Axenic Rearing of the Lawn Armyworm, *Spodoptera mauritia acronyctoides* (Guenee), and Synxenic Inoculation Techniques[1]

GERALD H. TAKEI AND MINORU TAMASHIRO
UNIVERSITY OF HAWAII
HONOLULU, HAWAII 96822

Inasmuch as diseases are advents of series of interacting processes, and may consist of complexes of organisms and conditions, pathological studies should not be undertaken without considering these interrelated factors. Vago (1963) in his work has emphasized the importance of interrelations in insect diseases. According to Steinhaus (1958, 1960), disease interrelations should be considered from the standpoint of the host insect, the infectious agent where applicable, the environment, and "stressors" or factors influencing the diseases in insects. In most cases the etiologies of diseases are obscured by the interrelations, and the physiopathological disorder leading toward the development of a disease is insufficiently known or is unknown. Furthermore, there is difficulty in distinguishing between the really predisposing physiological condition, the ecological factor ("stressor"), and the effect of the ecological factor. In order to isolate the interactions of the host and pathogen with a minimum of effect of other factors, axenically reared and/or axenically prepared host and pathogen are necessary. Synxenic arrangements of only the insect host and its pathogen may then be maintained so that the reactions between the two may be investigated to elucidate the mechanisms that may be involved in the infection.

This study describes the techniques developed for synxenic combinations of the lawn armyworm, *Spodoptera mauritia acronyctoides* (Guenee) and two pathogens found associated with it in Hawaii. The insect host, which was apparently introduced from the Pacific, was first recorded in Hawaii in 1953 by Pemberton (1955). The two pathogens, a protozoan, *Nosema sp.*, and a nuclear polyhedrosis virus (NPV), *Borrelinavirus sp.* were found infecting populations of the lawn armyworm soon after. The NPV is highly infectious, (Tanada and Beardsley, 1958; Tanada, 1960; Raheja, 1965; Laigo and Tamashiro, 1966) and is believed to have come to Hawaii along with its host (Tanada and Beardsley, 1957). The protozoan also has been reported to be highly infectious by Tanada and Beardsley (1958), Raheja (1965), and Laigo and Tamashiro (1967).

[1]Published with the approval of the Director of the Hawaii Agricultural Station as Journal Series No. 1638.

Oligidic Diet used to Rear Axenic Cultures of the Lawn Armyworm, *Spodoptera mauritia acronyctoides* (Guenée)[2]

Components of Diet	*Quantity in Grams*
Fresh leaves of napier grass	74.0 (wet mass)
Distilled water	175.0
Dried pinto beans	24.0
Dried brewers yeast	7.0
Salts, Wesson's	2.0
Soybean oil	0.9 (about 1.06 ml)
Tween—80	0.6 (about 0.6 ml)
Alfalfa leaf meal	3.0
Agar	4.0
Ascorbic acid	3.0
Formaldehyde (10%)	0.05 (about 0.5 ml)
Methyl p-hydroxybenzoate (15% w/v in 95% ethyl alcohol)	0.15 (1.0 ml)
Sorbic acid (7.5% in 95% ethyl alcohol)	0.075 (1.0 ml)
alpha-Tocopherol (2.5% in 95% ethyl alcohol)	0.025 (1.0 ml)
Aureomycin	0.03

[2]A modification of the diet used for the alfalfa caterpillar larva, Tanada and Chang (1968).

Materials and Methods

Laboratory cultures of *S. mauritia* were started from field collected, egg masses. The larvae were fed freshly prepared bouquets of tender, young shoots of napier grass, *Pennisetum purpureum* and were maintained in one half pint paper cartons covered with bottoms of standard petri dishes (Tanada and Beardsley, 1958). These served as stock cultures.

Armyworms from field collected egg masses which succumbed to outbreaks of nosemosis and virosis were separated and the cadavers were kept as sources from which inocula of *Nosema* spores and *Borrelinavirus* polyhedra could be prepared. Other stocks of both pathogens which had been collected over the past years were also available.

Axenic Host Cultures. Axenic cultures of *S. mauritia* were maintained by rearing the larvae on an oligidic medium which was a modification of the one used for the alfalfa-caterpillar by Tanada and Chang (1968). For the lawn armyworm, napier grass, *Pennisetum purpureum* Schumach was added to fortify the diet which was autoclaved. Among the antimicrobial agents which were included in the diet, formaldehyde and aureomycin appeared to have the greatest effects on the growth and development of the armyworms and quantities of these materials were reduced from those prescribed for the alfalfa-caterpillar. The other antimicrobial agents, methyl p-hydroxybenzoate and sorbic acid were also reduced.

The axenic diet medium was prepared by tearing blades of freshly cut napier grass to about 7.5 cm lengths and blending them with distilled water to a mull. Brewers yeast, Wesson's salts, dried pinto beans, alfalfa leaf meal, soybean oil and Tween-80 were then added and blended with the napier grass. Agar was added to the medium along with about 60 ml of distilled water to facilitate mixing, and the entire combine was blended rapidly before it thickened. The resulting mixture was sealed within the blender with aluminum foil and autoclaved. The vitamins and antimicrobial agents were blended into the medium after it cooled to 48°C. The diet was then quickly poured into sterile 8 dram vials to depths of 1.25 cm to 2.5 cm and was allowed to harden while the vials were held horizontally. They were capped with plugs of sterile cotton, covered with Saran wrap and stored in the refrigerator for later use. Best results were obtained when fresh batches of medium were prepared according to need, so that the vials of diet were not left standing in the refrigerator over long periods.

The egg masses were surface sterilized by placing them in 30 ml of 10% formaldehyde solution containing 0.05 ml of glacial acetic acid for at least 50 minutes. During this period they were periodically agitated and stirred. The masses were then rinsed in about 50 ml of sterile distilled water three times, over a period of 15 minutes after which they were placed in sterile 8 dram vials, one mass per vial, stoppered with sterile cotton, and allowed to stand in a cabinet until they hatched. Best results were obtained when the surface sterilization was performed 1 or 2 days after the eggs were laid. There was no advantage in separating eggs individually from a mass.

Freshly hatched larvae were placed on the axenic diet-medium. The rearing vials were first brought to room temperature, and sterilized, 10 to 2 mesh, vermiculite (Bot, 1967) was added to each vial so that the entire surface of the medium was covered. For the lawn armyworm, the vermiculite also appeared to provide cover under which feeding was enhanced.

A vial with freshly hatched larvae was first gently tapped while being held horizontally so that the larvae could be dislodged from the wall of the vial and be suspended by their silks. The suspended larvae could then simply be transferred by means of their silks with an applicator stick. For best results, one larva was reared individually in a vial with about 1.25 cm of medium. The entire larval growth and development to pupation occurred in this rearing vial without further transferrals or changes.

Autoclaved gallon jars with covers which were cut open and lined with very fine mesh screen served as oviposition chambers. They were first lined with sterilized paper towels, and 12 pupae were placed in each of them. When the adults emerged, 2 dram vials with sterilized honey water and cotton wicks were placed into the chambers as food,

and then crumpled, sterilized paper towels were added to provide resting and oviposition surfaces. All operations were performed within a sterilization box equipped with an ultra-violet germicidal lamp to ensure aseptic conditions. The egg masses which were oviposited on the paper towels were collected by cutting them out and placed in empty sterile 8 dram vials for eclosion of the next generation.

The rearing was done at an average temperature of 26.7 ± 2.2°C and an average relative humidity of 60 ± 10%. Smears of tissues from all stages of the cultured insect host and feces on plates of sabouraud dextrose agar fortified with 0.5% brewers yeast and on plates of nutrient agar resulted in negative microbial growth, as well as dips of the same tissues in fluid thioglycolate. These tests were applied periodically to verify axenic and/or aseptic conditions. Several lines of axenically reared *S. mauritia* were maintained with overlapping generations. Figures 1 to 3 show various phases during the axenic rearing of *S. mauritia.*

The addition of formaldehyde in the diet medium was found to be significant for the survival of *S. mauritia* cultures beyond a few generations. Larvae of a culture which were reared axenically on medium without formaldehyde appeared to grow to large size faster than their counterparts reared on medium with formaldehyde, and appeared slightly greener than usual. The difference in color apparently was caused by a hastened greening of the hemolymph within the larvae. The dura-

FIG. 1. Twenty-four day old lawn armyworms *Spodoptera mauritia acronyctoides* (Guenée) in their 8 dram rearing vials.

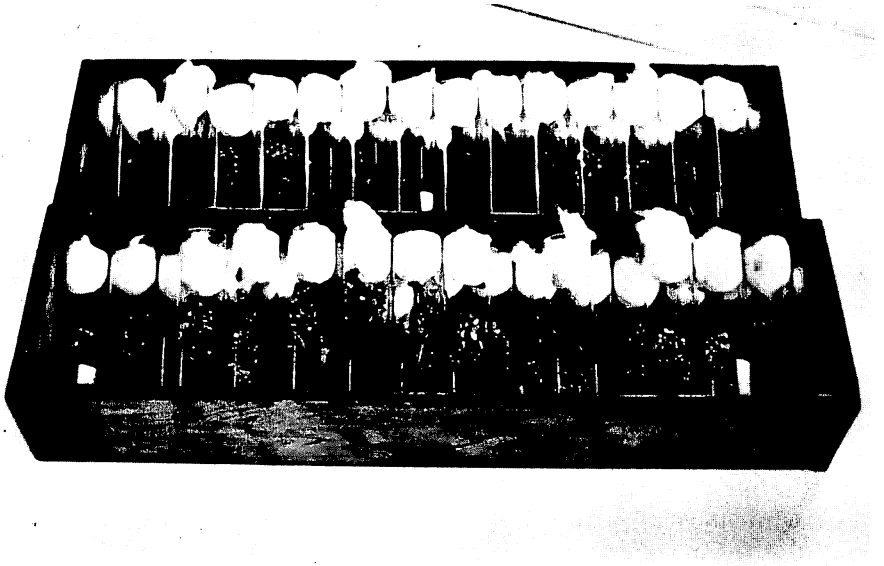

FIG. 2. Racks filled with axenic culture vials.

FIG. 3. Oviposition chamber; note egg masses laid on paper tissues.

tions of the larval and pupal stages, and the adult emergences were similar to those of larvae reared on media with formaldehyde. However, cultures without formaldehyde were not able to survive beyond 3 to 5 generations.

Preparation of Inocula. Spores and polyhedra were first separated from cadavers of infected host larvae by blending the cadavers in sterile distilled water and by filtering the resulting mixtures through a thickness of 3 tissues of Kimwipes.® The filtrates were stirred and centrifuged at about 500 rpm for 25 minutes. The sediments were resuspended in more relatively large volumes of sterile distilled water and were re-centrifuged at 800 rpm for 3-5 more times until they appeared white with pathogens. When these sediments were examined, they appeared free of other microbial contaminants, and gave negative microbial streak tests in fluid thioglycollate for at least 2 days.

Inoculation Procedure. The axenic larvae were first allowed to reach the 4th instar. A definite volume of the inoculum was injected into the vial, while the vial was held horizontal. The open end was flamed before and after the introduction. A volume large enough to wet the entire quantity of medium in a vial for one larva (0.5 ml), was applied so that the entire surface of the medium and the vermiculite particles were covered. Whether the pathogens were applied individually or in combinations, the total volume of inoculum did not exceed 1.5 ml. The controls were inoculated with an equal amount of sterile distilled water. Successful *per os* inoculations based on methods incorporating the pathogens into the insect diets have also been reported by Ignoffo (1964) and Chauthani (1968).

Microbial streak tests proved to be negative for bacterial and fungal contaminants for the entire duration of the tests. For synxenic virus inoculations of doses causing high mortality, the earliest symptoms of infection were observed on about the 3rd day after treatment. Infected larvae tended to move away from the media toward the cotton plug. Death and liquefaction usually occurred here, but carcasses were also observed attached to the glass walls of the vials (Fig. 4). Liquefaction of the carcasses progressed until only smudges of the remains of the larva were left. Sometimes the treated larvae could not be recovered at all and in such cases, microscopic examinations of the media and of washings of the vermiculite revealed polyhedral inclusion bodies, verifying that the disappearances of larvae were due to liquefaction and virosis. Sometimes this complete liquefaction apparently occurred overnight. The remains of the carcasses were odorless and gave negative streak tests for contaminating microorganisms. For low doses of virus polyhedra, symptoms of virus infection were observed in larvae during the later instars. These larvae were of relatively large size at the time infections were evident, and the carcasses that resulted darkened and were suspended from the cotton plugs by their prolegs (Fig. 5).

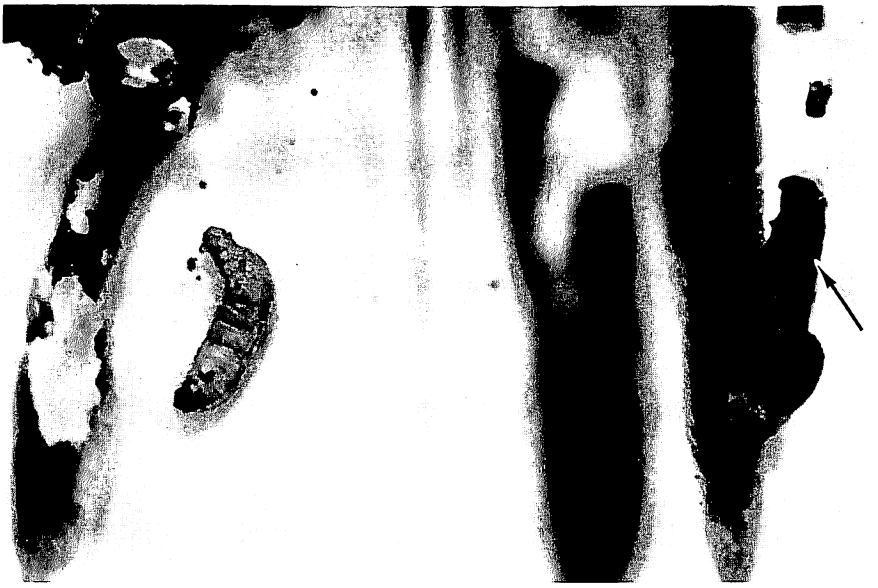

FIG. 4. Photograph of 2 separate vials with NPU killed lawn armyworms which are about 14 days old; death about 6 days after inoculation; carcass on left, pink with body enlarged and head remaining small below; carcass on right (arrow) dark brown smudge with head at top; both carcasses adhering to walls of vials; other dark smudges diet medium and vermiculite.

FIG. 5. Black, 26 day old lawn armyworm carcass; death 18 days after inoculation; suspended from cotton plug by prolegs.

Larvae reared on media without formaldehyde, did not react any differently to the challenges of varying doses of pathogens than their counterparts reared on media with formaldehyde. The antimicrobial level at which formaldehyde was included in the axenic diet appeared not to have affected the inoculated pathogens.

Discussion

Inoculating the host insect by applying the pathogens on its larval diet-medium is believed to be the most effective means of applying both *Nosema* and NPV. Ignoffo (1972), has made similar claims for microbial insecticides in general. The techniques which have been employed in this study are versatile and may not be restricted to only pathological work. Such techniques should also find utility in physiology and toxicology among other areas of study.

S. mauritia does not appear to require the association of bacteria, fungi and other microbial symbiotes for its survival. The need of formaldehyde in the diet for *S. mauritia* at low levels for its survival, suggests that this insect host in Hawaii may presently be subjected to some kind of slow acting disease, which apparently is subdued by the antimicrobial reagent. Other observations, moreover, support the presence of such a chronic, possibly endogenous disease within the host. The application of formaldehyde in such a manner suggests also that this reagent has chemotherapeutic as well as antimicrobial characteristics.

References

Bianchi, F.A. 1957. Notes and exhibitions. Proc. Hawaiian Entomol. Soc. 16: 184.

Bot, J. 1967. An artificial rearing medium for three noctuids of economic importance belonging to the genus *Spodoptera* (Lepidoptera). J. Entomol. Soc. S. Africa 29:157-160.

Chauthani, A.R. 1968. Bioassay technique for insect viruses. J. Invert. Pathol. 11: 242-245.

Ignoffo, C.M. 1964. Bioassay technique and pathogenicity of a nuclear-polyhedrosis virus of the cabbage looper, *Trichoplusia ni* (Hubner). J. Insect Pathol. 6: 237-245.

Ignoffo, C.M. 1972. An appeal for research. J. Invert. Pathol. 19: i-ii.

Laigo, F.M. and M. Tamashiro. 1966. Virus and insect parasite interaction in the lawn armyworm, *Spodoptera mauritia acronyctoides* (Guenẽe). Proc. Hawaiian Entomol. Soc. 19: 233-237.

Laigo, F.M. and M. Tamashiro. 1967. Interactions between a microsporidian pathogen of the lawn armyworm and the hymenopterous parasite *Apanteles marginiventris*. J. Invert. Pathol. 9: 546-554.

Pemberton, C.E. 1955. Notes and exhibitions. Proc. Hawaiian Entomol. Soc. 15: 373.

Raheja, A.K. 1965. The interrelation between two insect pathogens *Nosema sp.* and *Borrelinavirus sp.* in the lawn armyworm *Spodoptera mauritia acronyctoides* (Guenẽe) (Lepidoptera: Noctuidae). M.S. Thesis, University of Hawaii. 61 pp.

Steinhaus, E.A. 1958. Stress as a factor in insect disease. Proc. Intern. Congr. Entomol. 10th Montreal, 1956, 4: 725-730.

Steinhaus, E.A. 1960. The importance of environmental factors in the insect-microbe ecosystem. Bacteriol. Revs. 24: 365-373.

Tanada, Y. 1960. A nuclear polyhedrosis virus of the lawn armyworm, *Spodoptera mauritia* (Boisduval) (Lepidoptera: Noctuidae). Proc. Hawaiian Entomol. Soc. 17: 304-308.

Tanada, Y. and J.W. Beardsley. 1957. Probable origin and dissemination of a polyhedrosis virus of an armyworm in Hawaii. J. Econ. Entomol. 50: 118-120.

Tanada, Y. and J.W. Beardsley. 1958. A biological study of the lawn armyworm, *Spodoptera mauritia* (Boisduval), in Hawaii (Lepidoptera: Phalaenidae). Proc. Hawaiian Entomol. Soc. 16: 411-436.

Tanada, Y. and G.Y. Chang. 1968. Resistance of the alfalfa caterpillar, *Colias eurytheme*, at high temperatures to a cytoplasmic-polyhedrosis virus and thermal inactivation point of the virus. J. Invert. Pathol. 10: 79-83.

Vago, C. 1963. Predispositions and interrelations in insect diseases. In: Insect Pathology An Advanced Treatise, vol. 1 (ed, E.A. Steinhaus), Academic Press Inc., 339-379.