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V. SUMMARY

In the course of the present study on entomopathogens associated with certain naturally dead or moribund coleopterous pests, the following points could summarize the obtained findings :

A- Entomopathogens naturally occurred in moribund or dead individuals of 19 coleopterous pests

Surveys for naturally-occurring microbial control agents in 19 coleopterous pests attacking stored products and certain trees (palms, casuarina, navel orange, and fig trees) were conducted in Alexandria, Egypt between December 1998 and September 2001. The natural disease mortality among test insect pests was due, at least, to one of the following entomopathogens : - The bacteria, *Bacillus thuringiensis* var. *kurstaki, B.cereus, B.sphaericus,* and *Brevibacterium* sp.; polyhedrosis viruses; the protozoans, *Mattesia* sp. and *Nosema* sp. The association of each isolated entomopathogen with its naturally dead coleopterous host was occurred at a very low (0.1%) to moderate (28.6%) rate. On the other hand, natural microbial control rates among the subject 19 insect pests, due to all isolated pathogen (s) of each pest, varied between 1.2 and 31 %. Natural associations of all the above - listed entomopathogens with larvae of the 19 coleopterous pests (belong to 11 families) appeared here to be the first records in their test coleopteran hosts, except for the *Bt* isolate of the cigarette beetle , *Lasioderma serricorne*; the polyhedrosis viruses of the red palm weevil, *Rhynchophorus ferrugineus* and the

pubescent rose chafer *Tropinota squalida* ; *Bt* and *Nosema* sp. isolates of the red flour beetle, *Tribolium castaneum.*

B- Mass production of the entomogenous bacterium *Bacillus thuringiensis* var. *kurstaki*

1- From the standpoint of *Bt* large-scale production, two procedures were used and evaluated. The first procedure was carried out manually (for *BtTc*); while the second one was achieved by a semi-machinary technique (for *BtRf*). The present findings of the production of *B.thuringiensis* in marketable amounts and at competitive prices reveal that, the two adopted techniques, manual and semi-machinary, are principally characterized by ease, simplicity, and low technology which could be carried out by unskilled labourers. However, the manual technique for large scale production was faced with three major problems : (1) fermentation media components are numerous and frequently unavailable ; (2) contamination sometimes cannot be avoided, and (3) the exponential value of the estimated cell density, i.e., number of viable spores per gramme *Bt*-product, is significantly ($P = 0.05$, LS.D. test) much lower (10^4 viable spores/g) than the corresponding figures of *Bt*-products based on the adopted semi-machinary technique (10^{16} viable spores per gramme to 10^4 viable spores/g). These three problems appeared to be solved by adopting the procedures of the present semi-machinary technique.

2- In August 2000, the entomopathogoen *Bacillus thuringiensis* var. *kurstaki* was isolated from naturally

dead larvae of the curculionid *Rhynchophorus ferrugineus* in Alexandria, Egypt. It is the first naturally occurring *Bt* infection in the red palm weevil. Nine biopreparations based on such a naturally occurring strain of *Btk* were laboratory prepared in a large-scale production (Kilogrammes). Nine meals of cheaply available natural substances of vegetable or agronomical origin were used to mass produce *B.t.k.* by an easy, simple, and too much low cost semi-machinary procedure. The nine *Bt* products were laboratory standardized by adopting the plate count technique for estimating the approximate number of viable spores per gramme of each biopreparation .The *Bt* cell densities were extremely high and ranged from 1.5×10^{16} to 2.7×10^{4} viable spores/g *Bt* product. Test *Bt* products based on rice (1.5×10^{16} v.s./g) or millet meal (2.6×10^{15} v.s./g) recorded the highest cell densities followed by the black eye beans (7.8×10^{14} v.s./g), chick peas (2.5×10^{14} v.s./g), yellow lentils (1.7×10^{12} v.s./g), brown wheat (1.8×10^{11} v.s. /g), barley (1.3×10^{11} v.s./g), wheat bran (1.9×10^{6} v.s./g), and finally soybean meal (2.7×10^{4} v.s./g). The cost prices of one kilogramme of each test *B.t.* product were too much low and ranged from 1.80 to 6.65 Egyptian pound. On the other hand, the bioassay method was applied to evaluate the insecticidal activity of one of these *B.t.* products against its original host, *R. ferrugineus* larvae of different ages, i.e., young, middle aged, and old larvae. The daily mortality rates, and the patterns of virulence, based on the LC_{50s} and LT_{50s} were calculated. Diet- incorporation assays revealed that test *Bt* product of extremely high cell density of 10^{15}

viable spores/g had provided a considerable virulence to its natural host, *R.ferrugineus*..

C- The laboratory rearing and culture maintenance of the red palm weevil *R.ferrugineus*

The red palm weevil *Rhynchophorus ferrugineus* was successfully laboratory reared (over 3 generations to date) on a cooked diet based on carrot and sweet potato or potato, and on peeled sugarcane stem as well. In the case of larval rearing on the cooked diet, the life cycle from egg to adult emergence ranged 215 - 249 days with an average of 230.6 ± 3.6 days. The duration of the oviposition period was 81.5 ± 5.9 days (57-126 days), and each female oviposited 104 - 399 eggs with an average of 255.6±23.7 eggs. The daily oviposition mean was 2.7 eggs. Eggs hatched in two or three days (2.9±0.1 days), and hatchability averaged 95.3 ± 1.1 % (85.9–100%). The larval duration took 200-234 days with an average of 217.2 ± 4.0 days; while the pupal duration lasted 15 to 16 days with an average of 15.2 ± 0.1 days. Adult male longevity was 77-162 days (100.3 ± 9.7 days), while adult females survived for 80-180 days (113.9 ± 8.5 days), and preoviposition period was 2 - 4 days with an average of 2.5± 0.2 days. On the other hand, the corresponding figures for larval rearing on pieces of sugarcane internodes were, in respect, 108-196 days (137.3±11.6 days); 85.5± 8.2 days (37-118 days); 91-426 eggs (262±32.0 eggs); 2.8 eggs; 2-5 days(2.9±0.2 days); 91.6±3.3% (84.8-100%); 93-177 days (128±8.3 days); 15 to 17 days (15.7±0.1 days); 79-137 days (100.1±5.1 days), 44-174 days (106.9±5.7 days), and 2-4 days (3.2±0.2 days).

The life history parameters of oviposition, adult longevity (males and females), fecundity, the daily deposited eggs per female, egg incubation period, hatchability (%), and pupal duration of *R.ferrugineus* developed from larval rearing on either the cooked diet or sugarcane stem were statistically equal in their values. While other parameters such as preoviposition and postoviposition, larval duration, and life cycle period (egg to adult) were significantly different in their values and, in general, tended to be longer on carrot-sweet potato mash rather than on sugarcane. However, the larval rearing on the cooked diet seemed to be more preferable than on pieces of sugarcane stem because with the latter more precautions and attention must be taken into consideration, mainly infestation by fungi and *Drosophila*, as compared with the former diet which is characterized by simplicity, ease in preparation, availability of its components, and low cost.

D- Pathogenicity studies

In the course of the present study, three economically important coleopteran pests, the tenebrionid *Tribolium castaneum*; the cerambycid *Macrotoma palmata*; the curculionid *Rhynchophorus ferrugineus*, were subjected to bioassay trials using certain entomogenous bacterial isolates of *B.thuringiensis* var. *kurstaki* and *Brevibacterium* sp., and polyhedrosis viruses, which have been isolated from their host cadavers.

1-*Tribolium castaneum*

A laboratory produced biopreparation containing a naturally-occurring isolate of *B.thuringiensis* var. *kurstaki*, isolated from *T.castaneum* larvae, was evaluated in the laboratory for its pathogenicity to natural host larvae and adults of nearly a week-old. Based on the daily cumulative percentage mortality data and values of the median lethal time (LT_{50}) and concentration (LC_{50}), tested *Bt*-product of 1.9 x 10^4 viable spores per gramme showed a considerable pathogenic activity against test *T.castaneum* larvae and adults. A 100 % mortality among test larvae and adults was achieved with all tested concentrations, ranging from 7.6 to 1.9 x 10^4 viable spores per 5 g wheat bran, within 14 - 22 days or 18-22 days in the case of the treated larvae or adults, respectively. The estimated LC_{50} value for *T.castaneum* adults, at 13 days post-treatment, was higher (8.6 x 10^4 $V.S_s$/5 g wheat bran), but not significantly, than the corresponding value for test larvae (6.5 x 10^4 $V.S_s$/5 g wheat bran). Additionally, as test concentration increased from 1.9 to 7.6 x 10^4 viable spores/ 5g wheat bran (ca. 4 folds), the LT_{50} value was significantly decreased by nearly 1 - to 7 - day or 2 - to 6 - day for test larvae or adults, in respect; where with the lowest and highest dosages of 1.9 and 7.6 x 10^4 $V.S_s$/5g wheat bran, in respect, the calculated LT_{50s} for *T.castaneum* larvae were 16.4 and 9.2 days, respectively; while the corresponding values for test adults were, respectively, 18.0 and 12.3 days.

2- *Macrotoma palmata*

693 field-collected larvae of the cerambycid wood borer *Macrotoma palmata* F. were examined for any disease mortality and found to be naturally infected with three entomopathogenic bacteria, *Bacillus thuringiensis* var. *kurstaki* (6.1 %), *B.cereus* (0.1 %), and *Brevibacterium* sp. (10.1 %), and a polyhedrosis virus (2.7 %), during a 18-month period of investigation in Alexandria, Egypt, extended from April 2000 till September 2001. It is the first naturally occurring viral disease of *M. palmata* larvae, and the first record of a polyhedrosis virus infection in such cerambycid larvae. Also, it is the first record of naturally occurring bacterial disease due to the entomopathogenic bacterium *B.thuringiensis* var. *kurstaki*, *B.cereus* or *Brevibacterium* sp. Laboratory evaluation, based on the daily cumulative mortality data, and both the LC_{50} and the LT_{50} values,of these indigenous entomopathogen strains on larvae of different sizes (averaged 1.95 - 5.2 cm.) of their natural host, *M.palmata*, proved their moderate or considerable pathogenicity to the subject larvae. Also, another isolate of *B.thuringiensis* var. *kurstaki,* originally isolated from naturally dead larvae of the curculionid *Rhynchophorus ferrugineus,* was bioassayed against the subject larvae and showed to be remarkably less virulent to *M.palmata* larvae compared to *M.palmata-Btk* isolate. On the other hand, the results recorded in this study show that the described method for assaying test entomopathogens (tightly rolled filter paper- or ordinary paper – tunnels around each larva) gives reproducible results and can easily be adopted for

routine bioassay work of such cerambycid wood-borer *M.palmata* which is known, to a large extent, with its difficulty to be laboratory reared. The ease and simplicity of the bioassay procedure, and the reliability of the method described, as shown by the statistical evaluation of the obtained data, justify the use of the method adopted in the present study. The present study suggests that, baits based on these native entomopathogenic strains, or direct injection of any of the present pathogens into the tunnels in the *M. palmata*-host trees might have some value in *M. palmata* control procedure, curatively or preventively.

3- *Rhynchophorus ferrugineus*

In a 14-month survey for natural microbial agents of the red palm weevil *Rhynchophorus ferrugineus* in Alexandria, Egypt, 12.2 % of 353 larvae were found to be naturally infected by bacteria, *B.thuringiensis* var. *kurstaki* (8.8%), *B. sphaericus* (1.1 %), and *Brevibacterium* sp. (2.3 %) and 5.1 % by a polyhedrosis virus. The total natural mortality rate of red palm weevil due to all recorded entomopathogens was 17.3 %. The present bacterial isolates of *B.thuringiensis* var. *kurstaki*, *B.sphaericus*, and *Brevibacterium* sp. are reported from the red palm weevil for the first time in Egypt. In laboratory-conducted bioassays, the pathogenicity of the *Rhynchophorus-B.thuringiensis* var. *kurstaki* isolate and another isolate from the granary weevil *Sitophilus granarius*; *Brevibacterium* sp; the *Rhynchophorus* polyhedrosis virus and its isolate after passage through the

alternate host, *Spodoptera littoralis* larvae, to their natural host, *R.ferrugineus* larvae of different ages, was evaluated based on the computed LC_{50} and LT_{50} values.

Pathogenicity studies showed *R.ferrugineus* larvae to be moderately susceptible to their naturally-occurring entomopathogens, and the efficacy of the latter as newly isolated microbial control agents was proved, taking also into consideration the adverse side effects on survivors. On the other hand, the cross-infectivity studies revealed that the *S.littoralis* polyhedrosis virus did not cross-infect the early or late larval stages of *R.ferrugineus*. Therefore, such an alternate host, the cotton leafworm, which is relatively large and easy to mass rear, could be used to propagate the virus of the red palm weevil. Such an ability of the red palm weevil virus to infect cotton leafworm larvae will make microbial control with this entomopathogen much more feasible. The bioassay demonstrated that the *Rhynchophorus* polyhedrosis virus is cross-infective to larvae of cotton leafworm. In addition, based on the evaluated LC_{50} and LT_{50} values, the cross-infective virus isolate was more virulent to *R.ferrugineus* larvae than the original virus isolate. The present native natural pathogens are recommended to be applied in baiting of *Rhynchophorus* weevils and in direct injection into trunk of the date palm or other palms as preventative and curative procedures against red palm weevil. Hopefully, the discovery of these *Rhynchophorus* entomopathogens can serve in microbial-based control strategies for *Rhynchophorus* spp.

E- Standardization of the laboratory mass-produced *Bt* preparations (*Bt*-products)

1- *Bt* - products based on a semi-machinary procedure

The nine *Bt* products prepared in the course of present study were standardized by adopting the plate count technique for estimating the approximate number of viable cells (spores) per gramme of each *Bt* product, as a relatively quick and ease method for determining the potency of *Bt* preparations. The highest cell numbers of $1.5x10^{16}$ or $2.6x10^{15}$ viable spores per gramme *Bt*-product were achieved by *Bt*-products based on rice or millet meal, respectively. The lowest corresponding figures of $1.9x10^{6}$ and $2.7x10^{4}$ viable spores/g were recorded with *Bt* products based on wheat bran and soybean meal, in respect. Also, the bioassay method was used to evaluate the efficacy of one of the present *Bt*-products against its original host, *R.ferrugineus* larvae. The *Bt*-product based on millet (2.6×10^{15} viable spores/g) was laboratory bioassayed against *R.ferrugineus* larvae of different ages, by applying the diet-incorporation assay.

Based on the mortality rates and patterns of virulence, LC_{50} and LT_{50} values, tested *Bt*-product of extremely high cell density of 10^{15} viable spores/g showed a considerable virulence to its original host larvae of the red palm weevil. With the highest concentration of 13.0×10^{15} v.s./100g rearing diet, 2.5 or 7 days only were required to attain a 50% or a 100 % mortality among

young larvae of (0-30)-day-old, respectively; whereas 4.6 or 7 days were needed to achieve the same mortality levels, in respect, among old larvae of (58-79)-day-old. On the other hand 5.7 or 10 days were needed to produce the corresponding mortality levels, respectively, among middle aged *Rhynchophorus* larvae of (31-57)-day-old. Also, the LC_{50} values were comparatively high, ranging from 8.6×10^{15} to 11.6×10^{15} viable spores/100 g rearing diet. Larval mortality increased directly with dosage, while the relationship between dose and post-treatment period was reversed. In other words, the insecticidal activity of test *B.t.* product was not only a function of dosage but of time as well, and to a large extent, larval age.

2- *Bt*-product based on a manual procedure

The *Bt*-product which has manually been prepared, and containing a naturally-occurring isolate of *B.thuringiensis* var. *kurstaki* isolated from larvae of the red flour beetle *Tribolium castaneum*, was standardized by applying two methods: (1) the plate count method to determine the approximate number of viable spores ($V.S_S$) per gramme, which was 1.9×10^4 $V.S_S/g$, and proved to be significantly lower than the corresponding figures of the present *Bt* products based on a semi-machinary procedure; (2) the bioassay method to evaluate the efficacy of test biopreparation against the natural host larvae and adults of nearly a week-old. Based on the daily cumulative percentage mortality data and values of the estimated LT_{50} and LC_{50}, test *Bt* product showed a considerable pathogenic activity against the subject tenebrionid pest.

F- The noctuid cotton leafworm, *S.littoralis* as an alternate host for propagation of a polyhedrosis virus of the curculionid red palm weevil, *R.ferrugineus*, and as a test insect for bioassaying a *Bt*-product

The laboratory bioassays conducted in this study showed the noctuid *Spodoptera littoralis* (Boisd.) to be considerably susceptible to a polyhedrosis virus originally isolated from the curculionid *Rhynchophorus ferrugineus* (Olivier). On the basis of the computed LC_{50} and LT_{50} values,cross-infectivity assays revealed that the *Rhynchophorus* virus that passed through the alternate host *S.littoralis* (cross-infective virus) was more virulent to the natural host larvae than its original isolate. The *Rhynchophorus* polyhedrosis virus can therefore be mass-propagated in the cotton leafworm which is easily and cheaply mass reared , and the red palm weevil which is, to a large extent, difficult and expensive to rear, would not have to be used for virus propagation. On the other hand, the results presented in this study also reveal that *S.littoralis* is an availably good insect for bioassaying biopreparations based on the entomopathogenic bacterium *Bacillus thuringiensis* var.*kurstaki*.