THE LEAFHOPPERS AND PLANTHOPPERS

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Nutrition, Cell Culture and Symbiosis of Leafhoppers and Planthoppers

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8.1 INTRODUCTION

There are approximately 150 species of leafhoppers and planthoppers known as vectors of economically important plant pathogens, but less than two dozen of these vectors have been reared under conditions that permit analysis of their nutritional requirements and feeding behavior. A large number of Homoptera embracing many families have been examined with histological and ultrastructural techniques that detect the presence of intracellular microorganisms, but in only a few cases have the functions of these symbiotes been ascertained. Thus, the interrelated fields of rearing, nutrition, and symbiosis of an important group of insects offer many opportunities for further research.

8.2 REARING TECHNIQUES

Rearing on natural host plants is distinguished from rearing in small man-made containers in which the diet and other factors are controlled.

8.2.1 Containment and Feeding for Mass Rearing

Mass rearing on natural host plants in greenhouses has been practiced for many years. The principal purpose is to have a year-round supply of insects for

investigations on plant pathogen transmission and for subjects in a teaching laboratory. Propagation and biology of parasitoids can be studied also in greenhouse circumstances that resemble field conditions. The technique is tedious if not simple, and fraught with complications. Field-caught insects are released in mesh-screened enclosures over healthy host plants. There must always be a source of healthy replacement plants, which probably have been sprayed with insecticides or fungicides that may affect the physiology of the leafhoppers and planthoppers. Commonly entomopathogenic fungi (*Entomophthora* species) infect the insects and destroy the colony (2). In transmission studies there is a degree of uncertainty regarding the freedom from accidental contamination of the plants and insects when they are cultured in close proximity to other plants. Another drawback is the time required to wait for the development of symptoms of viral or mycoplasmal infections in plants. To avoid undesired infections, plants must be grown under closely controlled conditions and insects must be well-ventilated.

8.2.2 Artificial and Chemically Defined Diets

Artificial diets on which the insects can be reared in the laboratory under more closely controlled and isolated conditions solve many of the problems of environmental contaminants and uncertainty of infections. Nevertheless, rearing on artificial diets poses a new set of complications that will be discussed.

Containment. The design of satisfactory rearing vessels for planthoppers and leafhoppers has interested numerous investigators. Probably more time and effort have been expended on designing rearing vessels than on formulating nutrient media. A comprehensive, illustrated summary of the research on rearing vessels was provided by Mitsuhashi (54). Early experiments were initiated by Carter (5) to see how long the beet leafhopper, *Eutettix tenellus* Baker, would survive on a dilute sugar solution and the smaller brown planthopper, *Laodelphax striatellus* Fallén, on distilled water contained by thin, stretched animal membranes (fish bladder or beef mesentery). Other investigators found that insects would feed on semisolidified nutrients covered with a thin film of paraffin, or on liquids covered with cheesecloth or plastic film. In some cases insects probed directly on sitting or hanging drops of nutrient solutions, or rested directly on cotton wicks saturated with solution.

The first to achieve growth of a leafhopper on a complex synthetic diet, although the life cycle was not completed, were Mitsuhashi and Maramorosch (61) who fed *Macrosteles fascifrons* (Stål) on sterile cotton gauze wicks. In the numerous modifications in rearing technique that followed, several features were common to many of them. The food was almost always a fluid contained in a material that could be probed by the insects' mouthparts. The rearing vessel was small (no larger than a petri dish or a 50-mL beaker) and entirely or partially transparent because of the necessity to observe the small insects closely. The component parts were easily assembled and disassembled, and

they were inexpensive (test tubes or glass cyclinders) or disposable (small plastic cups). Small numbers (12–20) of insects were placed in each container so that they were not crowded—they should neither touch when feeding nor bump into one another when jumping.

The variety of means of successfully feeding the insects suggests that probing through a membrane is not an essential factor in feeding behavior. Nevertheless, diet contained in a membrane is easier to protect from contamination and desiccation, and smaller amounts are needed. Mittler and Dadd (63) described the use of an artificial, stretchable membrane with a waxy texture (Parafilm, Marathon, American Can Co., Neenah, Wisconsin) for research on aphid nutrition. It was soon found that leafhoppers and planthoppers also will probe and feed through this membrane or a similar one (Sealon, Fuji Photo Film Co., Tokyo, Japan). If the plastic membranes, which resemble the cuticle of plants, had been available years ago, it might never have been known that the insects would probe animal membranes as well.

These stretchable synthetic membranes that adhere to one another or to glass, plastic, or metal containers have simplified handling of the diet and keeping it aseptic (see Section 8.4). Furthermore, more than one membrane-covered diet can be made available in a single rearing vessel so that choice tests can be carried out. The general practice is to stretch one membrane over the top of a cylinder or cup, stretching it to four times its original area so that it becomes thin enough for a newly hatched nymph to pierce it. A few drops of nutrient solution are placed on it, and a second stretched membrane is applied over the first. The membranes are sealed together around the periphery of the fluid with finger pressure. The volume of fluid must be small enough so that it does not interfere with sealing of the membranes and cause leaking. The completed assembly is known as a *sachet*. Given an acceptable food, the insects usually remain attached to the sachet in an inverted orientation, their stylets probing the membrane much of the time. Using a sachet, Mitsuhashi and Koyama (57) were the first to rear continuously a planthopper, *L. striatellus*, on a synthetic medium. By applying a sachet to both ends of a cylinder, feeding choice tests were carried out (57) and cholesterol emulsions were offered separately (34) in experiments with the green rice leafhopper *Nephotettix cincticeps* Uhler and the zigzag-striped leafhopper *Inazuma dorsalis* Motschulsky.

Seemingly the insects are not very demanding in their physical requirements, considering that a number of species have accepted food in such a variety of devices. The continued interest in improving the rearing vessels stems from the need to improve survival of the insects, prevent losses by their escape during transfer, minimize deterioration of the medium, and reduce the amount of handling of the system. Even on an optimal diet, mortalities occur when the nymphs become stuck in honeydew. Nymphs leave the sachet occasionally, especially to molt, resting on the wall of the floor of the vessel. An accumulation of honeydew dropping from the insects feeding on the ceiling is viscous and sticky enough to trap those coming in contact with it unless

precautions are taken to remove or absorb it. When the diet sachets need to be replaced, attached insects can be lost or those in the cylinder can jump away. The system was modified to alleviate some of these problems by Anderson (1) who introduced two major improvements. By lining the cylinder with blotting paper and placing two thicknesses of filter paper on the floor, the honeydew was absorbed better. The sachet was replaced by a single sheet of Parafilm stretched over the top of a small plastic cup that was then inverted over a circular opening in the ceiling. This resulted in a solid ring surrounding the feeding membrane so that the insects were prevented from attaching directly above the wall and therefore did not drop honeydew on it. Stretching one membrane over a cup was less time consuming than preparing a sachet, and since more diet could be put in the cup, it did not have to be changed quite so frequently. With these improvements, more young nymphs survived and handling time was reduced. However, escape of the insects when the container was opened could not be entirely prevented.

Phagostimulation. Before artificial, synthetic media can be satisfactorily evaluated, a feeding stimulus for the insects must be provided. Mittler and Dadd (63) reported using sucrose at 18% (w/v) for the green peach aphid, *Myzus persicae* (Sulzer). Sucrose and nine other sugars have been tested in water or in soft agar, in concentrations of 1–20%, for feeding preference and survival of leafhoppers and planthoppers. For *L. striatellus* sucrose at 5% in water was preferred and it supported the longest survival time (56). Glucose, fructose, and maltose at 0.1*M* were only slightly usable by first instar *M. fascifrons* (18). Sucrose over a wide range of low concentrations, 0.0075–0.4*M*, was phagostimulatory for the potato leafhopper, *Empoasca fabae* (Harris), becoming phagoinhibitory at 1*M* (12). Glucose and fructose, separately or combined, were not as stimulatory for these two insects. When given a choice of 10% sucrose or glucose and fructose at 10%, *I. dorsalis* preferred sucrose (32). Offered ^{32}P labeled sucrose in concentrations ranging from 5–25%, the brown planthopper *Nilaparvata lugens* (Stål) took up maximum quantities at 20%, and its consumption was further improved by adding amino acids, vitamins, and mineral salts to the sucrose (74). *N. lugens* was stimulated to probe deeply and repeatedly through a Parafilm membrane into 2% sucrose solution containing dilute concentrations of salicylic acid, 0.004*M* being most effective (75). Salicylic acid is a normal component of rice plant sap, but it was quite specific for the brown planthopper, since it was less stimulatory for the other two rice planthoppers, *Sogatella furcifera* Horvath and *L. striatellus*, and without effect on probing by the two rice leafhoppers, *N. cincticeps* and *I. dorsalis*. The extent of probing by the insects was determined by removing the Parafilm membrane, staining it with 0.1% rhodamine B, and microscopically examining the stylet sheaths. With this technique, rice plant flavonoids also were shown to be probing stimulants for *N. lugens*. The Parafilm membrane technique was used to demonstrate that a plant, barnyard grass, contains an extractable antifeedant against *N. lugens* (28). The extract added to 15%

sucrose tagged with ^{32}P caused this planthopper to starve to death but was effective neither against *L. striatellus* nor *S. furcifera*. The antifeedant was isolated and identified as *trans*-aconitic acid (29). Aromatic amines (43) and chlordimeform, an acaricide (16), also inhibited fluid uptake through a Parafilm membrane. Sōgawa (78) distinguished two gustatory stimuli, one for probing and one for sucking, and stated that observing the depth of the probes is a better criterion for probing than for sucking. To measure sucking, resulting in actual fluid uptake, ^{32}P in the insect bodies was counted (16); or the amount of colored diet remaining after a feeding period was estimated by diluting it to a known volume and spectrophotometrically measuring its absorbancy (16). Other research on the feeding physiology of *N. lugens* was reviewed by Sōgawa (79).

Physical Factors. Investigations have been done on the effects of height of the rearing vessel, population density, temperature, relative humidity, photoperiod, direction of the light source, and color of illumination for only a few species (54). For first instar *L. striatellus* the height of the vessel was critical, since the youngest insects must be able to jump to the ceiling if the membrane is stretched across the top of the vessel. In a vessel no higher than 25 mm survival was normal and this was not affected by the population density up to a maximum of 40 nymphs per vessel. Temperatures ranging from 10 to 30°C were tolerated. The direction of the light had no effect on orientation of nymphs with respect to the food source. Relative humidity was very important, especially for first instars, the best survival occurring at 100% relative humidity (58). Providing such a moisture-saturated atmosphere introduces other problems, however. If free water droplets condense in the vessel, the nymphs get stuck in them and drown; so some means of absorbing or wicking away the water must be found (1). *L. striatellus* first instars were indifferent to photoperiod, and even reached the food in complete darkness (58). However, when growth of the entire immature life stage was compared in short and long days, short days (8L:16D) considerably prolonged nymphal life, especially the fourth instar, of *L. striatellus*; but *Sogatella longifurcifera* Esaki and Ishihara and *L. furcifera*, on the other hand, were not affected by photoperiod (41). Other experiments indicate that planthoppers and leafhoppers grew equally well in constant light or 18-hr photoperiod (1). The color of illumination did have a highly significant effect on feeding and therefore on survival, with yellow being the most attractive. Colors were tested by passing light through monochromatic filters or covering the vessels with sheets of colored acetate. Preference, in decreasing order, was for yellow, orange, red, and blue, with green being repellent for *L. striatellus* (58); yellow, green, red, and blue, with purple being repellent for *I. dorsalis* (33); and yellow, orange, yellow-green, green, red, and blue for *M. fascifrons* (1). Synthetic diets, which contain riboflavin, are yellow when fresh and this may account in part for their attractiveness; but attachment of nymphs to the membrane in total darkness must be a result of random movement.

Those species of leafhoppers and planthoppers that have been reared suc-

cessfully seem to respond to nearly identical physical conditions, but there must be other unknown factors that are deterring efforts to colonize more species. Probing and sucking stimulants need to be identified. For instance, the planthopper *Peregrinus maidis* (Ashmead) was placed in the same situation used to culture *M. fascifrons* but it failed to feed satisfactorily (Brooks, unpublished).

Oviposition Stimulus. Continuously rearing a viable colony depends on a satisfactory rate of egg production. In general, females oviposit through the Parafilm membrane into the diet, and the eggs must be removed to moist filter paper before they hatch. When given a two-way choice between diet and sucrose, with a sachet at each end of a horizontal cylinder, mated female *L. striatellus* favored sucrose (37). When given a two-way choice between distilled water and a carbohydrate solution, *L. striatellus* laid more eggs per female in sugars, and the preference was for sucrose over glucose, raffinose, fructose, trehalose, and maltose. By counting the number of eggs oviposited in a series of concentrations of the sugars, it was found that the optimum concentration of sucrose for oviposition was 10% (60). Since there was only a slight decrease in egg deposition in sucrose down to 5%, the latter concentration was recommended because 10% is too viscous to be handled conveniently.

To investigate the effect of diet compared to sucrose, each major diet group was added separately to 5% sucrose and it was found that only the amino acid solution was inhibitory (37). By adding each of the 23 amino acids separately to 5% sucrose, a significant depression in oviposition was found to occur with arginine, glutamic acid, tyrosine, and valine, while cystine was the only amino acid that stimulated oviposition.

Salicylic acid, a probing stimulant for *N. lugens*, also stimulated oviposition in this species but not in *L. striatellus* (75).

8.3 NUTRITION

The rearing of plant-sucking insects is based on use of solutions made with chemically identified components. Such diets are known as *holidic*. They may not bear much resemblance to plant sap on which insects feed in the wild. Although the principle of holidic diets and the technique of providing them encased in a membrane have been available since 1963, there are data on practical application for only a few species of leafhoppers and planthoppers.

8.3.1 Holidic Diet Formulation

Reagent grade chemicals are used. Five major diet groups are made separately, some of them in concentrated stock solutions, and kept frozen until needed. This permits experimental adjustment of proportions and dilutions of the constituent groups, which are sugar (preferably sucrose), L-amino acids, B

vitamins, mineral salts (and trace minerals), and lipogenic factors. DL-amino acids may be used if the amounts are twice those of the L forms. Homoserine, because of its expense, should be omitted unless there is evidence that it is required by the species under investigation (54). Ascorbic acid is included, but there is doubt as to its necessity for some species since it is rapidly lost through oxidation. Riboflavin, while it is included, readily degrades in light as is indicated by fading of the yellow color of the diet, which raises some question as to its need. Cholesterol is necessary for adult development and egg production in some species but its universal requirement has not been tested. The final pH of the diet is usually adjusted to 6.5 or 7.0, but various species have imbibed fluids where the pH ranged from 5.5 to 8.5. The diet formulae and detailed procedures for preparing them have been reviewed by Mitsuhashi (52, 54). Generally the rearing system is clean but not aseptic. The diet is sterilized by filtration, the eggs from which a new generation is started are surface-sterilized, and all of the plastic or glass ware is clean and sterile. Nevertheless, bacterial contamination of the diet and fungal contamination of the honeydew often develop rather quickly, necessitating frequent changes of the diet. It is possible to achieve truly aseptic conditions with additional effort, and this has been done (see Section 8.4).

The basis for the composition of diets for leafhoppers and planthoppers was the pioneering work on aphid diets (reviewed by Hou, 19; Mitsuhashi, 54). The first success in rearing a leafhopper for part of its life cycle on a synthetic medium under aseptic conditions was that of Mitsuhashi and Maramorosch (61). *M. fascifrons*, reared aseptically on plants, was fed on a sterile wick saturated with a liquid designed for tissue culture. Following numerous adjustments, four diets have been developed and are used extensively in attempts to culture leafhoppers and planthoppers. The diets are designated as MED-1, which was modified from Ehrhardt's diet (13) for aphids (52); MED-4, a further modification in which some of the amino acids were reduced and a few changes were made in the trace minerals (52); MMD-1, a modification of the aphid diet of Mittler and Dadd (63) in which sucrose was greatly reduced (52); and HB, derived by further reductions in amino acids and B vitamins but with the important addition of cholesterol and lecithin (21). The compositions of these diets were given by Mitsuhashi (54).

In comparing the individual ingredients of holidic diets fed to two aphids, a planthopper, and a leafhopper, Hou (19, 20) emphasized that the diets for the latter two insects are much reduced in sucrose content, and for the leafhopper the entire formulation is less concentrated.

8.3.2 Determining Specific Requirements

There are no known cases of instant success in continuous rearing on an artificial diet. The work has proceeded in a stepwise fashion. The first step is finding a suitable photoperiod and a diet that the nymphs will accept, while survival and growth rate are essential criteria. Proper adult development and

emergence often require further adjustments in the diet. Oviposition in the medium or in sucrose may be stimulated or inhibited by various factors that for the most part are unknown. Finally, the viability of embryos or nymphs of the next generation may not be the same as in the first generation. By addressing these problems one at a time, it is now possible to rear several species through successive generations.

8.3.3 Species Reared Continuously on Synthetic Diets

Six species of leafhoppers and three species of planthoppers have been studied intensively, and of these only three leafhoppers and one planthopper have been reared continuously on synthetic diet. The three leafhoppers are *Macrosteles orientalis* Vilbaste and *I. dorsalis* reared on MED−1 supplemented with a second sachet containing a saturated suspension of cholesterol in water; and *M. fascifrons* reared on HB, which contains a colloidal suspension of lecithin and cholesterol. The planthopper is *L. striatellus* reared on MED−1 without cholesterol. All four of these species adapt to any kind of vessel and they oviposit in the diet or 5% sucrose (54).

Only partial development without reproduction has resulted from numerous efforts to rear the remaining five on MED-1 (54). Nymphs of *S. furcifera* survived on MED-1 or MMD-1 better than on rice seedlings, but the adults did not reproduce (40). Similarly, nymphal development period of *S. longifurcifera* was prolonged on MED-1, MED-4, or MMD-1. Although survival was better than on rice seedlings, it failed to lay eggs (42). *Balclutha viridis* Matsumura and *N. cincticeps* used cholesterol provided in a separate container to develop to the adult stage, but neither species oviposited. *N. lugens* responded better when the diet sachet was placed on the floor of the vessel rather than the top, and oviposited to a slight extent in the diet. If 5% sucrose containing 0.002*M* salicylic acid was available, oviposition improved slightly but it has not been possible to rear this planthopper continuously. Its survival was only 10% for 15 days on MED-1 (35). *Euscelis plebejus* Fallén could reach the adult stage on artificial medium only if it was started in the third or fourth instar (26). An effort was made to rear *N. cincticeps* on the HB leafhopper medium (24). Although the rate of nymphal growth and percent of adult emergence were similar to development on rice seedlings, adults failed to oviposit in the medium. Females had normal eggs in the ovarioles and would oviposit in rice seedlings. On the other hand, gravid females collected from rice paddies also would not oviposit in the feeding vessel. All attempts failed to induce oviposition in sucrose with stimulating amounts of amino acids, or in different physical arrangements.

It is quite obvious that a major obstacle in continuous rearing is failure of oviposition to occur in the artificial conditions. At present there is no universal diet that can be used. MED-1 has been used most successfully, but improvements are needed. Once a diet is found that does permit rearing, it is possible to analyze the effects of different combinations and new ingredients in an effort to

improve the diet. For instance, Mitsuhashi and Koyama (59) identified folic acid as the factor affecting wing morph of *L. striatellus*. Koyama and Mitsuhashi (36, 37) found that arginine, glutamic acid, tyrosine, and valine, in 5% sucrose markedly reduced the number of eggs produced by *L. striatellus* while cystine stimulated oviposition. Essential vitamins and trace metals have been determined for this planthopper (38, 39). Without iron, copper, or zinc nymphs do not develop, but they are indifferent to a wide range of concentrations and do not require manganese or calcium. The optimum ranges, in milligrams per 100 mL diet, were reported as $FeCl_3 \cdot 6H_2O$, 1.1–71.3; $CuCl_2 \cdot 2H_2O$, 0.017–2.14; and $ZnCl_2$, 0.2–12.7. Iron, copper, and zinc are essential for *M. fascifrons* (23), with the Fe^{3+} requirement being no more than 6.60 μg/100 mL (1).

The role of cholesterol in the nutrition of planthoppers and leafhoppers is enigmatic. *L. striatellus* can be reared continuously without cholesterol, whereas this sterol is required for adult development and viable egg production by *I. dorsalis*, *M. fascifrons*, and *M. orientalis* (54). Attempts to rear *N. cincticeps* with cholesterol added have failed (24). For *M. fascifrons*, cholesterol can not be replaced by lecithin or linoleic or linolenic acids (22).

8.4 MICROBIAL INTERACTIONS

Several reasons make aseptic rearing desirable. The first is simply a matter of economics. The diet will not deteriorate so quickly if it is protected from microbial degradation. The second purpose is to distinguish the contributions of adventitious microorganisms from dietary factors or endogenous enzymes. In an otherwise aseptic condition, pure suspensions of viruses or spiroplasmas can be fed to the vector by incorporating them in an artificial diet (77). A third purpose is to study the interaction of vector tissues with plant viruses and mollicutes *in vitro*. For this, it is essential to rear the insects aseptically to obtain uncontaminated cells for culture.

8.4.1 Aseptic Rearing

Mitsuhashi and Maramorosch (61) reared leafhoppers aseptically, doing so by placing insects developed from surface-sterilized eggs on a wick saturated with an aseptic, synthetic diet. *M. fascifrons* reared from third instar on the wick molted once but did not complete its life cycle. Stickiness of the diet-saturated wick was a large part of the problem.

Aseptic conditions can be managed on natural host plants, on plant callus, and in rearing vessels. Procedures for growing sterile plants and introducing surface-sterilized eggs have been detailed by Mitsuhashi (54). Plants need to be cultured aseptically starting with surface-sterilized seeds. Callus needs to be produced with tissue culture methods. For insects that can be reared on artificial diet, this is the simplest method, although not necessarily the least

expensive. The methods are the same as outlined above with the addition of sterilizing all components of vessels with steam or dry heat, and the Parafilm can be sterilized by soaking in ethyl alcohol. The diet must be changed every 2 or 3 days even under aseptic conditions because of oxidation and autobiodegradation, and the lining of the vessels must be replaced to remove the honeydew.

The following insects have been raised on sterile plant seedlings: *Agallia constricta* (Van Duzee), *Agalliopsis novella* Say, *Dalbulus maidis* (DeLong and Wolcott), *I. dorsalis*, *L. striatellus*, *M. fascifrons*, *Nephotettix apicalis* Motschulsky, *N. cincticeps*, and *N. lugens*. On plant callus, *M. fascifrons* and *N. cincticeps* grew to a limited extent but did not complete the life cycle (54).

Very little has been done with aseptic rearing on artificial diet. Mitsuhashi (54) mentioned unpublished work on rearing of *L. striatellus* this way. Wei and Brooks (84) reared *M. fascifrons* aseptically on synthetic diet for five successive generations, during which time growth rate, survival, and adult emergence were all the same as normal. Gram-negative rods, gram-positive cocci, and some fungi were recovered from the surface of eggs that had not been surface sterilized, but sterility tests revealed no microorganisms in the insects or in the diet at the conclusion of aseptic rearing. The diet sachets had been probed repeatedly by the feeding and ovipositing insects but were still sterile.

Although Mitsuhashi (51) recommended the use of aseptically reared leafhoppers for starting cell cultures, there has been little application of this method, most investigators relying on embryos or, rarely, organs of adults.

8.5 CELL CULTURE IN VITRO

With the intent of developing tissue cultures from leafhoppers and planthoppers for studying interactions of vector cells with plant pathogens, several culture media have been designed. A useful medium is MM (62).

8.5.1 Primary Cultures

Primary cultures in MM medium were initiated from embryos of *N. cincticeps*, *N. apicalis*, *I. dorsalis*, *L. striatellus*, and *A. constricta* (reviewed by Maramorosch, 47). The cultures were successfully inoculated with plant viruses, although viruses did not propagate well and cultures could not be maintained for long.

8.5.2 Continuous Cultures

A medium was designed by Chiu and Black (10) containing very nearly the same ingredients as the MM medium but the proportions of sodium and potassium to one another were significantly altered through the use of five times as much K^+ and one-fifth as much Na^+. Cultures from six leafhoppers

were initiated in the medium of Chiu and Black. These were *A. constricta* and *Agallia quadripunctata* (Provancher) (10), *Aceratogallia sanguinolenta* (Provancher) (11), *A. novella* (85), *Colladonus montanus* (Van Duzee) (73), and *M. fascifrons* (Tsang and Brooks, unpublished). All the cultures were started from minced or trypsinized embryos. In time, they became adapted and were passaged more than 55 times, thus qualifying as continuous cell lines. In addition, McIntosh and co-workers (50) established lines of *M. fascifrons* and *D. maidis* in mammalian tissue culture medium.

The Chiu and Black medium was improved, mainly by increasing the glucose, yeastolate, and lactalbumin hydrolysate and by adding histidine (48). A medium with an extremely high sodium content was used to initiate cultures from embryos of *N. lugens* and *Nephotettix nigropictus* (Stål) but they failed to survive (76). A series of 29 different primary cultures of *P. maidis* embryo cells, in MM medium and in Chiu and Black medium with various modifications, were tried but none of the cultures survived beyond the third passage (Tsang and Brooks, unpublished). These efforts were made under the same conditions and with the same media used routinely for maintenance of *M. fascifrons* cell lines.

For the most part, the species from which cell cultures have been established are the same that have been reared on artificial diet. This is probably a reflection of the preferences of investigators rather than a unique property of the insects.

In comparing the formulae for various diets and in comparing different cell culture media, it is clear that as improvements were made, the potassium was elevated and the sodium was lowered. Vago and Flandre (81) drew attention to the fact that insects are of two types with respect to the sodium and potassium levels in their hemolymph, and that the leafhoppers belong to the category whose hemolymph is rich in potassium and magnesium, poor in sodium. This distribution of the major cations may have some influence on survival *in vitro*, if not growth, of mycoplasma-like organisms (MLO) and spiroplasmas (helical mollicutes), which rank with viruses as causative agents of many serious plant diseases transmitted by leafhoppers and planthoppers.

8.5.3 Plant Pathogens Cultured In Vitro

Plant viruses have been successfully inoculated into leafhopper cell cultures (46, 47). Lines of at least six species have been inoculated, including *A. constricta*, *A. quadripunctata*, *A. sanguinolenta*, and *A. novella*. The *in vitro* method is useful in establishing the minimum infectious dose for cells in a monolayer, and constitutes a rapid and sensitive method for bioassaying virus titer in vectors, rather than depending on the development of symptoms in plants. The comparative infectivities of virus strains can be established in leafhopper cell monolayers.

With mycoplasmas and spiroplasmas it is a different story. No insect cell

lines have been infected with plant pathogenic spiroplasmas (47) or MLO (3), despite that Hirumi (17) and Quiot and co-workers (72) discovered insect cell lines inadvertently and unknowingly may be infected with mycoplasma. Hirumi (17) found, among 12 contaminated cell lines from three insect orders, that cultures of *A. constricta* and *M. fascifrons* were infected with *Acholeplasma laidlawii* (Sabin) and other related species of mammalian origin. He speculated that the source of infection was the bovine serum in the culture medium. Quiot and co-workers (72) believed the MLO that destroyed their cell cultures had been in the original insect (cockroach) tissues.

Giannotti and co-workers (15) maintained slightly broken tissues and organs of *E. plebejus* embryos in a few drops of culture medium resting on an agar base, and contaminated them with suspensions of clover phyllody MLO taken from infectious organs of adult leafhoppers or infected plants. A brei harvested from the embryo tissues 15 days later was injected into healthy clover where disease symptoms appeared.

Richardson and Jensen (73) inoculated MLO causing Western X-disease of peach into an established cell monolayer of the vector, *C. montanus*, but the MLO did not multiply. Attempts were made to protect the MLO during isolation and purification by adding sucrose to the cell culture medium (Chiu and Black), yet infectivity disappeared within a few days (64). Adding cells of the monolayer to the medium decreased its suitability with respect to maintaining infectivity (65).

The effect of sodium and potassium on the culture of MLO in cell-free medium was investigated by Caudwell and co-workers (6). These authors found that the yellows agent from *Vicia faba* or from infectious *E. plebejus* survived longer, possibly with some multiplication, in a medium with elevated potassium and lowered sodium, rather than in the inverse ratios. Although the effect of the cations was not discussed, McGarrity and co-workers (49) found that *Spiroplasma citri* Saglio and co-workers grew to high titers in the medium of Schneider's *Drosophila melanogaster* Meigen cell line (*Dm*-1), but not in the cells, which died. In *A. constricta* cell line (*Ac*-20), *S. citri* grew to high titers but not in the medium, which suggested that the *Ac*-20 cells elaborated a growth promoting factor for *S. citri*. Preliminary thin section electron micrographs indicated that spiroplasmas can be found intracellularly in both *Dm*-1 and *Ac*-20.

Steiner and McGarrity (80) deliberately infected the Drosophila cell line (*Dm*-1) cultured in a spiroplasma medium with *A. laidlawii* and *Mycoplasma hyorhinis* Switzer, then evaluated the available diagnostic procedures feasible for the detection of mycoplasmal infection. They found that *A. laidlawii* was infectious with a very small inoculum and multiplied at temperatures ranging from 22 to 37°C. The infection caused no cytopathology at 25°C, yet killed cells within 2 wk at 30°C. *M. hyorhinis*, on the other hand, established at 30°C but not 25°C. The use of mouse embryo fibroblast cell cultures, an indicator for mammalian cultures, is of unknown value for insect cell cultures; other recog-

nized detection methods are unreliable for insect cell cultures. The temperature of incubation, types of media, and characteristics of the insect cells are all expected to influence the growth and physiology of vertebrate mycoplasmas in insect cell culture. It is very likely that these or similar problems affect the ability to culture or detect plant MLO or spiroplasmas. The development of an efficient indicator cell culture for assay of insect cell cultures for mycoplasmal infections would contribute greatly to reliability and standardization of procedures (80). A cell culture assay would significantly speed up the diagnosis of infection in vectors.

8.6 SYMBIOSIS

With few exceptions (14), Homoptera are hosts to microorganisms that probably serve beneficial roles in every aspect of the insects' biology (4). Very little progress has been made in culturing microorganisms isolated from hosts, and therefore it is assumed that the two partners are mutually dependent. The term *symbiosis* is applied to the relationship in the sense of *mutualism*. The microsymbiotes are of various morphological kinds and are almost completely unclassified. Histological examinations of hundreds of homopteran species revealed that 4.3% of them have a single type of symbiote; 55.0% have two types; 30.5%, three types; 4.3% four types; 1.7%, five types; and 0.5%, six types (4). In some families of Homoptera the different types inhabit distinct cells, while in other Homoptera they may be found in both extracellular (hemocoele) and intracellular locations. When intracellular, the cells in which they reside usually are specialized fat body cells known as *mycetocytes*; a collection or body of mycetocytes, found in some species, is known as a *mycetome*. The symbiotes are transmitted from generation to generation by passing through the follicular epithelium of the posterior pole of oocytes still in the ovary and then infecting the developing embryo. The symbiotes multiply throughout nymphal life and are present in both sexes, but the number is always higher in females, and tends to decline with age in adults.

Considering the great number of variations in structure and possibly in function, searching for a common thread in homopteran symbiosis is a formidable task. A detailed review of the subject by Houk and Griffiths (25) presents facts known for aphids and leafhoppers, comparing these wherever possible with symbiosis in other species.

8.6.1 Leafhopper Symbiosis

Although over 436 species of leafhoppers have been examined (31), most of our information on leafhoppers derives from work done on the European little cicada, *E. plebejus*. The following brief summary was condensed from the 1980 review of Houk and Griffiths (25).

Ultrastructure and Life Cycle of *E. plebejus* Symbiotes. *E. plebejus* nymphs and adults contain two kinds of pleomorphic, prokaryotic microorganisms in a pair of abdominal mycetomes, each kind being restricted to a distinct region of the mycetome. The microorganisms, for want of a scientific classification, are designated as *a* and *t* symbiotes. Both of these symbiotes occur in two forms: a vegetative, non-dividing form in the mycetocytes and a transitory, infectious, dividing form that passes from adult mycetocytes through the follicular epithelium of the posterior pole of the egg and enters the periplasm. The infectious forms of *a* pass through an intermediate stage before entering the final mycetocytes of the embryo, while the infectious *t* forms enter the mycetocytes directly. The two forms remain segregated once they have entered the appropriate mycetocytes where they become invested with a membrane (M3) provided by the host tissue. Beneath M3 there are two other membranes representing the outer part of the cell wall (M2) and the cytoplasmic membrane (M1). Only the two forms of symbiotes that are infectious lack M3 at any time, and once they become invested with it, they retain it until the next cycle of egg penetration. Except for the very thin cell wall and the bizarre morphology, the symbiotes have internal organization and organelle structure characteristic of prokaryotes in general, bacteria in particular. Forms that divide do so by binary fission (without the formation of cross walls). The symbiotes have ribosomes that resemble the ribosomes of bacteria and have DNA strands with less than 40% guanine + cytosine, as in all other intracellular microorganisms. Lamellar bodies and paracrystalline arrays, thought to represent mesosomal structures, are present. However, there are variations in details in the two kinds of symbiotes. Freeze-fracture comparison of the structures of their inner membranes suggest different functions in osmoregulation. The mycetocytes, on the other hand, contain a complement of organelles typical of eukaryotic cells, such as a large, multilobate nucleus, rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, glycogen granules, mitochondria, and free and bound ribosomes.

Function of *E. plebejus* Symbiotes. Investigators have eliminated *E. plebejus* symbiotes without killing the hosts by injecting lysozyme, by treating eggs or nymphs with X-rays, or by removing, isolating, or displacing the symbiote ball in embryos with surgery, ligation, or centrifugation. All such treatments are followed by developmental delays and morphogenetic disruptions. Mortality is high in nymphs and fecundity is reduced in surviving adults. In spite of indications that several biochemical reactions are missing as a consequence of aposymbiosis, there is no conclusive evidence comparable to that obtained from aphids for amino acid and sterol synthesis, or vitamin conversion. Thus the functions of *E. plebejus* symbiotes remain unknown. This is largely due to the lack of a suitable *in vitro* culture system for biochemical studies with isolated symbiotes.

Other Leafhopper Symbiotes. *N. cincticeps* was studied by Mitsuhashi and Kono (55) who found that it also has *a* and *t* symbiotes, both infectious and

vegetative forms, similar to those in *E. plebejus*. The symbiotes occur in the cytoplasm of mycetocytes arranged in mycetomes in the fat body, and in the pedicels of ovaries where they infect the eggs. Attempts to culture the symbiotes were unsuccessful. In addition, rickettsia-like microorganisms were found in all tissues, including eyes and sperm! The rickettsia were present in every individual examined from three different prefectures, and there was no evidence of pathogenicity.

In *M. fascifrons* paired mycetomes, easily recognized in full-term embryos, persist throughout life (83). Both mycetomes have two components, the *t* organ and the surrounding *a* organ, and thus are structurally similar to the mycetomes of *E. plebejus* (30). The individual symbiotes, on the other hand, bear a greater resemblance to those of *Helochara communis* Fitch (7) or *N. cincticeps* (55). In their reactions to several stains and physiological solutions the two types of symbiotes differed (83). The *a* symbiotes were sensitive to the ionic composition of saline solutions used for dissection and examination, changing form and disintegrating within 20 min in solutions with a high Na+ content. In Kurtti's symbiote isolating medium with a Na:K ratio of 0.24 and osmotic pressure of 405 mOsm/kg, the symbiotes retained their appearance of twisted cylinders for a longer period of time. Thus these symbiotes seem to have little osmoregulatory capability. How this observation relates to function as derived from studies of membrane ultrastructure awaits additional investigation.

Wei (82) attempted to identify the function of *M. fascifrons* symbiotes by eliminating them. Leafhoppers were fed concentrations of chlortetracycline ranging from 1 to 100 ppm incorporated in synthetic diet. At high antibiotic levels, insect mortality was high and no reproduction occurred. At lower levels, hatching rate was poor and all progeny died. Histology of these moribund progeny gave the appearance of the normal complement of symbiotes, which in turn suggested that death was caused by toxic effects of the antibiotic carried over from the mother rather than by lack of symbiotes. Only at 1 ppm was there growth, development, and reproduction, but this was accompanied by a normal distribution of symbiotes to the progeny. Thus it was impossible to obtain aposymbiotic strains by feeding chlortetracycline because at the highest concentration that permitted survival the symbiotes were still present.

Kaiser (27) found two different types of symbiotes in the mycetomes of *Graphocephala coccinea* Forstier, distinguishable on the basis of morphology as well as ultrastructure, which were designated as *a* and *s* symbiotes. Two morphological forms of each symbiote, believed to be the infectious and vegetative forms, became recognizable in the mycetomes of the embryo just before hatching.

8.6.2 Planthopper Symbiosis

Three species of planthoppers, *L. striatellus*, *N. lugens*, and *S. furcifera*, were examined histologically by Chen and co-workers (9). Planthopper symbiosis does not resemble that of leafhoppers. There is no distinct body such as the mycetome. Rather the symbiotes are scattered throughout the abdominal fat

body with a tendency, in females, to clump in particular cells forming syncytia. As the insect ages, the number of symbiotes declines, especially in males. The symbiotes are eukaryotes with the form of yeasts. In electron micrographs of *P. maidis* embryos, the yeast-like characteristics of the symbiotes were noted (E. D. Ammar, personal communication).

The feature shared in common by leafhopper and planthopper symbiotes is their transovarial transmission. They enter the posterior pole of the egg through the epithelial plug and colonize the abdominal fat body of the late embryo (68). Yeasts of *N. lugens* and *L. striatellus* were quantified in eggs and embryos as well as in nymphs and adults (9, 67). The yeasts increased steadily with host development but the number of symbiotes per unit of fresh body weight remained nearly constant. Exposing first instars to 35°C for 3 days greatly reduced the yeast population, which was followed by poor growth of nymphs, a high rate of adult molting failure, and poor reproduction by surviving adults (8, 69).

Noda and co-workers (71) analyzed carcasses of heat-treated *L. striatellus* for sterols and found they contained smaller amounts of cholesterol and markedly reduced amounts of 24-methylenecholesterol compared to untreated insects. If heat-treated insects were fed on rice plant sheaths immersed in emulsions containing sterols, the percent of adult emergence was much improved (70). It was concluded that symbiotes play a vital role in sterol metabolism through synthesis of 24-methylenecholesterol. If ecdysone was topically applied to heat-treated insects at a concentration of 10^{-2} μg per insect, molting followed successfully in 3 or 4 days.

8.6.3 Culture of Symbiotes

There seem to be no reports of continuous culture of symbiotes of any leafhopper. Mitsuhashi and Maramorosch (62) initiated cell cultures of postembryonic stages of *M. fascifrons*, *D. maidis*, and *A. constricta* in MM medium where active bacteria were observed for a limited time in spherical envelopes and in the cytoplasm of a few cells. Even though the cells multiplied for a time, the symbiotes did not become established *in vitro*.

Mitsuhashi (53) cultured yeasts from symbiote balls of the planthopper, *L. striatellus*, in leafhopper tissue culture medium, MGM-401. The symbiote balls, isolated from surface-sterilized eggs, were cultured initially in liquid medium, then transferred to solidified medium where budding and mycelium formation resulted in the development of large colonies. Fungal, but not bacterial, antibiotics inhibited growth of the microorganisms. The cultures were not maintained for long. Grace's medium permitted Kusumi and co-workers (44) to isolate two forms of yeasts from this planthopper. The insect origin of the cultured yeasts was confirmed with immunological techniques. The yeasts differed in their sensitivity to high temperature and to cycloheximide (45). Applying the same cultural and serological techniques to *N. lugens*, Nasu and co-workers (66) discovered that these yeasts have a common antigenicity with those isolated from *L. striatellus* and *S. furcifera*, raising the

question of whether all rice planthoppers carry the same two immunologically distinct microorganisms. Yet Chen and co-workers (9) reported that the antibiotic and fungicidal sensitivities of the yeasts of *N. lugens* are not the same as those in *L. striatellus*, leaving their interspecific relationships unresolved.

Yeasts of *P. maidis* embryos were inoculated with the embryo cells into various modifications of the Chiu and Black leafhopper tissue culture medium, where they persisted for some weeks but failed to multiply (personal observation).

8.7 CONCLUSIONS

A few species of economically important leafhoppers and planthoppers can be reared continuously on completely defined synthetic liquid diets offered in Parafilm membranes. The ease with which these species adapt to rearing vessels of various design suggest that containment does not pose a problem for the insects—only for the experimenter. For other species that have not adapted to feeding on artificial diet, it is quite likely that essential phagostimulatory substances are lacking. The diets incorporate a complete array of amino acids, B vitamins, and other components, many of which probably are not required and may be inhibitory to feeding. Omission techniques with some species demonstrate that only a small number of amino acids are essential. Similarly little is known of the chemistry of oviposition stimulants. A search for molecules that promote feeding and oviposition, and an effort to simplify the diet, would seem to be desirable at this point.

It is possible to rear some species aseptically on either sterile plants or sterile synthetic diet. Tissues of the insects can then be used to inoculate cell cultures *in vitro*, expanding opportunities otherwise available only from the use of embryonated eggs.

The technique of insect tissue culture *in vitro* was developed with the hope of using this system to propagate and study plant viruses and mollicutes vectored by insects. Some success with virus infection has been achieved, but no plant pathogenic mollicute has yet been grown in cultured insect cells *in vitro*.

The variety of intracellular prokaryotes in leafhoppers and eukaryotes in planthoppers that live symbiotically with the insects is dazzling. It is probably because of the biochemical activities of the symbiotes that the insects have minimal nutritional requirements, yet proof is lacking. The prokaryotic microorganisms are refractory to culture and yeasts of only two species of planthoppers have been cultured. The latter have been implicated in the synthesis (or conversion) of a sterol precursor of ecdysone.

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LITERATURE CITED

1. B. L. Anderson, The effect of dietary iron on the biology of the aster leafhopper, *Macrosteles fascifrons* (Stål) (Homoptera: Cicadellidae). M.S. thesis, University of Minnesota, Minneapolis, 1984.
2. S. Balazy, J. J. Lipa, and W. Nowacka, An epizootic caused by the fungus *Zoophthora radicans* in an insectary rearing of *Aphrodes bicinctus* (Homoptera, Cicadoidea). *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **28**, 121–124 (1980).
3. E. E. Banttari and R. J. Zeyen, Interactions of mycoplasma-like organisms and viruses in dually infected leafhoppers, planthoppers and plants. In, *Leafhopper Vectors and Plant Disease Agents*, K. Maramorosch and K. F. Harris (eds.) Academic, New York, 1979, pp. 327–347.
4. P. Buchner, *Endosymbiosis of Animals with Plant Microorganisms* (revised English version), Wiley-Interscience, New York, 1965.
5. W. Carter, A technic for use with homopterous vectors of plant disease with special reference to the sugar-beet leafhopper, *Eutettix tenellus* Baker. *J. Agric. Res.* **34**, 449–451 (1927).
6. A. Caudwell, C. Kuszala, and J. Larrue, Sur la culture in vitro des agents infectieux responsables des jaunisses des plantes (MLO). *Ann. Phytopathol.* **6**, 173–189 (1974).
7. K. P. Chang and A. J. Musgrave, Multiple symbiosis in a leafhopper, *Helochara communis* Fitch (Cicadellidae: Homoptera): Envelopes, nucleoids and inclusions of the symbiotes. *J. Cell Sci.* **11**, 275–293 (1972).
8. C. C. Chen, L. L. Cheng, and R. F. Hou, Studies on the intracellular yeast-like symbiote in the brown planthopper, *Nilaparvata lugens* Stål. II. Effects of antibiotics and elevated temperature on the symbiotes and their host. *Z. Angew. Entomol.* **92**, 440–449 (1981).
9. C. C. Chen, L. L. Cheng, C. C. Kuan, and R. F. Hou, Studies on the intracellular yeast-like symbiote in the brown planthopper, *Nilaparvata lugens* Stål. I. Histological observations and population changes of the symbiote. *Z. Angew. Entomol.* **91**, 321–327 (1981).
10. R. J. Chiu and L. M. Black, Monolayer cultures of insect cell lines and their inoculation with a plant virus. *Nature Lond.* **215**, 1076–1078 (1967).
11. R. J. Chiu and L. M. Black, Assay of wound tumor virus by the fluorescent cell counting technique. *Virology* **37**, 667–677 (1969).
12. D. L. Dahlman, L. A. Schroeder, R. H. Tomhave, and E. T. Hibbs, Imbibition and survival response of the potato leafhopper, *Empoasca fabae*, to selected sugars in agar media. *Entomol. Exp. Appl.* **29**, 228–233 (1981).
13. P. Ehrhardt, Nachweis einer durch symbioteische Mikroorganismen bewirkten Sterinsynthese in künstlich ernährten Aphiden (Homoptera, Rhynchota, Insecta). *Experientia* **24**, 82–83 (1968).
14. M. J. Fletcher and D. T. Anderson, Unusual features in the embryonic development of *Scolypopa australis* (Walker) (Homoptera: Fulgoroidea: Ricaniidae). *Intern. J. Insect Morphol. Embryol.* **9**, 129–134 (1980).
15. J. Gianotti, J.-M. Quiot, W. Schwemmler, and C. Vago, Multiplication des mycoplasmes de la phyllodie en culture organotypique de l'insecte vecteur. *C. R. Acad. Agric. France* **56**, 59–62 (1970).
16. M. Hirata and K. Sōgawa, Antifeeding activity of chlordimeform for plant-sucking insects. *Appl. Entomol. Zool.* **11**, 94–99 (1976).
17. H. Hirumi, Viral, microbial, and extrinsic cell contamination of insect cell cultures. In, *Invertebrate Tissue Culture: Research Applications*, K. Maramorosch (ed.), Academic, New York, 1976, pp. 233–268.

18. R. F. Hou, Artificial rearing and nutrition of the aster leafhopper, *Macrosteles fascifrons* (Stål) (Homoptera: Cicadellidae). Ph.D. thesis, University of Minnesota, Minneapolis, 1976.

19. R. F. Hou, Artificial rearing of leafhoppers. *Bull. Soc. Entomol.* **11**, 27–33 (1976).

20. R. F. Hou, Dietary requirements for sugars in plant-sucking insects. *Bull. Soc. Entomol.* **12**, 47–53 (1977).

21. R. F. Hou and M. A. Brooks, Continuous rearing of the aster leafhopper, *Macrosteles fascifrons*, on a chemically defined diet. *J. Insect Physiol.* **21**, 1481–1483 (1975).

22. R. F. Hou and M. A. Brooks, Effects of cholesterol on growth and development of the aster leafhopper, *Macrosteles fascifrons* (Stål) (Hemiptera: Deltocephalidae). *Appl. Entomol. Zool.* **12**, 248–254 (1977).

23. R. F. Hou and M. A. Brooks, Trace metals especially Fe^{3+} as dietary factors for leafhoppers. *Experientia* **34**, 465–466 (1978).

24. R. F. Hou and L.-C. Lin, Artificial rearing of the rice green leafhopper, *Nephotettix cincticeps*, on a holidic diet. *Entomol. Exp. Appl.* **25**, 158–164 (1979).

25. E. J. Houk and G. W. Griffiths, Intracellular symbiotes of the Homoptera. *Annu. Rev. Entomol.* **25**, 161–187 (1980).

26. D.von Hülbert, and G. Schäller, Der Einfluss künstlicher Ehrnährung auf Entwicklung und Formenbildung bei *Euscelis plebejus* Fall. (Homoptera Auchenorrhyncha) unter verschiedenen photoperiodischen Bedingungen. *Zool. Jahrb. Syst.* **99**, 545–560 (1972).

27. B. Kaiser, Licht– und elektronenmikroskopische Untersuchung der Symbionten von *Graphocephala coccinea* Forstier (Homoptera: Jassidae). *Intern. J. Insect Morphol. Embryol.* **9**, 79–88 (1980).

28. M. Kim, H.-S. Koh, T. Ichikawa, H. Fukami, and S. Ishii, Antifeedant of barnyard grass against the brown planthopper, *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **10**, 116–122 (1975).

29. M. Kim, H. Koh, T. Obata, H. Fukami, and S. Ishii, Isolation and identification of *trans*-aconitic acid as the antifeedant in barnyard grass against the brown planthopper, *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **11**, 53–57 (1976).

30. H. K. Körner, Die embryonale Entwicklung der symbionten-führenden Organe von *Euscelis plebejus* Fall. (Homoptera: Cicadina). *Oecologia Berlin* **2**, 319–346 (1969).

31. H. K. Körner, On the host-symbiote-cycle of a leafhopper *(Euscelis plebejus)* endosymbiosis. *Experientia* **32**, 463–464 (1976).

32. K. Koyama, Choice experiments of *Inazuma dorsalis* (Hemiptera: Deltocephalidae) on some sugars. *Jpn. J. Appl. Entomol. Zool.* **15**, 269–271 (1971).

33. K. Koyama, Preference for color of diets in *Inazuma dorsalis* (Hemiptera: Deltocephalidae). *Jpn. J. Appl. Entomol. Zool.* **17**, 49–53 (1973).

34. K. Koyama, Rearing of *Inazuma dorsalis* and *Nephotettix cincticeps* on a synthetic diet. *Jpn. J. Appl. Entomol. Zool.* **17**, 163–166 (1973).

35. K. Koyama, Rearing of the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) on a synthetic diet. *Jpn. J. Appl. Entomol. Zool.* **23**, 39–40 (1979).

36. K. Koyama and J. Mitsuhashi, Essential amino acids for the growth of the smaller brown planthopper, *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **10**, 208–215 (1975).

37. K. Koyama and J. Mitsuhashi, Differences in oviposition of the smaller brown planthopper, *Laodelphax striatellus*, in response to various amino acid solutions (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **11**, 33–37 (1976).

38. K. Koyama and J. Mitsuhashi, Essential vitamins for the nymphal growth of the smaller brown planthopper, *Laodelphax striatellus*. *Jpn. J. Appl. Entomol. Zool.* **21**, 23–26 (1977).

39. K. Koyama and J. Mitsuhashi, Essential trace metals for the nymphal growth of the smaller brown planthopper, *Laodelphax striatellus* Fallén. *Jpn. J. Appl. Entomol. Zool.* **23**, 173–177 (1979).

40. K. Koyama and J. Mitsuhashi, Rearing of the white-backed planthopper, *Sogatella furcifera* Horvath (Hemiptera: Delphacidae) on a synthetic diet. *Jpn. J. Appl. Entomol. Zool.* **24**, 117–119 (1980).

41. K. Koyama and J. Mitsuhashi, Rearing of the planthoppers (Hemiptera: Delphacidae) on a synthetic diet under a short photoperiod. *Jpn. J. Appl. Entomol. Zool.* **27**, 69–71 (1983).

42. K. Koyama, J. Mitsuhashi, and S. Nasu, Rearing of the planthopper, *Sogatella longifurcifera* Esaki et Ishihara (Hemiptera: Delphacidae) on a synthetic diet. *Jpn. J. Appl. Entomol. Zool.* **25**, 198–200 (1981).

43. S. Kurata and K. Sōgawa, Sucking inhibitory action of aromatic amines for the rice plant- and leafhoppers (Homoptera: Delphacidae, Deltocephalidae). *Appl. Entomol. Zool.* **11**, 89–93 (1976).

44. T. Kusumi, Y. Suwa, H. Kita, and S. Nasu, Symbiotes of leafhoppers: I. The isolation of intracellular symbiotes from the smaller brown planthopper, *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **14**, 459–463 (1979).

45. T. Kusumi, Y. Suwa, H. Kita, and S. Nasu, Properties of intracellular symbiotes of the smaller brown planthopper *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **15**, 129–132 (1980).

46. K. Maramorosch, New techniques for the study of insect-transmitted plant viruses. In, *Proceedings of the Twelfth International Congress on Entomology*, 8–16 July 1964, London, 1965, pp. 712–713.

47. K. Maramorosch, Leafhopper tissue culture. In, *Leafhopper Vectors and Plant Disease Agents*, K. Maramorosch and K. F. Harris (eds.), Academic, New York, 1979, pp. 485–511.

48. G. Martinez-Lopez and L. M. Black, New medium and conditions for the culture of monolayers of Agallian leafhopper (*Agallia constricta* Van D.) cells used in studies of certain plant viruses. *Phytopathology* **64**, 1040–1041 (1974).

49. G. McGarrity, D. Phillips, T. Steiner, M. Garnier, and J. Bove, Spiroplasma (helical mycoplasma) infections of invertebrate cells in vitro. (Abstr.) *In Vitro* **20**, 242–243 (1984).

50. A. H. McIntosh, K. Maramorosch, and C. Rechtoris, Adaptation of an insect cell line *(Agallia constricta)* in a mammalian cell culture medium. *In Vitro* **8**, 375–378 (1973).

51. J. Mitsuhashi, Use of invertebrate cell culture for the study of plant viruses. In, *Invertebrate Tissue Culture*, Vol. 2, C. Vago (ed.), Academic, New York, 1972, pp. 343–360.

52. J. Mitsuhashi, Methods for rearing leafhoppers and planthoppers on artificial diets. *Rev. Plant Prot. Res.* **7**, 57–67 (1974).

53. J. Mitsuhashi, Cultivation of intracellular yeast-like organisms in the smaller brown planthopper, *Laodelphax striatellus* Fallén (Hemiptera, Delphacidae). *Appl. Entomol. Zool.* **10**, 243–245 (1975).

54. J. Mitsuhashi, Artificial rearing and aseptic rearing of leafhopper vectors: Applications in virus and MLO research. In, *Leafhopper Vectors and Plant Disease Agents*, K. Maramorosch and K. F. Harris (eds.), Academic, New York, 1979, pp. 369–412.

55. J. Mitsuhashi and Y. Kono, Intracellular microorganisms in the green rice leafhopper, *Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae). *Appl. Entomol. Zool.* **10**, 1–9 (1975).

56. J. Mitsuhashi and K. Koyama, Survival of smaller brown planthopper, *Laodelphax striatellus* Fallén, on carbohydrate solutions (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **4**, 185–193 (1969).

57. J. Mitsuhashi and K. Koyama, Rearing of planthoppers on a holidic diet (Delphacidae, Deltocephalidae). *Entomol. Exp. Appl.* **14**, 93–98 (1971).

58. J. Mitsuhashi and K. Koyama, Artificial rearing of the smaller brown planthopper, *Laodelphax striatellus* Fallén, with special reference to rearing conditions for the first-instar nymphs. *Jpn. J. Appl. Entomol. Zool.* **16**, 8–17 (1972).

59. J. Mitsuhashi and K. Koyama, Folic acid as a dietary factor affecting wing morph of the planthopper, *Laodelphax striatellus* (Hemiptera, Delphacidae). *Entomol. Exp. Appl.* **17**, 77–82 (1974).

60. J. Mitsuhashi and K. Koyama, Oviposition of smaller brown planthopper, *Laodelphax striatellus*, into various carbohydrate solutions through a Parafilm membrane (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **10**, 123–129 (1975).

61. J. Mitsuhashi and K. Maramorosch, Aseptic culture of four virus transmitting species of leafhopper (Cicadellidae). *Contrib. Boyce Thompson Inst.* **22**, 165–173 (1963).

62. J. Mitsuhashi and K. Maramorosch, Leafhopper tissue culture: Embryonic, nymphal, and imaginal tissues from aseptic insects. *Contrib. Boyce Thompson Inst.* **22**, 435–460 (1964).

63. T. E. Mittler and R. H. Dadd, Artificial feeding and rearing of the aphid, *Myzus persicae* Sulzer, on a completely defined synthetic diet. *Nature London* **195**, 404 (1962).

64. S. Nasu, D. D. Jensen, J. Richardson, R. J. Chiu, and L. M. Black, Extraction of Western X mycoplasmalike organism from leafhoppers and celery infected with peach Western X-disease. *Appl. Entomol. Zool.* **9**, 53–57 (1974).

65. S. Nasu, D. D. Jensen, J. Richardson, R. J. Chiu, and L. M. Black, Primary culturing of the Western X mycoplasmalike organism from *Colladonus montanus* leafhopper vectors. *Appl. Entomol. Zool.* **9**, 115–126 (1974).

66. S. Nasu, T. Kusumi, Y. Suwa, and H. Kita, Symbiotes of planthoppers: II. Isolation of intracellular symbiotic microorganisms from the brown planthopper, *Nilaparvata lugens* Stål, and immunological comparison of the symbiotes associated with rice planthoppers (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **16**, 88–93 (1981).

67. H. Noda, Preliminary histological observation and population dynamics of intracellular yeast-like symbiotes in the smaller brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **9**, 275–277 (1974).

68. H. Noda, Histological and histochemical observation of intracellular yeastlike symbiotes in the fat body of the smaller brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **12**, 134–141 (1977).

69. H. Noda and T. Saito, Effects of high temperature on the development of *Laodelphax striatellus* (Homoptera: Delphacidae) and on its intracellular yeastlike symbiotes. *Appl. Entomol. Zool.* **14**, 64–75 (1979).

70. H. Noda and T. Saito, The role of intracellular yeastlike symbiotes in the development of *Laodelphax striatellus* (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **14**, 453–458 (1979).

71. H. Noda, K. Wada, and T. Saito, Sterols in *Laodelphax striatellus* with special reference to the intracellular yeastlike symbiotes as a sterol source. *J. Insect Physiol.* **25**, 443–447 (1979).

72. J.-M. Quiot, J. Giannotti, and C. Vago, Infection and degeneration of primary cell cultures and cell lines of invertebrates by *Mycoplasma*. In, *Proceedings of the Third International Colloquium on Invertebrate Tissue Culture*, 22–25 June 1971, Smolenice near Bratislava. Slovak Academy of Science Bratislava, Czechoslovakia, 1973, pp. 467–475.

73. J. Richardson and D. D. Jensen, Tissue culture of monolayer cell lines of *Colladonus montanus* (Homoptera: Cicadellidae) a vector of the causal agent of Western X-disease of peach. *Ann. Entomol. Soc. Am.* **64**, 722–729 (1971).

74. T. Sakai and K. Sōgawa, Effects of nutrient compounds on sucking response of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). *Appl. Entomol. Zool.* **11**, 82–88 (1976).

75. S. Sekido and K. Sōgawa, Effects of salicylic acid on probing and oviposition of the rice plant-

and leafhoppers (Homoptera: Delphacidae and Deltocephalidae). *Appl. Entomol. Zool.* **11**, 75–81 (1976).

76. T. Senboku and E. Shikata, Embryonic tissue culture of *Nephotettix nigropictus* Stål and *Nilaparvata lugens* Stål. *Hokkaido Univ. Mem. Fac. Agric.* **12**, 101–108 (1980).

77. K. E. Sherman and K. Maramorosch, Feeding of the leafhopper *Macrosteles fascifrons* on an artificial diet containing the aster yellows spiroplasm. (Abstr.) *Proc. Am. Phytopathol. Soc.* **4**, 197 (1977).

78. K. Sōgawa, Studies on the feeding habits of the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) V. Probing stimulatory effect of rice flavonoid. *Appl. Entomol. Zool.* **11**, 160–164 (1976).

79. K. Sōgawa, The rice brown planthopper: Feeding physiology and host plant interactions. *Annu. Rev. Entomol.* **27**, 49–73 (1982).

80. T. Steiner and G. McGarrity, Mycoplasmal infection of insect cell cultures. *In Vitro* **19**, 672–682 (1983).

81. C. Vago and O. Flandre, Culture prolongée de tissus d'insectes et de vecteurs de maladies en coagulum plasmatique. *Ann. Epiphytes* **14** (new Series III), 127–139 (1963).

82. L.-Y. Wei, Symbiotes and nutrition of the aster leafhopper, *Macrosteles fascifrons* (Stål). M.S. thesis, University of Minnesota, Minneapolis, 1978.

83. L.-Y. Wei and M. A. Brooks, Intracellular symbiotes of the aster leafhopper, *Macrosteles fascifrons* (Stål). *Bull. Inst. Zool. Acad. Sinica*, **17**, 61–66 (1978).

84. L.-Y. Wei and M. A. Brooks, Aseptic rearing of the aster leafhoppers, *Macrosteles fascifrons* (Stål), on a chemically defined diet. *Experientia* **35**, 476–477 (1979).

85. I. Windsor, Clover clubleaf: A possible rickettsial disease of plants. Ph.D. thesis, University of Illinois, Urbana, 1972.