was formed by Mr. Gilbert Fuentes (entomologist) and Dr. Franklin Jimenez (molecular biologist).

The first assignment of the Israeli team was to establish an insectory for rearing *H. armigera* and *S. littoralis*. For these two pests, EIP developed a useful rearing method as well as innovative bioassay techniques. As soon as the Costa-Rican team joined the program, they initiated an extensive program of Bt-isolate collection with the impetus of finding better local antagonists for the control of *P. xylostella*. Following these activities, the teams moved forward to the second part of the program which involved molecular studies and genetic manipulations of the outstanding isolates. The purpose of the manipulations were to develop an ideal recombinant Bt strain with a broader pesticidal spectrum as well as enhanced rates of insect control.

METHOD AND RESULTS

Insect Rearing

As a guideline it was agreed between the teams that the entire operation of insect rearing will rely on artificial diets. It was a significant undertaking for quite sometime to select the most appropriate diet for each of the test insects. The first responsibility of the collaborating teams was to establish an insectory for *S. littoralis* and *H. armigera* in Israel and a similar insectory for rearing of *P. xylostella* in Costa-Rica. The significant achievement in that regards was that within a short period of time, an artificial diet was developed for all three pests. The compositions of these diets were improved and slightly modified several times until a final version was reached. The specifications are listed in Tables 1-5 as follows:

Table 1: A Diet for artificial rearing of *Heliothis armigera:*

	Nutrient fraction (g/3 liter)
White beans	1200
Wheat germ	200
Fibrous cellulose powder	25
Yeast powder	160
Cholesterol	3
Ascorbic acid	16
Methyl-p-hyrdoxybenzoate (nipagin)	16
Sorbic acid	5
Formaldehyde 37%	16 ml
Chloromycetine	6
Agar	70

Table 2: A diet for artificial rearing of *Spodoptera littoralis:*

	Nutrient fraction g/5.36 liter
Soybeans	900
Wheat germ	170
Fibrous cellulose powder	40
Yeast powder	300
Ascorbic acid	26
Milk powder 28%	80
Methyl-p-hyrdoxybenzoate	16
Sorbic acid	10
Formaldehyde 37%	25 ml
Chloromycetine	6
Agar	70

Table 3: A diet for artificial rearing of *Plutella xylostella:*

Method I

	Part 1/liter[1]
Wheat germ premix	560g/l
Vitamine Free Casein	28.00 %
Sucrose	27.75 %
Wheat germ	24.00 %
Alphacel,Non-nutritive Bulk	12.00 %
Cholesterol	0.05 %
Linseed Oil	0.20 %
Salt mixture Wesson	8.00 %
Calcium Carbonate	21.000 %
Copper Sulfate $5H_2O$	0.039 %
Ferric Phosphate	1.470 %
Manganous Sulfate.anhydrous	0.020 %
Magnesium Sulfate.anhydrous	9.000 %
Potassium Aluminium Sulfate	0.009 %
Potassium Chloride	12.000 %
Potassium Dihydrogen Phosph	31.000 %
Potassium Iodine	0.005 %
Sodium Chloride	10.500 %
Sodium Fluoride	0.057 %

Tricalcium Phosphate	14.900%
Sorbic acid	2g
Methyl paraben	4g
Formaldehyde	2ml
KOH 4M	16ml

	Part 2 / liter(1)
Agar	60 g
Vitamine Premix	60 g
Alpha-Tocopherol	80 gm/kg
Ascorbic acid	270 gm/kg
Biotin	20 mg/kg
Calcium Pantothenate	1 gm/kg
Choline Chloride	50 gm/kg
Crystalline folic acid	250 mg/kg
Inositol	20 gm/kg
Niacinamide	1 gm/kg
Pyridoxine Hydrochloride	250 mg/kg
Riboflavin	500 mg/kg
Thiamine Hydrochloride	250 mg/kg
Aureomycine	0.6 g
Kanamycine sulphate	0.4 g

Phosphoric/propionic acid mix	10 ml (mix of 159 ml propionic acid and 16 ml phosphoric acid, 205 ml dH_2O
Linseed oil	26 ml
d H_2O	3000
tetracycline	2 g
tenox	0.4 g

(1) Part 1 and part 2 should be mixed separately and then combined only prior to the pouring of agar into plates.

Table 4: A diet for artificial rearing of *Plutella xylostella*
Method 2:

	Diet Composition /4 liter
Casein	126 g
Sugar	135 g
Wheat germ	175 g
Wessalon salt	36 g
Alphacel	25 g
Sorbic acid	4 g
Methyl paraben	5.4 g
Vitamin mix	36 g
Benylate	3 g
Agar	12.5 g

Table 5: Artificial diet for Bioassay:

Beans	180 g/l
Fat-milk powder 28%	29 g/l
Choline chloride	1.4 g/l
Cholesterol	0.4 g/l
Cellulose fibers	22 g/l
Ascorbic acid	1.4 g/l
B-vitamines	140 ml
Myo-Inositol	4.600 g/l
Panthotenic acid	0.850 g/l
Niacin (amide)	0.330 g/l
P-aminobenzoic acid	0.154 g/l
Riboflavin	0.048 g/l
Pyridoxine	0.038 g/l
Thiamine	0.028 g/l
Folic acid	0.012 g/l
Biotin	0.008 g/l
Agar	22 g/l
Methyl-p-hydroxybenzoate nipagin	3.5 g/l

Two methods were developed for *P. xylostella* simply because the life cycle of the pest is very complicated and once every few months we had to alternate between the two different diet versions.

The diets for each of the three insects as well as the bioassay diet were incorporated into the rearing protocols. The latter were presented in several of the annual reports which we submitted to A.I.D. To avoid repetition, these are not included herein.

II. Screening of new Bt isolates and their efficiency against lepidopteran pests.

In our previous reports we have presented a list of Bt isolates which were collected from soil and crops in Costa Rica. All of these isolates and several others that were collected at later dates, have been processed according to a standard protocol for isolation of Bt strains from soil. All of the well-defined single-colony derived isolates (i.e. originated from single colonies) were initially tested for their larvicidal activity against *H. armigera* and *S. littoralis* using our standard bioassay systems. The results of these screening tests are presented in Table 6.

Table 6: List of Bt strains that were isolated from soils in Costa-Rica

Isolate No.	Crystal morphology	% mortality 2ug Bt powder/g food	
		H. armigera	*S. littoralis*
14.3	amorphous	n.t.[1)]	21.1
19-3	bipyramidal	23.8	14.6
41-1	bipyramidal	38.6	8.3
48-2	amorphous	19.6	23.2
52-1	amorphous	n.t.	7.0
53-1	amorphous/cuboid	39.6	9.5
59-3	bipyramid/cuboid	15.7	13.0
60-2	bipyramidal	4.8	n.t.
61-3	bipyramidal	18.7	9.9
62-2	amorphous	8.3	11.8
194A	amorphous	1.2	2.4
163A	bipyramidal	0	0
177A	amorphous	18.4	3.6
165A2	amorphous	7.5	4.8
170B	amorphous	15.4	n.t.[(1]
201A1	bipyramidal	11.1	1.2
172A	bipyramidal	9.4	1.2

cont.

170A2	bipyramidal	0	0
168B2	amorphous	2.4	1.2
170B	amorphous	1.2	4.8

(1) n.t. - not tested.

Three Bt isolates were then subjected to a toxicity test against *P. xylostella* and the results are presented in table 6a.

Table 6a: Larvicidal activity of several Bt strains against *P. xylostella*

Strain	LC_{50} (1)	PLC_{50} (2)
61-3	17	2.7
62-2	3.4	0.8
186-B2	14	3.2
EG2346 (HD1 like)	4.1	1.4

(1) LC_{50} values are mg powder per $175mm^2$ diet surface.
(2) PLC_{50} values are mg total crystal protein per $175mm^2$ diet surface.

A detailed molecular analysis indicates that 62-2 has a protein profile identical to HD1 which is the most commonly used strain in commercial Bt-products. The other, namely 186-B2 and 61-3 are identical and unique in some features. The rest of the listed isolates failed to be extensively screened due to mortality problems that the *plutella* colonies encountered too frequently.

As a result of the extensive screening program we chose to focus on some prominent strains. As was reported numerous times during the program period, two isolates being 7-29 and HD263 were selected to address the control program of *S. littoralis* and *H. armigera* respectively. Derivative isolates 61-3 and 62-2 were selected as our candidates for further development of a biopesticide against *P. xylostella*.

III. Genetic manipulations of the Bt toxin genes-construction of the Bacillus vector

In previous reports we described methods for the isolation of clones containing Bt toxin gene fragments from the 7-29 and HD-263 strains. The clone PH729-2796 (7-29 strain) was found to be most effective against *S. littoralis* and clone PH263-D62 (HD-263 strain) was prevalent for larvicidal activity against *H. armigera*. To facilitate the introduction of Bt-Cry genes into Bacillus hosts, we constructed a shuttle plasmid incorporating the stable Bacillus replicon PBC16 (J. Bacteriology 133:897, 1978). As already published by scientists from Ecogen Inc., a 3.0Kb EcoRI fragment of PBC16 was blunt ended with the Klenow fragment of E.Coli DNA-polymerase and was ligated to SspI-digested puc18. The resulting plasmids PUB186 and PUB187, which differ with respect to the orientation of the Bacillus replicon, encode resistance to amplicillin and tetracyline in E. Coli and preserve the lac complementation system for cloning in E. Coli. One plasmid, PUB187 has the orientation such that the tetracycline resistance tet^r gene was close to the Bla gene of PUC 18; this plasmid was selected for further construction.
A unique 320bp DNA. of *B. megaterium* which contains a promoter sequence was cloned into the EcoRI-BamHI sites in the multiple cloning sites of PUB187. Meanwhile, a 26bp sequence was synthesized to serve as a stop codon in three reading frames and with a SmaI and KmnI restriction sites. The sequence of the oligonucleotide was delineated and found to be as follows:5'AGCTTGATTGATTGATTGACCGGGGTACC-3'. This fragment with one flushed and one HindIII digestion site, was ligated to the HindIII site of PUB187 in the correct orientation and after additional Klenow treatment and ligation it was finally cloned into the plasmid.

The resulting plasmid, designated PUBPS87 (Appendix II) is a shuttle vector containing a Bacillus promoter and a stop codon for better expression. It also expresses a tetracycline resistance gene for better selection in Bacillus hosts. The HindIII fragment of PH263-D62 was subcloned into HindIII digested PUBS87. The resulting plasmids PH263-D62-1 and PH263-062-2 represent the two orientation reading frames. Both plasmids as expressed in E. coli, gave consistant larvicidal activity (Table 7). The limitation of those clones resides with the fact that they are transformants of E. coli which is prohibited from use in the open-field. Therefore we had to reclone the vectors in a more friendly host. Our ultimate choice has been an isolate of *B. megaterium*.

Transformation of PH263-D62-1, PH263-D62-2 and PUB187 into *B. megaterium* as the Bacillus host was conducted by the protoplast fusion, using PEG and regeneration medium. The larvicidal activity of *B. megaterium* containing these clones is also presented in Table 7. Two clones namely BM.PSD62-1 and BM.PSD62-2 were subjected to extensive series of bioassays. Since they contain *H. armigera* toxin-genes, the rank order of mortality was 62-1 > 62-2 on *H. armigera* and both were inactive against *S. littoralis*.

Table 7: The larvicidal activity of parental and genetically manipulated derivative Bt strains.

Bacteria (1)	*H. armigera*		*S. littoralis*	
	mg bacteria powder/g food	% mortality	mg bacteria powder/g food	%mortal.
Bt.HD263	0.002	~50	nt	nt
Bt. 7-29	nt(3	nt	0.05	~50
Bt.HO-1-5-1980	0.05 - 0.1	30-75	0.06-0.8	40-65
TG1.pH263-D62	0.2	~40	nt	nt
TG1.PUBPS87	2.0	0	nt	nt
TG1.PS87D62-1	0.5-1.0	35-75	nt	nt
TG1.PS87D62-2	0.25-0.5	35-75	nt	nt
TG1.729-2756	nt	nt	0.2-0.4	38-52
TG1.729-2799	nt	nt	0.4	45
TG1.729-2796(2	nt	nt	0.1-0.4	30-80
TG1.729-2799(2	nt	nt	0.1-0.8	35-75
TG1.PHG 171	2.0	1-2	0.8	0
TG1.PS87-27-1	nt	nt	nt	nt
BM.PUBPS87	2.0	0	2.0	0
BM.PSD62-1	0.2-0.4	30-70	2.0	5
BM.PSD62-2	2.0	30-70	2.0	5

1) A list of different Bt clones in E.coli (TG1) or in *B. megaterium* (BM)
2) Addition of IPTG
3) nt - not tested

Two clones were derived from the Bt collection of *S. littoralis* active transformants, namely; PH729-2796 and PH729-2799 which contain the 2.7 Kb DNA of a Cry gene of strain 7-29; these reflect the two orientations of reading frames. These clones exhibited similar toxicity when expressed in E. coli without the inducer IPTG. Addition of IPTG caused higher percentage of mortality only with the PH729-2796 clone. (Table 7). This finding indicates that there is an influence of the lac promoter on the expression and orientation of the Cry gene in the clone.

In summary, the larvicidal activity of the Bt toxin gene following expression in *B. megaterium* is very encouraging and due to the vector construct and the nature of the host we can expect to get expression of several toxin genes in a single and a highly-productive host. In the future we intend to continue and follow the guidelines of the project program and try to accomplish the goal of inserting two mutually independent genes in one *B. megaterium* vector. It is our expectation that these systems will give rise to fusion gene-products under the control of strong promotors.

In order to further elucidate the unique features of some of the cloned genes, we extended our efforts of sequencing the D-62 gene (more details appear in previous annual reports). The main results of recent analyses highlight the fact that the plasmid is devoid of restriction sites for the enzymes ClaI, HpaI, SmaI and ScaI. However in contrast to their absence, restriction sites exist for the enzymes KpmI and PstI (1 restriction site), EcoRI (2 sites), BamHI (4 sites), BstyI and XbaI (5 sites) and HindII (6 sites). This additional information is extremely instrumental when we come to construct the fusion gene-products.

IMPACT AND RELEVANCE

The project program served as an useful trigger and initiative for the Costarican research teams to adopt modern technologies for the development of biological pesticides. The time is now ripe to aggressively extend their activity in that field of research because, as already stated in the executive summary, they are positioned in the cross-road of supply of fresh vegetable and fruit to the U.S.A.,Canada and certain countries in Latin-America. As a prominent supplier, they have to comply with market demands which among other elements, include also retreat from the use of chemicals that leave residues on the commodities. Any alternative that may offer reduction in the use of chemical pesticides is most welcome.

The impact on the individual scientists is primarily in the area of molecular biology since Dr. Franklin Jimenez and his research team intends to carry on the program from the stage that it reached when we had to terminate the R&D funded project. They will continue to collect Bt strains from different sites in Costa Rica and will request that Ecogen screen such strains on a broad spectrum of lepidopteran insects. Moreover, all the technology that was required for manipulation of the Bt toxin-gene has been transferred from the EIP's laboratory in Jerusalem to that of Dr. F. Jimenez in San Jose. This technology transfer took place during his trip to Israel in February of 1992. The entire period was devoted to the practicing of techniques and at the end of his stay in Jerusalem all necessary reagents, clones and plasmids were delivered to his laboratory for further development.

The common interest of the Israeli and the Costa Rican team led to a successful collaboration. Significant results in terms of basic understanding of gene expression as well as establishment of the grounds for development of useful biopesticides were accomplished.

Project Productivity and Future Work

The project accomplished a significant fraction of all its goals. The main achievements were in the area of Bt-strain collection against major pests, the development of molecular tools for genetic manipulation of Bt strains toxin genes and the establishment of detailed bioassay systems which in the future will be used for product quality assurance.

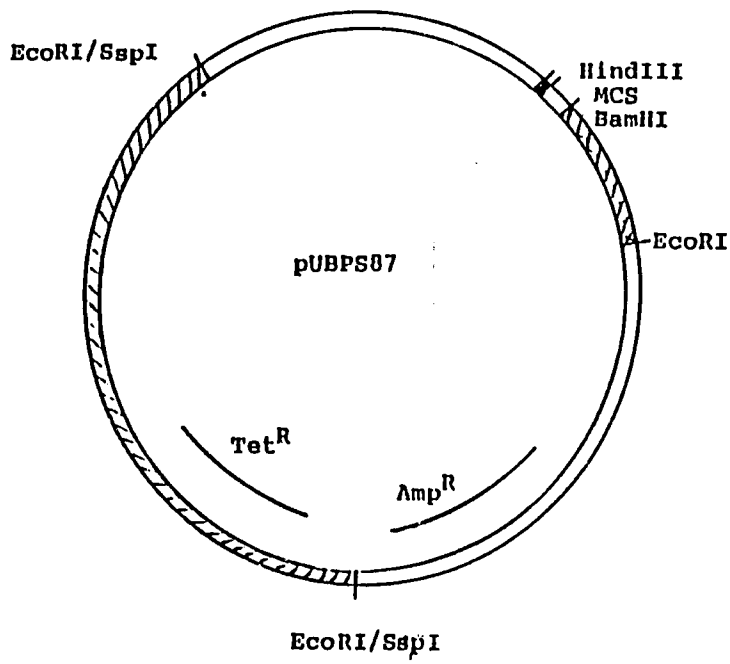
The project did not accomplish the goal of expression of fusion genes with broader larvicidal activity simply because it took much longer to overcome the hurdles which were encountered during the design of the recombinant and the shuttle vectors. Another aspect of the program which suffered delays was that of mass screening of Bt strains against *P. xylostella*. The delays were imposed due to a viral disease that inflicted the colony in Costa Rica several times during the program period. Recently the problems have been relieved and in the future, based on the know-how that exists to date and provided that the proper personnel is available and budgets can be allocated, the program can be extended satisfactorily.

PROJECT ACTIVITIES - LIST OF MEETING ATTENDED

Name	Date	Location	Length	Purpose
Dr. B. Fridlender+ Dr. R. Hofstein	Dec. 89	San Jose	1 week	Final arrangements prior to program launch
Mr. G. Fuentes	April 90	Jerusalem	1 month	Training on insect-rearing using artificial diets
Dr. M. Keren-Zur	Oct. 90	San Jose	1 week	Annual evaluation of progress
Dr. F. Jimenez	Feb. 92	Jerusalem	1 month	Training on genetic engineering technology - improvement of Bt strains
Dr. O. Carmi	May 92	San Jose	1 week	Solving problems in P. xylostella insectory

APPENDIX II

Plasmid pUBPS07



Sequence derived from pBC16

Sequence derived from PUC18

Sequence of Bacillus promoter

Sequence of stop codon in three reading frames

MCS Multiple cloning site