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

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The insecticidal δ-endotoxin produced by sporulating cells of different strains of Bacillus thuringiensis is characterized by a very specific spectrum of activity. This activity is limited to certain Lepidoptera, Diptera and Coleoptera species of insects. We are interested in achieving a better control of the pest Heliothis armigera which has one of the widest crop distribution of any agricultural pest. This pest attacks a wide range of crops, and the rate of damage is therefore very high. Our initial approach is based on the screening of B.t. strains for selection of the best candidates for control of the pest. The next step is to isolate the gene responsible for the toxic activity and manipulate it genetically in order to have a better control.

The screening was done on nine strains of B.t. which we received from the Pasteur Institute Culture Collection Bank.

- B.t.t. Berliner 1-1
- B.t.t. 1202 1-220
- B.t.alesti HD104 3-37
- B.t.k. Jha-1a 30-174
- B.t.k. Jha-2a 30-175
- B.t.galleriae HD-29 5-31
- B.t.galleriae 119-3 5-70
- B.t.entomocidus 60-1
- B.t.dendrolimus 40-1

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and three other strains from FRM bacterial bank:

- B.t.k. HD263 NRRL
- B.t.k. HD-1 NRRL
- B.t. entomocidus 24B.

More strains for the screening were ordered from different sources.

There is a need to increase pesticidal potencies of B.t. against Lepidopteran larvae in order to reduce the costs of microbial products to a level compatible with that of chemical insecticides. Any such development of microbial pest management would rely on bioassays with artificially reared larvae of Heliothis armigera.

We have established an "insectarium" where the insects were reared in a room maintained at 25±1° C, 60-70% relative humidity and 16 hrs photophase. The room was illuminated by natural and fluorescent lights.

The rearing conditions were as follows:
Ten pairs of moths were placed in a 3l-glass-jar lined with toweling paper and was closed with a white cotton cloth, serving as an oviposition site. After 1-2 days the eggs on the cloths were collected each day during 3-4 days and sterilized in 10% aqueous formaldehyde solution, washed in running tap water and dried. This disinfection procedure was needed to prevent nuclear polyhedrosis virus in the insect colony.

A piece of cloth containing 200-300 eggs were fixed to the polyethylene cloth serving as cover of a 0.5 liter-glass-jar lined with sterile filter paper. When the larvae hatched after 2-3 days, artificial diet cubes were placed inside the jar.

The ingredients of the rearing diet were as follows:

Nutrient fraction (g/3 liter):

Beans	1200
Alfalfa meal	200
Yeast powder	160
Cholesterol	3
Ascorbic acid	16

Antimicrobial fraction (g/3 liter):

Methyl-p-hydroxybenzoate (nipagin)	16
Ascorbic acid	5
Formaldehyde 37%	16 ml
Chloromycetine	6
Agar	70

The neonates migrated from the cloth to the diet and were fed in the jar for ~10 days. Then, the larvae were bred individually in cavities of jelly trays. In each cavity a cube of diet was offered to the larva for feeding until pupation (10-12 days), an empty tray with holes for ventilation was placed upside down on top of the rearing unit as a lid. Pupae were collected from the trays, disinfected in 0.2% aqueous hypochlorite solution followed by washing with tap water. Then the pupae were sexed and kept in emergence boxes. Fresh females were paired with males either freshly emerged or 24 hrs old.

Preliminary screening of B.t. strains was based on comparison with the LC_{50} of the B.t. standard HD-1-S-80 (concentration of 2 µg B.t.- powder/g food), and at rates causing 80-100% mortality in the larvae. The bioassay potency protocol for screening B.t. strains was achieved by standardized diet taking into consideration both the nutrional requirements of the larvae and the procedures of a pesticidal bioassay for which were used 3rd instar larvae and/or newly hatched neonates. In the neonate-bioassay the mortality range was close to the LC_{50}-rate indicating that it was more accurate than the official bioassay. The short bioassay time of 72 hrs saved costs in terms of rearing procedures as well as diet and fermentation ingredients. It should be stressed that the neonate larval test would not always reflect the susceptibility of mature larvae but it will be useful for the preliminary screening.

The ingredients of the bioassay diet were:

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
Agar	22 g/l
Methyl-P-hydroxybenzoate (nipagin)	3.5 g/l

The milk powder instead of alfalfa gave yellow color and an useful contrast to the dark color of the larva.
B.t. strains were grown in sporulation medium NZB.

NZB (g/l), pH 7.0:

Glucose	10
Glycerol	10 ml
Corn-steep-liquor	10 ml
N-Z-Amine B	10
Yeast extract	5
$MgCl_2 \cdot 6H_2O$	4.2
$CaCO_3$	2

After 2-3 days the pellet of cells, spores and crystals of the different strains were dried and mixed with the artificial diet for potency bioassay.
In the neonate bioassay, 25 ml of the diet and the B.t. mixture was poured to 9 cm diam. plastic petri-dishes into which a plastic grid containing 21 units was inserted before diet setting. In each of the units a single neonate larva was placed by a fine hair brush. A cover was put above in such a way that the larvae could not migrate between and outside the grid units.

The H. armigera larva (84 larvae x 4 replications) sized 1.0-1.2 cm (neonate before any feeding) were bred separately for the bioassay, this because of their canibalistic behaviour.

The rearing period of the neonates in the bioassay was 3 days. Mortalities in the control (without bacteria) exceeding 10% were rejected. The mortalities were corrected according to Abbott-equation (1925). Twelve B.t. strains were already screened and three were selected for potency determinations (B.t.HD-263, B.t.k. Jha-1a, B.t.k. Jha-2a). We will soon start also to test the pesticidal efficacy of recombinant strains.

In parallel with the bioassay screening we also screened several strains for plasmid DNA-segment in which the toxin gene is located. We tested different methods for screening and isolation of the relevant plasmids.
The DNA of B.t.k. HD-1, HD-263, B.t.t.-1202, B.t.k. and Jha-1a strains were cut with different restriction enzymes: Bam HI, Bgl II, Eco RI, Hind III, Pst I, Sal I. The vector pBR322 was chosen for preparation of the DNA-fragments library. This vector has two unique restriction sites: Bam HI and Sal I in the gene for tetracycline resistance and Pst I site in the bla gene.

A great variety of plasmid arrays were found within the B.t. strains. Because of the large degree of homology shown among the published B.t. endotoxin DNA-sequences, we decided to use known toxin genes and oligonucleodides as probes.
We have prepared two oligonucleodide probes, the N-terminal probe RB-9 5'-CTAGTTGATATAATATGG (18-mer) and the C-terminal probe RB-182 5' AATACTTCCCAGAAACCGATA (21-mer) (Appl. Environ. Mircobiol. 53: 2808, 1987).

The preliminary screening for δ-endotoxin gene was done by hybridization on gels or on Nitrocellulose membranes of the RB-9 probe to DNA of B.t.k. HD263, Jha-1a, B.t.t.-1202. A good hybridization was obtained with HD-263 which was cut with different restriction enzymes. Jha-1a gave a slight band and B.t.t. 1202 did not hybridize with this probe.

Two libraries of HD-263 plasmid DNA fragments cloned in the Bam HI and Pst I sites of pBR 322 were screened with the two oligonucleotides probes, a few colonies came-up positive for incorporation of the endotoxin gene. These colonies now await testing on the bioassays which we are currently developing, and biologically active colonies will be subjected to cloning processes and further genetic manipulations.