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JAN. 12-14, 1976, BROWNSVILLE, TEXAS

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REPORT ON THE WORKING CONFERENCE ON SPECTRA OF ACTIVITY OF BACILLUS THURINGIENSIS DELTA-ENDOTOXINS BROWNSVILLE, TEXAS JANUARY 12-14, 1976

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Recent controversy on the efficacy of Bacillus thuringiensis for spruce budworm control has resulted in the general belief that while the potential for the use of this pathogen is still high, there are still some significant problem areas which warrant research and development before this control agent can be recommended. Among these are formulation and application technology problems, sunlight inactivation problems, development of control strategies specific for the budworm and a search for the most lethal form of the pathogen for this insect species. There are some 350 strains or isolates of B.t. available for testing from USA, France and USSR. Tortricids, the group in which the spruce budworm falls, are generally highly susceptible to B.t. The toxicity of the pathogen, however, is not necessarily related to the number of spores and crystals in the product but also to the biological activity of the delta-endotoxin which can vary markedly with culture medium constituents, bacterial strains and gut pH of the target insect.

The HD-1 strain of B.t. used in commercially available products may not be the strain of choice for maximum efficacy in spruce budworm control. Yamvrias and Augus (J. Invertebr. Pathol 15, 92-99, 1970) have

shown that the B.t variety alesti was more toxic for the budworm than berliner, entomocidus, galleriae, sotto, kenyae, dendrolinus or finitimus and that a combination of spores and crystals were more lethal than either alone.

Thus, with 350 isolates known, it seems imperative at this point that some of them be tested against the budworm either as univalent (single strain) or as polyvalent preparations.

Over the past 2 years there has been an international co-operative project organized by Dr Howard T. Dulmage, Fermentation Microbiologist, USDA, to study the delta-endotoxins from a variety of isolates of B.t. This working conference was called to review the available data and to organize future research. Meetings on January 12 and 13 were restricted to scientists. On January 14, the meeting was opened to industry representatives at which time the results of the screening program were discussed and plans were drawn for possible co-operative work with industry in the development of those endotoxins of particular interest. Observers and/or co-operators from various countries numbered: Japan 3, England 1, Netherlands 1, Canada 3, France 1, U.S.A. 37.

The agenda included (a) Techniques used in the program (production of formulations, identification of cultures, serological procedures, statistical programming, (b) Results of bioassays, and (c) Discussion on significance of results. The points discussed are presented as an appendix of this report.

At the end of the meeting on Wednesday afternoon, I called a group of participants together to discuss the eastern spruce budworm problem with a view to getting some lead as to the best isolates for

testing against this species. The group included myself, Drs Paul Fast and Janina Krywiewczyk (Insect Pathology Research Institute), Drs Howard Dulmage (USDA, ARS), Normand Dubois and Clarence Thompson (USFS), H. de Barjac (Pasteur Institute, Paris).

Reports of the Co-operators

1. Data Processing Service - Mr R. De Clark, USDA, Beltsville, Maryland.

Mr De Clark reported on the data processing system used in the co-operative program and supplied each delegate with a 2 inch thick compilation of all available records of bioassay to date. The record has susceptibility data for 25 insect species including the gypsy moth, tussock moth and fall web worm. No data is as yet available for eastern or western spruce budworm. In addition, he distributed "Search Request Forms" which may be used by anyone interested in getting a computer print-out from him on the susceptibility of the various insect species to the 350 B.t. strains. I have asked for print-outs on the above 3 insect species.

2. Bioassay Technique - Mr T. Penna, USDA, Brownsville, Texas.

Penna described the bioassay techniques used at Brownsville to measure the activity of various B.t. strains or isolates. The response of insects to a test isolate is compared with the response to a reference standard. The toxins are administered in artificial diet. The primary reference standard is E61 maintained at the Pasteur Institute, Paris

with an assigned potency of 1000 international units (IU). At Brownsville, 4 day old larvae of Trichoplusia ni are used in bioassay. This bioassay is adopted as the official one by EPA for measuring potencies of B.t. in the USA. The primary US standard approved by EPA is the HDI-S-1971 strain with a potency of 18,000 IU/mg. This standard is presently used in the co-operative program. Essential criteria for getting accurate results include (1) disease free insects, (2) proper diet preparation (agar should boil vigorously before adding to diet mixture). To compute potency in IU, the LD_{50} of a sample is computed by a potency probit analysis (samples with non-parallel slopes are rejected). The ratio - LD_{50} standard is then computed with 95% confidence limits surrounding it. Assays should be replicated at least 3 separate days. Dry formulations of B.t. which are generally very stable are used.

3. Production of Formulations - Mr J.A. Correa, Brownsville. Correa reported that the spectra of activity may be influenced not only by the isolates but also by the fermentation conditions under which the isolate is grown. Thus the Brownsville laboratory undertook to produce formulations of each isolate under the same fermentation conditions. Each isolate is also serologically identified before testing by the co-operators. He described lyophilization of each isolate prior to storage. The medium used in the program is a Proflo based one with code B4. Fermentation and recovery procedures were described.

4. Serology or Serotyping of B.t. - Dr H. de Barjac, Pasteur Institute, Paris.

de Barjac described preparation of antisera, agglutination technique for positive and title determinations and biochemical tests for determining varieties or subspecies. A key was presented for biochemical determinations of 17 different varieties of B.t. Dr de Barjac uses a flagella antigen technique.

5. Serology of the delta-endotoxin - Dr J. Krywienczyk - IPRI, Sault Ste. Marie.

Krywienczyk presented a list of 319 isolates identified by toxic crystal characteristics based on crystal antigen dissolved in Bombyx mori gut juice or alkali. This classification approach was generally considered to be of great significance since all these isolates may now be classed according to the serological properties of the toxic crystal. Of the 319 isolates, 37 were identified by this method as HD-1 (the present commercial strain), 29 as var. thuringiensis, 62 as galleriae, 14 as aizawai, 13 as alesti, 19 as HD73, and a few mixtures of two different strains. Antibodies for the tests were produced in rabbits.

6. Screening of 237 B.t. isolates against Porthetria dispar (gypsy moth) - Normand Dubois, USFS, Hampden, Conn.

Dubois described his preliminary and definitive screening procedures. Preliminary bioassay of each isolate was run at 25 ug of material/ml of diet. Definitive assays to determine

LC_{50}, slope and fiducial limits were run at appropriate dose ranges if the preliminary assay gave greater than 80% mortality in 4 days. His results showed a full spectrum of activity from very active to moderately to inactive for the different isolates. The kurstaki and galleriae groups had the largest proportion of very active preparations followed by berliner and aizawai. One preparation from the alesti and one from dendrolimus group were considered very active. Isolates giving Trichoplusia/Heliothis ratios higher than 1.2 were always highly toxic to P. dispar but not to Orgyia pseudotsugata. Isolates with an I.U. of 3000 or above for T. ni were always very active against P. dispar. HD1 strain was the most pathogenic of all those tested.

7. Bioassays against Pieris brassicae - L.P.S. Van der Geest, University of Amsterdam.

A total of 314 isolates were tested against this species but LC_{50} values were available for only a few isolates mainly due to limited supply of larvae. About 12 of the 35 isolates of var. thuringiensis appeared to be highly toxic as were 14 of 23 kurstaki, 11 of 51 galleriae, 1 of 9 dendrolimus, 4 of 9 alesti, 5 of 23 aizawai, and 1 each of var. tolworthi, morrisoni and sub-toxicus. Var. kurstaki, thuringiensis and alesti were the most effective in descending order.

8. Bioassays against Galleriae mellonella - H.D. Burgess, Glasshouse Crops Research Institute, Sussex, England.

Dr Burgess gave a historical review of the spectrum of activity of B.t. endotoxins. He described the 3 host groups with examples (Group 1 - B. mori; Group 2 - Trichoplusia ni; Group 3 - G. mellonella). In Group 1, high doses of crystal toxin caused paralysis of body, mouth parts and gut leading to rapid death. Group 2 do not suffer body paralysis but otherwise react similarly to Group 1. Group 3 react most sensitively to a 1:1 ratio of spores and crystals. Forty-four (44) of the 319 isolates tested were highly active against the wax moth with LC_{50} about 0.002 ug/ml compared with the international standard (E61) of 0.23 ug/ml. The most active varieties based on crystal toxin serology were galleriae (31), alesti (5) and aizawai (8). Typical CL was 85-117%, typical slope 4.2.

9. Bioassays against Bombyx mori, Hyphantria cunea and Spodoptera litura K. aizawa, Institute of Biological Control, Fukuoka, Japan.

Of the 56 isolates of B. thuringiensis var. galleriae screened, 48 were highly active against the silk-worm but only one (1) was less active than the US standard. Of 25 var. aizawi tested 22 were highly active and 15 were less pathogenic than the US standard. These varieties were also the most toxic for Hyphantria cunea but var. thuringiensis, alesti and kurstaki were also highly active. Twelve (12) of the 25 isolates of var. aizawai tested against Spodoptera litura inhibited larval growth but their toxicities were generally lower than the US standard.

On the whole, isolates from var. kurstaki were the most active against all three insect species. B.t. showed large fluctuations in LC_{50} with time over a 2 year period or even with one year but these fluctuations were caused by variation in the insects since all the test isolates were compared with US standard.

10. Bioassays against European Corn Borer (Ostrinia nubialis).

L.C. Lewis, Aukeny, Ohio.

Rearing technique and bioassay method were presented. Neonate larvae were used throughout. The LC_{50} of the US standard for this species is 12 ug/ml of diet. Of the 16 varieties tested only var. kurstaki, tolworthi and galleriae gave more than 50% mortality at 25 ug/ml of diet with mortalities varying from 54 to 61%. This insect appears to be generally resistant to B.t. endotoxin. The variety kurstaki was the most toxic. The commercially used HD-1 strain was as good as or better than any other.

11. Bioassays against the black army worm (Agrotis ipsilon) and the army worm (Pseudaletia unipuncta).

C. Beegle et al, Iowa State University.

Diet preparation and screening procedures were given. A total of 318 isolates were tested at 500 ug of B.t. powder per ml of diet. Neonate larvae were used throughout. The results indicate that Agrotis ipsilon larvae are not affected by the spore-crystals of any of the isolates screened. Some variations in degrees of activity of the different isolates were apparent

but the author believed these to be due to a heat tolerant, water soluble, small molecular weight exotoxin. One (1) var. thuringiensis, 1 var. kurstaki and 1 var. galleriae appeared to be active against the army worm on the basis of very limited data.

12. Bioassays against Plodia interpunctella and Cadra cantella.

Wm. H. McGaughey and E.B. Dicke, US Grain Marketing Research Centre, Manhattan, Kansas.

Insect larval diet, bioassay procedures and determination of LC_{50} were presented. Fifty six (56) of the 319 isolates tested were about as toxic as the present commercial strain (HD-1). The most active varieties were aizawai, darmstadiensis and kurstaki.

13. Bioassay against Douglas fir tussock moth - C. Thompson, USFS, Corvallis.

Field applications of less than 6 billion IU per acre were unlikely to give acceptable control. Of the 258 isolates tested, 27 gave better than 88% mortality at 25 ug/ml of diet. Varieties thuringiensis, kurstaki and galleriae were the most toxic. The HD-1 strain (commercial product) gave only 65% mortality at 25 ug/ml indicating that there are better strains which should be researched for this species. Isolates designated as HD 109 and HD 149 were highly effective.

14. Bioassays against larvae of the horn fly (Haematolua irritaus)

R.E. Gingrich, USDA, Kerrville, Texas.

Data against this species are very incomplete but it appears that of the 17 varieties tested only a few galleriae isolates showed relatively high toxicity due most likely to an exotoxin rather than delta endotoxin.

15. Bioassays against mosquito larvae - I.M. Hall, University of California, Riverside.

Data presented for Aedes aegypti, A. triseriatus, Culex pipiens, C. quinquefaciatus and C. tarsalis. Of 225 isolates of var. thuringiensis tested, 8 were effective against Aedes sp. but not against Culex sp. One of var. alesti, 3 kurstaki out of 28, 3 gallariae of 55, 1 kenyae of 6, 1 entomocidus of 5, 2 aizawai of 26, and 4 tolworthi of 8 were toxic for Aedes sp.

16. Bioassay by H. Dulmage - General Comments.

Dr Dulmage (like Dr Aizawa) reported fluctuations in LC_{50} of various B.t. isolates against Trichoplusia ni and Heliothis zea due to changes in the physiology of test insects. Changes went from 22 to 7 to 30 ug/ml of diet, the latter level due to changes in rearing techniques. There was a large range of activity within a single B.t. variety which agrues for a choice of at least 2 sub varieties in any bioassay. The variety kurstaki was generally potent against loopers. He suggested that one should consider applying a variety of strains in the

field against any particular insect species. Diet assays with greater than 19% confidence limits should be repeated. LC_{50}, standard errors and confidence limits should always be reported. Strain HD 241 appears to be specially active against spruce budworm.

CONCLUSIONS AND RECOMMENDATIONS OF THE MEETING

"Many strains of B. thuringiensis have been classified bacteriologically and tested in a wide range of insects. The work is about half complete. Great differences have been found in the susceptibility of individual insect species to different bacterial strains. Equally, there are great differences in the susceptibility of different insect species to individual bacterial strains. It is therefore necessary to find bacterial strains that are most potent against the most important insect pests. It will probably be necessary to utilize commercially a number of different strains for particular pests".

"There is scope for improvement in the production yield of toxin of particular strains by manipulation of fermentation conditions. From the scientific viewpoint, the bacterial strains have been classified logically and subgroups have been formed based on groupings of susceptible insects".

"At this point, the program shows great potential in further development of B.t. for commerce and in improving our scientific understanding of the B. thuringiensis and B. cereus groups of spore forming bacteria. It should be continued as far as possible beyond the preparation of a combined publication".

Results of the Meeting on Choice of Strains to be Tested against the eastern spruce budworm.

(1) Choice of strains should be made primarily within group that are active against other forest insects (P. dispar, O. pseudotsugata Choristoneura occidentalis) when data available.

(2) A representative of each type of toxin.

(3) A representative of each bacterial serotype.

(4) A representative of each crystal type.

(5) List to be sent for perusal to each member of the budworm meeting group.

(6) Final choice to be given to C. Thompson and N. Dubois for testing against western spruce budworm and eastern budworm in Maine.

(7) Use potency ratios to express activity, e.g. T. ni or H. zea/C.f.

My own conclusion is that a preliminary bioassay should be carried out against the eastern spruce budworm with about 50 strains at 50 and 25 ug of B.t./ml of diet. This should be narrowed down to about 5 or 10 strains or isolates for a definitive bioassay using at least 5 dosage ranges per strain.

APPENDIX

PROPOSED QUESTIONS FOR DISCUSSION ON TUESDAY, JANUARY 13

1. Is there a relationship between the serotype of the B. thuringiensis isolate and the spectrum of activity of the δ-endotoxin it produces?
   a. The preliminary survey made it appear that there is a close relationship, but there appears also to be some evidence that a group of serotype 1 (thuringiensis) cultures may produce the same δ-endotoxin as a group of serotype 3a,b (kurstaki) cultures. Is this true?
   b. If the serotype and the δ-endotoxin do correspond, what about the cases where more than one type δ-endotoxin is produced by cultures within the same serotype? Should this have any influence on the classification of the variants?
   c. Is there any way to decide if the spectra we observe are due to only individual δ-endotoxins? Is there any chance that some of these spectra are due to a mixture of δ-endotoxins? If so, how do we demonstrate this?
   d. What further work needs to be done to decide the serotype-spectrum relationship? What about those cases where only one or two isolates of a particular serotype have been tested? Should we try to test more members of these serotypes? Are they available?
2. How reliable is the T/H ratio? Can this be used to identify serotypes? What is the relationship of the T/H ratio to activity against other insects? What can be done to determine this experimentally?
3. How reliable is the C/M ratio? Can this be used to identify serotypes? What is the relationship of the C/M ratio to activity against other insects? What can be done to determine this experimentally?

4. What other ratios can and should be developed?
5. Var. galleriae has long been known to produce toxins with very high activity against Galleria mellonella. What is the relationship of the galleriae activity to the activity against other insects? What insects respond like trichoplusia ni? Do they respond like T. ni to other toxins? What insects respond like Heliothis virescens? Do they respond like H. virescens to other toxins? What about those cultures that are classified as galleriae, but have high activity against Heliothis? What is the relationship of their activity to the activities of the toxins produced by var. kurstaki?
6. The silkworm, B. mori is a "Type 1" insect. What similarities are there between the response of B. mori to the various toxins and the responses of other insects in this study?
7. Pieris brassicae is very sensitive to δ-endotoxins. Is the response of this insect to the various toxins unique, or does its reaction correspond to the reactions of any other insects in this study?
8. Ostrinia nubilalis is sensitive to the endotoxins from many different serotypes. Does this response correspond to the response of any of the other test insects?
9. While it was too early to draw definite conclusions, the preliminary analyses indicated that the relative responses of Cadra cautella and Plodia interpunctella to the various toxins were usually similar, but in at least one case might differ. Was this true? Is there any similarity between the responses of these two insects and other insects in the study?

10. What is the relationship of the toxicities of these formulations against mosquitoes and the toxicities against lepidopterous insects? Does it appear that mosquitoes are sensitive to the δ-endotoxin? Or is this activity due to a different toxin? Do all mosquitoes respond similarly? Does the mosquito activity correlate to the activity against any of the other insects in this study?
11. Does the activity measured against Hyphantria cunea correspond to the activity measured against any of the other insects in the survey? What relationships can be discerned in the activities measured against B. morin, T. ni, H. virescens, and H. Cunea?
12. What about the activity against Spodoptera litura? Is this due to a δ-endotoxin or due to another toxin? Is there any association of the activity against S. litura and activities against any other insect in this study?
13. What about activities against the forest insects? Is there any relation between the sensitivities of Hemerocampa pseudotsugata and Lymatria dispar? Or between either of these insects and others in the program?
14. Can the serology of the crystal be used to predict the spectrum of activity of the toxin? Is there a relationship between the serology of the crystal and the serology of the culture?
15. Is the same toxin always produced by a given culture? How can we best determine this?
16. What is the relationship of the δ-exotoxin to the activities observed?
    a. Some of the data indicate that the activities observed against Agrotis ipsilon, Spodoptera exigua, and Spodoptera frugiperda

may be only due to δ-exotoxin. Is this true? Is there any evidence to the contrary?

b. There was indication in the preliminary analysis that C. cautella and P. interpunctella were very sensitive to δ-exotoxin. Is this true? If so, how does this interfere with the interpretation of other test results against these two insects?

c. What other insects may be sensitive enough to δ-exotoxin that the presence of this toxin might interfere with interpretation of results?

d. There is evidence that there may be another toxin present that may, like δ-exotoxin, kill the horn fly. Is this true? Is there any evidence that this other toxin is active against any of the other test insects?

17. Based on all the present data, what cultures are the most important as possible sources of useful insect control formulations? What other cultures are important to help us learn more about these toxins?