C8-048

US-ISRAEL CDR PROGRAM

Second Progress Report

Development of Microbial Pesticides to Protect Grain from Harvest Time till Reaching the Consumer

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for period ending December 31, 1989

During the period covered by this report, research undertaken by our laboratory was focussed mainly on applied subjects requiring immediate attention, such as establishment of additional laboratory colonies of several target insects, development of rearing techniques and artificial diets for improved mass rearing when material food was not satisfactory, and screening of microbial entomopatheogenes.

Establishment of laboratory colonies

The following store-product pests are presently reared in our laboratory:

Coleoptera

1) Bostrichidae: Lesser grain borer, Rhyzopertha dominica (F.)

2) Cucujidae: Flat grain beetle, Cryptolestes pusillus (Schon)

3) Curculionidae: Rice weevil, Sitophylus oryzae (L.)

4) Silvanidae: Saw-toothed grain beetle, Oryzaephilus surinamensis (L.)

5) Tenebrionidae: Red flour beetle, Tribolium castaneum Hbst.

6) Tenebrionidae: Yellow meal warm Tenebrio molitor.

Lepidoptera

1) Gelechiidae: Angoumois grain moth, Sitotroga cereallela (Oliv)

2) Pyralidae: Mediterranean flour moth, Ephestia Kuehniella (Zell.)

3) Pyralidae: Indian meal moth, Plodia interpunctella (Hbn.)

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Most of the above listed species are cosmopolitan and exist both in Israel and in the Philippines.

Rearing methods

When preparing the rearing media for the cultures of Rhyzoperta, Sitophylus and Sitotroga precautions were taken to avoid or prevent insect and mite infestestion of grain by sterilization. Sterilization has been accomplished by heating the grain and the ground grain to 60°C for 90 minutes in an autoclave.

Grain was tempered to the desired moisture level by mixing grain of different moistures in the proper proportions. The moisture content was determined by the electronic Motomco Moisture Meter. The calculation used to determine the amount of water to add to grain for tempering are as follows:

$$\frac{100\% - \%\text{ of present moisture}}{100 - \%\text{ of desired moisture}} - 1 = F$$

F x gram of grain = ml of water needed to bring the grain to the desired moisture.

The external feeders Tribolium and Tenebrio were reared on the selected culture media based on cracked or broken kernels of grain, or in flour of milled grain. The confused flour beetle and the red flour beetle were reared on media consisted of white flour and corn meal (47.5% each) and brewer's yeast (5%). Before mixing these components the flour was sifted through a N-14 sieve and then the flour and corn meal were autoclaved 90 minutes at 110°C to kill infesting insects or mites. After the materials cooled to room temperature, and were mixed in the proportions by weight as stated above and sored at 4°C in sealed 10 l jars until needed.

The confused flour beetles were collected at the infested storage site in a nearby settlement, by means of trap described by Graham (Trop. Agr. 39, 53-56, 1962). The trap consisted of a glass vial enclosed in an envelope of wire gauze 70-80 mesh. The top edge of the gauze was folded tightly over the lip and into the mouth of the vial. The vials were placed upright in small samples of infested grain. The traps work on the principle that the insects congregate at the highest

accessible point and attempt to fly. The highest point is at the top of the vial into which they eventually fall.

The Indian meal moth, Plodia was reared on medium containing 4 parts of corn meal, 4 parts of whole wheat flour, 1 part of dried yeast, 1 part honey, 1 part glycerine, 1 part wheat germ, 1 part of oatmeal. The whole wheat flour and corn meal portions were autoclaved for 90 minutes at 110°C. This treatment eliminates also any mites and insects that might be present and at the same time conditions the ingredients to a favorable moisture level.

In our laboratory 1 liter wide-mouth jars were used for mass rearing containers, with two-piece screw top with filter paper lid to provide adequate air exchange. Approximately 150 gm of rearing medium was placed in each 1 liter jar, to which 50 seeding adults of 1-3 days old adults were used for egg-laying...

All test insects were reared in the insectary maintaining constant temperature of 27°C ± 1°C and at relative humidity of 70% ± 5%.

Isolation of Spore-Forming Bacteria

The screening for new potent pathogens is one of the objectives of this project. The applied method for isolation of spore forming bacteria was based on the know-how accumulated in our laboratory during the past ten years. A protocol has been developed for isolation of spore-forming material from soil and insect debris material, around silos and open grain storage sites as well as soils taken from bottoms of dry desert river beds (where loes top-soil accumulates). In cases where dry soil samples were collected, the material was taken from below the exposed surface where bacteria have been protected from direct sun light.

All isolation procedures were carried out in a sterile laminar air flow cabinet located in a separate laboratory in which no experimental work on either B.t. or B. sphaericus had ever been performed. Aseptic techniques were strictly observed to prevent contamination of the source material and of the isolation media.

Insect debris or soil samples were placed in tubes containing 4.5 ml of sterile phosphate-buffered saline (PBS) solution and vigorously agitated with a vortex. The tubes were heated at

78°C for 15 min in a water bath to kill non-spore-forming organisms and vegetative cells. A sample (0.075 ml) of the heated suspension was aseptically pipetted onto the surface of LB solid medium containing, per liter, 5 g yeast extract, 10 g tryptone, 5 g NaCl, and 20 g agar (Miller, 1972). The LB plates had been previously incubated at 30°C for 48 hr to test their sterility. The suspension was spread evenly over the agar surface, and the plates were incubated at 30°C.

If insect material was used, the samples were homogenized aseptically and the homogenate was transferred to a tube of sterile PBS. This tube was then shaken vigorously for 24 hr. Such action serves to thoroughly aerate the medium and promotes the formation of spores by bacteria which in the insect cadaver may have been in a vegetative state. After shaking, the tubes were heat-shocked at 78°C for 15 min and samples of the suspension plated in the manner outlined above.

Plates thus prepared were examined for bacterial colonies after 48 and 72 hr. By means of this method of inoculation, single colonies, easily distinguishable from each other, developed on the agar. Furthermore, the individual growth of the colonies facilitated their removal from the plates by means of an inoculation loop when the bacteria were subcultured onto LB plates. These plates were incubated for 48 hr and the purity of the resulting cultures was confirmed.

Isolates were examined microscopically and tentatively identified according to their morphology. Sporulating cells were examined for the presence of parasporal inclustions -- and indication of their status as B.t. isolates. Most isolates were large spore forming rods, motile in their early stage of development and of a typical Bacillus appearance. Their growth characteristics and microscopic appearance suggested that they were B.t. types. Crystals were observed within the sporulating cells which further demonstrated their B.t. status. During the second and third year of this project the most promising isolates will be tested by the serum agglutination test with antiserums prepared against most of the known strains of B.t. to ascertain their subspecies.

Initially the pathogenic isolates were cultured on LB slants and stored at 4°C. Later, samples were preserved as powders by both the lactose coprecipitation method and lyophilization.

Samples of the liquid cultures were frozen at -- 70°C following incorporation with a 5% solution of glycerol (ratio 2:1, respectively).

Lyophilized powder preparations were produced from cultures in PGSM after 48 hr incubation. They were centrifuged, washed twice in sterile water, and lyophilized. The resulting powders were stored in the presence of a desiccant under refrigeration and vacuum.

Bioassays

Protocols for bio-assays were applied for evaluation of activity of several B.t. preparation against adults and larvae of the store-grain pests. For the lesser grain borer, R. dominica and the rice weevil S. oryzae, lyophilized spore/crystal mixtures were mixed with hard wheat grains at the desired proportions.

For the flat grain beetle C. pusillus; the red flour beetle T. castaneum and the saw-toothed grain beetle D. surinamensis, the spore/crystal mixtures were mixed with a blend of wheat flour, corn meal, and brewer's yeast at the proportions 49: 49: 2. Each treatment was replicated three times, and compared to the untreated control.

Samples containing 150 gr of the treated commodities were placed in 1 l rearing jars and consequently infested with 7-14 days-old adults of the respective species. After 7 days the adults were removed by sieving and the numbers alive and dead noted. The commodities were held for additional 8 weeks and sieved to remove the F_1 progeny for counting. Since it was unlikely that any effects on adults would be found, the percent reduction in F_1 adult progeny was used as the primary measure of activity.

B.t. is known to delay larval development in both lepidopteran and coleopteran species of grain storage pests. When appreciable effects on larval development were noted, the infested samples were sieved twice weekly, beginning when F_1 adults first appeared and estimates of the time to 50% adult emergence were obtained.

The new strains of B.t. were tested initially at two doses, in (100 and 200 mg per kg of diet) order to obtain an indication of whether any activity was present. The precise magnitude of their toxicity will be determined during the second and third year of the project, and compared to

the formulations of B.t. var. kurstaki, B.t. var. darmstadiensis, B.t. var. aizawai and B.t. var tenebrionis obtained from Dr. H.T. Dulmage. In addition commercial preparations of Dipel, Thuricide and Bactospane will be tested as well, against the same target insect pests.

So far about 200 samples from 129 diverse environs throughout southern Israel were collected, during the course of this survey. Of these samples a total of 82 isolates of Bacillus thuringiensis producing parasporal inclusions were obtained from soils, dust and insect debris. Of them 26 were toxic to insects tested: 12 Lepidoptera-toxic, 14 were mosquito-toxic and none of them were toxic to Coleopetera. Although 56 isolates of B.t. (68, 3%) produced parasporal inclusion with various morphologies (bipiramidal and irregular shapes), they did not demonstrate any toxicity against insects of three orders, (Lepidoptera, Coleoptera, Diptera).

Toxicity of Bacillus thuringiensis isolates against
Lepidoptera: Ephestia kuehniella (EK), Plodia interpunctella (PI) and Sitotroga cerealella (SC) Coleopetera: Tribolium (TC), Cryptolestes pusillus (C.P.), Oryzaephilus surinamensis (OS), Rhyzopertha dominica (RD), Sitophylus oryzae (SO); Diptera: Aedes aegypti (AE).

Number of isolates tested*	Number of isolates toxic to									Number of non-toxic isolates
	Lepidoptera			Coleoptera					Diptera	
	EK	PI	SC	TC	CP	OS	RD	SO	AE**	
3	0	0	0	0	0	0	0	0	1	2
1	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	3	2
3	2	1	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	2	2
2	0	0	0	0	0	0	0	0	1	1
5	0	0	0	0	0	0	0	0	5	0
6	3	1	0	0	0	0	0	0	0	2
5	0	0	0	0	0	0	0	0	2	3
4	3	1	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	44
82		12				0			14	56

The serotypes of the new isolates will be identified during the second and third year of this study.
The toxicity against Aedes aegypti larvae were tested at the concentrations of 10^{-4} and 10^{-5} of the B.t. strains

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