INDUCTION OF MAIZE WALLABY EAR DISEASE BY *CICADULINA BIMACULATA* AND ITS INCIDENTAL INFECTION BY LEAFHOPPER A VIRUS

by

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SUMMARY

1. Wallaby ear disease (WED) of maize has been shown to be phytotoxemia caused by the leafhopper, *Cicadulina bimaculata* and not a virus with reovirus-like particles as previously claimed by Grylls (1975). This conclusion is supported by experiments in which it was shown that: (a) insects free of leafhopper A virus (LAV), a member of the Reoviridae structurally similar to Fijivirus (Boccardo *et al.*, 1980) were capable of inducing WED just as efficiently as insects infected with the virus; and (b) plants at early stages of disease development recovered following the removal of infesting *C. bimaculata*.

2. The severity of WED in maize seedlings was shown to be dependent on a number of factors including: (a) age of the seedling at the time of infestation by *C. bimaculata*; (b) numbers of insects infesting the seedling; and (c) the duration of exposure of plants to insects. Maize seedlings which were infested by large numbers of *C. bimaculata* for prolonged periods and which became severely diseased were no longer able to recover following removal of the insects. However, the disease could reach such severity that the plants died.

3. LAV-infection of *C. bimaculata* was shown to have no significant effect on their time of nymphal development or their fecundity. However, virus infection did shorten the life span of the adult insects.

4. LAV was shown to be transmitted both vertically and horizontally in *C. bimaculata*. Vertical transmission was through eggs but not sperm. Although the virus could not be transmitted horizontally by contact between

insects or from virus contaminated surfaces, it could be acquired by *C. bimaculata* feeding on maize plants co-infested with LAV-infected leafhopper. However, the availability of the virus from plants failed to persist after the removal of the LAV-infected insects. These data support the following conclusions: (1) that LAV does not replicate in maize plant tissue as observed previously by Boccardo *et al.* (1980); and (2) that the maize plant can act as a non-persistence vector of LAV.

STATEMENT

This thesis contains no material which has been accepted for the award of any degree or diploma in any University and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

F. OFORI

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CHAPTER 1

GENERAL INTRODUCTION

1. Early report on maize wallaby ear disease (MWED)

Maize (*Zea mays* L.) wallaby ear disease was first reported from Queensland by Tryon (1910) and it was ascribed to adverse environmental conditions (Blackford, 1938; Simmond, 1939). Subsequently Schindler (1942) observed that the disease developed in maize plants on which *Cicadulina* (Cicadula) *bimaculata* (Evans) [Fig. 1A] had fed and suggested that it was caused by a leafhopper-borne virus. A similar disease observed in the Philippines was attributed to a toxic salivary secretion of the leafhopper, *Cicadulina bipunctella* (Mat.) (Maramorosch *et al.*, 1961). Agati and Calica (1949, 1950) had earlier incriminated *C. bipunctella* as a vector of a virus which infects maize and rice inducing galls on the leaves.

2. Previous work on the etiology of MWED

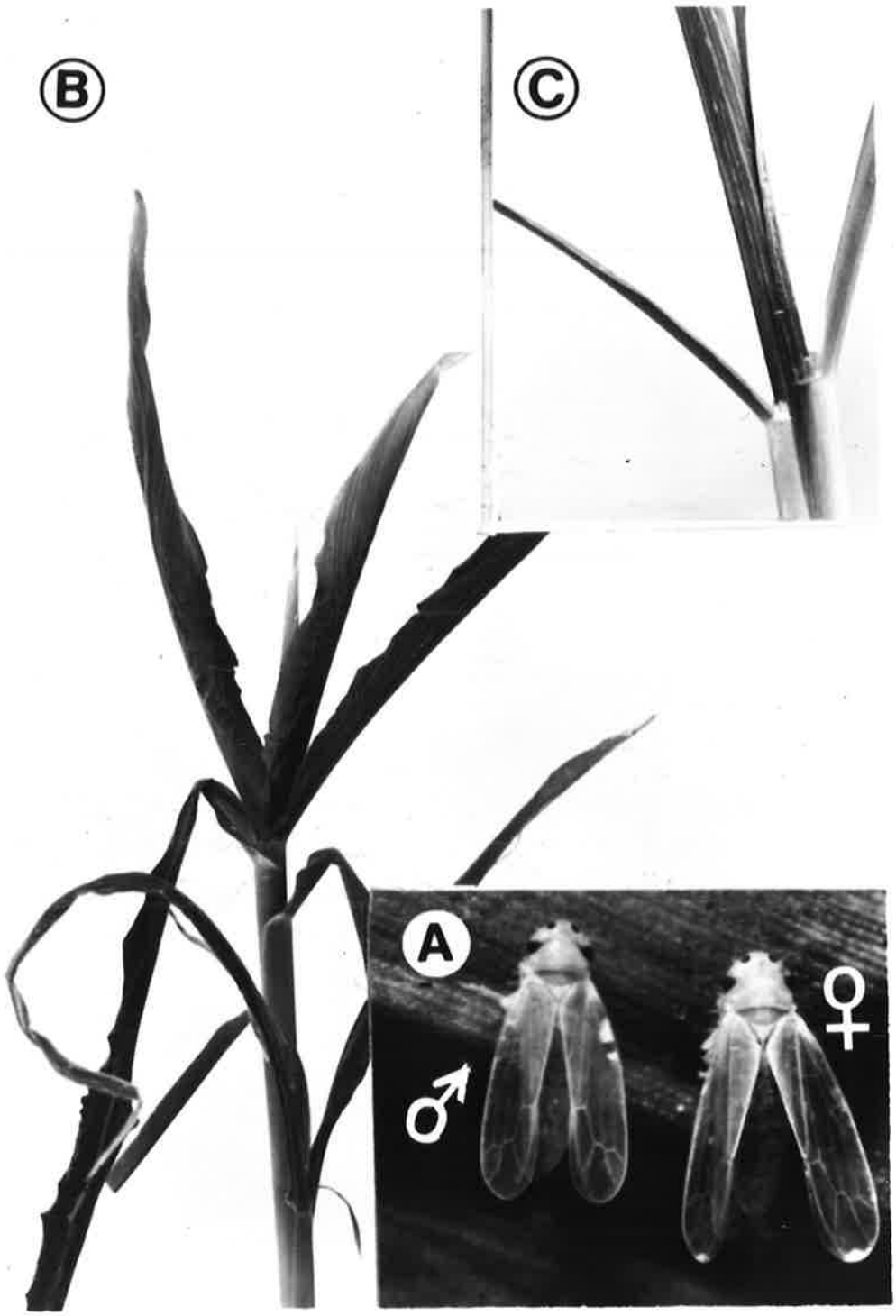
Grylls (1975) reported his extensive experimental results and concluded that MWED was caused by a plant virus transmitted by both *C. bimaculata* and *Nesoclutha pallida* (Evans). He observed that, *C. bimaculata* capable of inducing the disease carried reovirus-like particles about 85 nm in diameter with densely stained cores about 50 nm in diameter. Particles similar to the cores were also detected in partially purified extracts from maize plants showing wallaby ear disease symptoms. However, no such particles were detected in leafhoppers incapable of inducing disease symptoms in maize plants. Diseased plants had stiff erect leaves [Fig. 1B] which also developed galls on the under-

FIG. 1:

A. Adult *Cicadulina bimaculata*.
Length: Male 3.18 mm (S.E. = 0.019, n = 8),
Female 4.40 mm (S.E. = 0.013, n=32)
After Evans.

B. Wallaby ear diseased maize plant showing stiff erect leaves

C. Galls on the undersurface of a leaf along the veins of a wallaby ear diseased maize plant.


B
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surfaces. [Fig. 1C]. Affected plants were pre-disposed to other soil-borne fungal diseases and according to Grylls (1975) the host range of the virus included over a dozen species in the Gramineae.

Boccardo *et al*. (1980) confirmed the presence of reovirus-like particles in *C. bimaculata* capable of inducing wallaby ear disease in maize. The structure and size of the particles answered the description of that found by Grylls (1975) and it was shown that the particles contain double-stranded RNA(dsRNA) similar to that reported by Reddy *et al*. (1976). The RNA was resolved into 10 segments by polyacrylamide gel electrophoresis (PAGE) suggesting that the virus might belong to the genus Fijivirus, the members of which induce galls on their host plants (Matthews, 1982).

Structurally the reovirus-like particles detected in *C. bimaculata* appeared similar to those of Fijivirus but intact particles (IPs) detected in *C. bimaculata* reacted differently to some chemical and physical treatments from some members of Fijivirus (Boccardo *et al*., 1980). Histological studies by Hatta *et al*. (1982) also revealed that the anatomy of galls from maize plants affected by wallaby ear disease differed from those infected with Fijiviruses. In wallaby ear disease the vascular tissue was not involved in gall formation [Fig. 2; see also Fig. 3] and neither virus-like particles nor viroplasms were detected in any cells of infected plants. However, viroplasms have been observed in *C. bimaculata* infected with LAV (Boccardo *et al*., 1980).

In galls induced by members of the Fijivirus the vascular tissue is always involved in the gall formation and both virus-like particles and viroplasms are found in the vascular tissue (Teakle *et al*. 1969; Hatta *et al*., 1976; Giannotti *et al*., 1977; Hibino *et al*., 1979 and Milne, 1980).

FIG. 2: Transverse sections of vein from maize leaves as revealed by light microscopy.

A. Transverse section of a leaf vein on a healthy maize plants.

B. Transverse section of a vein through a gall on the leaf of a wallaby ear diseased plant.

(X, normal xylem, normal phloem (arrowed) gt, neoplastic cells).

Bar represents 10 µm

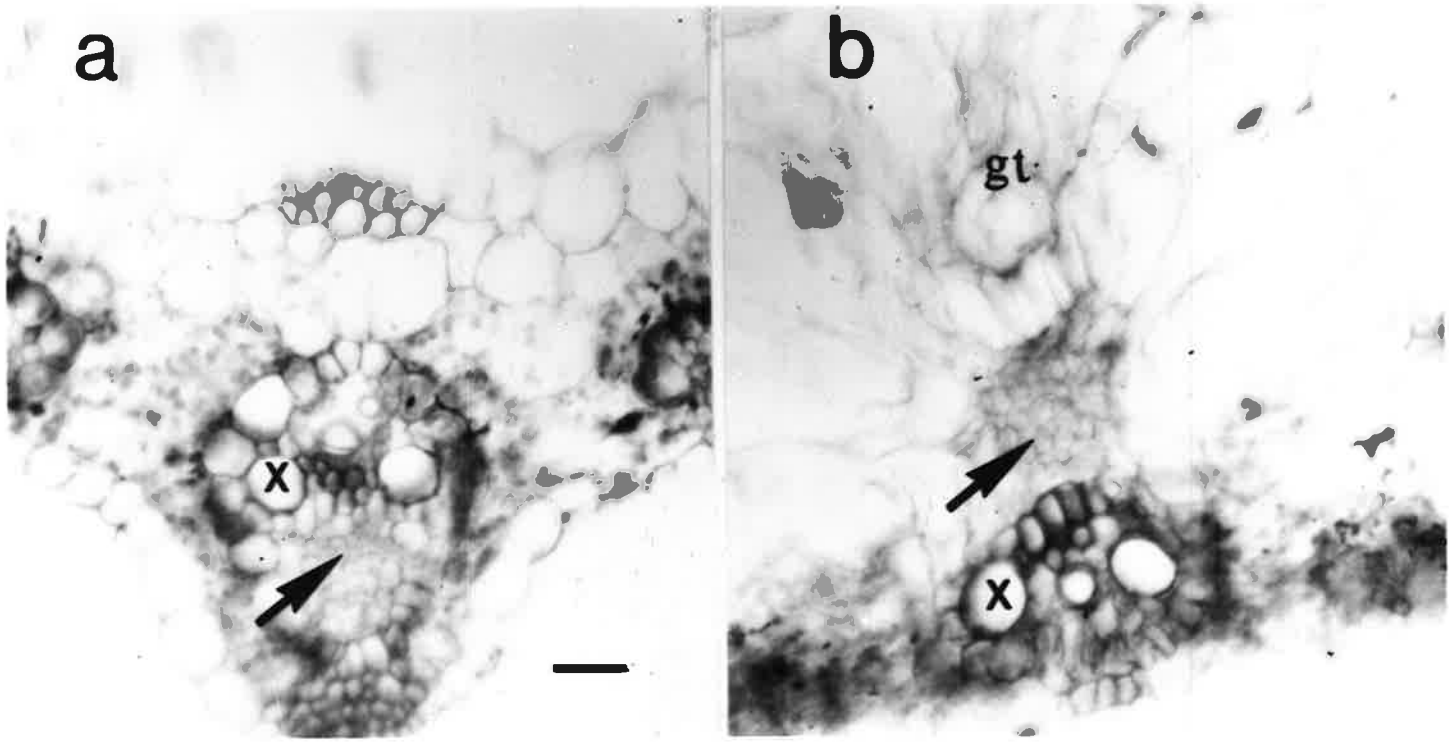

a
x
b
gt
x

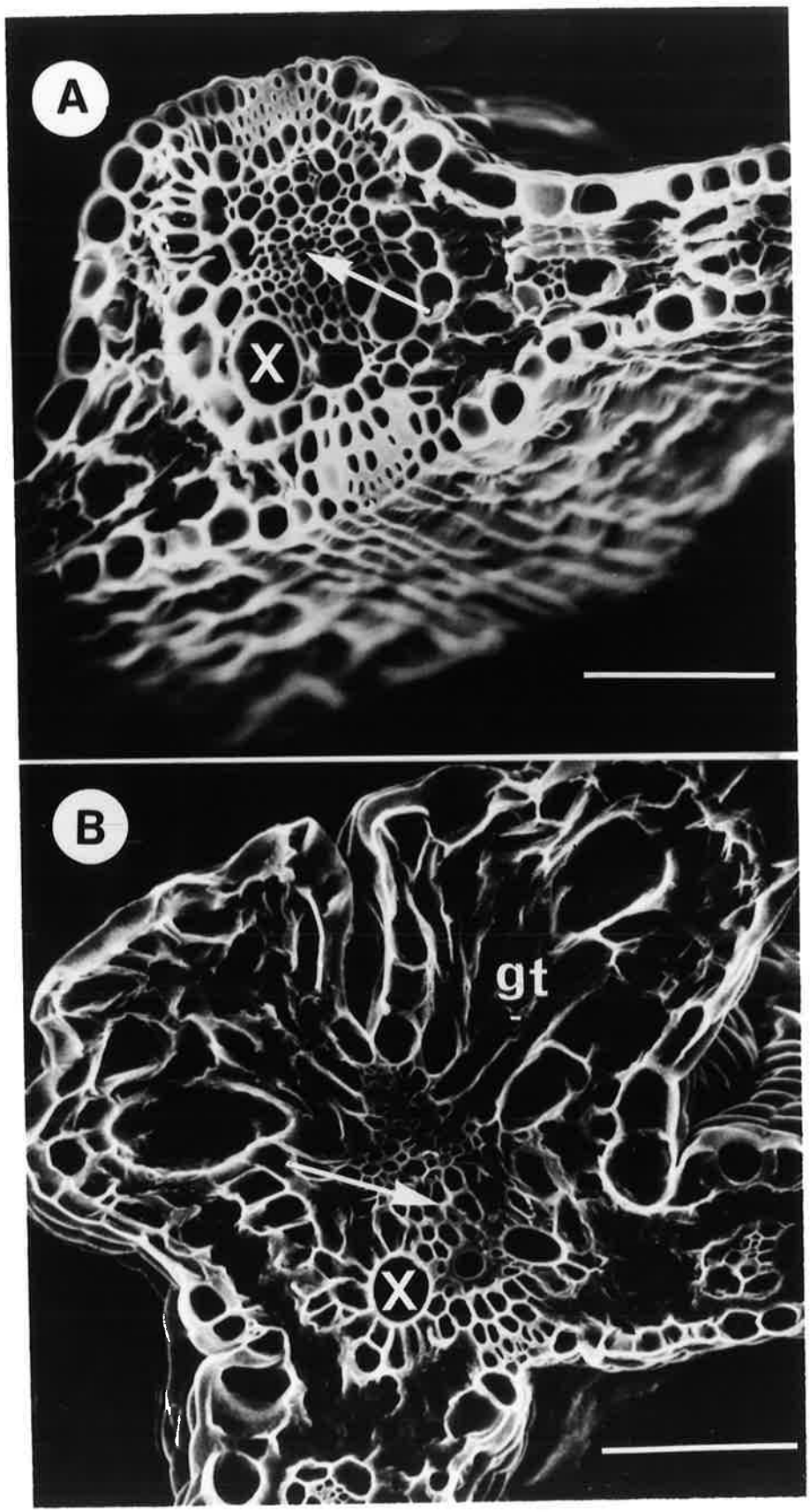
FIG. 3: Transverse sections of vein from maize leaves as revealed by scanning electron miscroscopy.

A. Transverse section of leaf vein on a healthy maize plant.

B. Transverse section of a vein through a gall on the leaf of a wallaby ear diseased plant.

(X, normal xylem, normal phloem (arrowed) gt, neoplastic cells).

Bar represents 100 µm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Despite the occurrence of the reovirus-like particles in *C. bimaculata* Boccardo *et al.* (1980) failed to find any evidence suggesting multiplication of the virus in diseased maize plants. They also observed that, symptom development was arrested and plants recovered from the disease after removal of the infesting insects suggesting that the disease was caused by an insect toxin rather than a plant virus infection. It was further observed that individual *C. bimaculata* carried three types of virus-like particles, two of which were detected only in leafhoppers capable of inducing the disease in maize. Since there was no evidence to suggest that any of the three types of particles detected in leafhoppers infected maize plants they were referred to as (i) Leafhopper A virus-like particles (LAV); (ii) Leafhopper B virus-like particles (LBV); and (iii) Leafhopper C virus-like particle (LCV). LAV which has reovirus-like particles was detected together with LCV, a rhabdovirus-like particle, in leafhoppers capable of inducing MWED. The third particle (LBV), a small polyhedral type was detected in both disease inducing and non-disease inducing *C. bimaculata*.

3. Plant virus infection and phytotoxemia

Distinction of systemic phytotoxemia caused by toxicogenic insects from plant virus infection is usually difficult to determine (Carter, 1973). According to Carter (1973) where only local lesions are involved in phytotoxemia, determination is not complicated. Usually a single insect will produce the lesion which is specific to the insect and no new lesions arise after removal of the insect from the host. However, where tissue malformations are involved, as in wallaby ear disease, the criteria for separation of phytotoxemia from plant virus infection may differ from that of local lesions. According to Carter (1973) a reasonable conclusion

may be reached by considering the following factors in phytotoxemia involving tissue malformation:

(a) In phytotoxemia the degree of injury to plants is related to the length of time and number of insects feeding. Such conditions do not apply to plant virus infection.

(b) In phytotoxemia, plants recover once the insects are removed but a disease can progress to the point of no return if the attack is sustained long enough. In plant virus infection plants are not known to recover.

4. Objectives of this Thesis

Before the project was started there were two schools of thought as to the etiology of MWED. Grylls (1975) concluded that the disease was caused by a virus transmitted by both *C. bimaculata* and *N. pallida* and Boccardo *et al.* (1980) on the other hand, observed that the reovirus-like particles did not multiply in the maize plant and that the disease was caused by an insect toxin probably secreted by leafhoppers as a result of LAV infection.

The question as to the cause of the disease was resolved by experiments reported in this thesis. It is shown that LAV is not involved in the etiology of maize wallaby ear disease (Ofori and Francki, 1983). The second part of the project deals with the effect of LAV an insect virus, on *C. bimaculata* and its mode of transmission from insect to insect.

CHAPTER II

GENERAL MATERIALS AND METHODS

1. Plant Materials and Culture

(a) *Plants maintained in glasshouse:*

Seeds of maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) were germinated in plastic pots and the seedlings were maintained in a glasshouse compartment isolated from insect colonies.

(b) *Cultivation of barley seedlings under asceptic conditions:*

Barley seeds were surface-sterilized in 1% Hyamine [50% (w/v) aqueous solution of quatenary ammonium chloride : Rohn and Hass Co., Philadelphia, USA] and rinsed thrice in sterile distilled water. Two seeds were germinated in each test tube on 3% sterile agar in Hoaglands nutrient solution. The seedlings were maintained at 25°C and continuous illumination of 3,800-4,300 lumens/m^2.

2. Establishment and maintenance of insect colonies

(a) *Culture of LAV-infected Cicadulina bimaculata:*

Impregnated females collected from the field (Queensland) were cultured on maize seedlings grown in 15 cm plastic pots in an isolated glasshouse compartment kept at 25-35°C. The colony was maintained continuously by replacing pots of old diseased plants with ones containing young vigorously growing seedlings. The insects were shown by immunoelectron microscopy to be infected with LAV as described later in the section dealing with virological techniques. The maize plants developed characteristic wallaby ear disease symptoms within 6-8 days of exposure to the insects.

(b) *Culture of LAV-free C. bimaculata*:

Cultures of LAV-free *C. bimaculata* were established from pairs of insects raised under aseptic conditions. Nymphs hatched from surface-sterilized eggs were raised to adults on barley seedlings grown under aseptic conditions. Males from each pair were retrieved seven days after pairing and the females after the emergence of their progeny. All parent insects were assayed for the presence of LAV by immunoelectron microscopy. Only colonies derived from both parents shown to be free from LAV were maintained further. Each colony was maintained in a large insect cage in a glasshouse compartment.

(c) *Culture of Nesoclutha pallida*:

Impregnated females collected from the field (Queensland) were cultured on Rhodes grass (*Chloris gayana* L.) grown in 15 cm plastic pots placed in a large wooden cage. The colony was maintained under glasshouse conditions. Old plants in the cage were periodically replaced by young vigorous growing ones. The colony was shown to be free from LAV by immunoelectron microscopy of concentrated insect extracts.

(d) *Culture of mixed colonies of C. bimaculata and N. pallida on barley plants*:

Neither maize nor Rhodes grass on which *C. bimaculata* and *N. pallida* were cultured respectively, could serve as a suitable common host plant for the two species of leafhoppers. *C. bimaculata* adults transferred onto Rhodes grass failed to reproduce and the insects died out in about two weeks. A similar result was observed when *N. pallida* was transferred to maize plants. However, barley plants served as an acceptable common host plant to both *C. bimaculata* and *N. pallida*.

Impregnated females of both species of leafhoppers were pooled and raised on barley seedlings placed in a large wooden cage. Fifty percent of the insects of the donor *C. bimaculata* colony were shown to be LAV-infected whereas no LAV was detected in insects of the *N. pallida* colony. The mixed colony was maintained for four months and the barley seedlings supporting the insects developed typical wallaby ear disease symptoms. The plants were stunted and developed galls.

3. Manipulation of Insects

(a) *Collection and Storage of Insects:*

Bulk collection of insects for LAV extraction was done with a suction pump device [Fig. 4] and the insects were either immediately used for virus extraction or stored at -20°C until required. Adult insects and nymphs for other experimental work were collected into vials with a camel brush which ensured minimum injury to the insects.

(b) *Immobilization of insects for easy handling:*

Lower temperatures were used to immbolize insects for easy handling. Single or groups of insects collected into vials were cooled at 4°C for 20-30 mins and the vials were then kept in an ice bucket for 3-5 mins. Insects immobilized in this manner regained their normal activity after 1-2 mins after transfer to a temperature of about 25°C.

(c) *Collection and hatching of C.bimaculata eggs:*

Pieces of infested maize leaves were teased in water under a binocular dissecting microscope. The eggs were usually lodged

FIG. 4: A suction pump and insect chamber for bulk aspiration of leafhoppers from infested plants.

along the mid-rib [Fig. 5A; see also Fig. 5B]. Eggs encountered in leaf tissue were of three kinds, newly laid eggs were flexible and without shells, those that were at an intermediate stage of development had hard outer shells but no eye spots. Well developed eggs had clearly defined eye spots and these were selected for hatching.

Another method used in obtaining eggs at a similar stage of embryonic development was to cage groups of impregnated females on a single maize leaf for 24 hours. The mother insects were then released and the plants kept at 25°C for 9-10 days for the eggs to mature. They were then recovered by teasing the leaf tissue as described above. Mature eggs obtained in this manner hatched within 1-2 days when placed on moist filter paper.

Surface-sterilized eggs in 1% Hyamine followed by two rinses in sterile distilled water, were hatched singly or in bulk on moist filter paper. Single eggs were hatched on filter paper strips set in 3% aqueous agar in vials [Fig. 5C(a)]. For bulk hatching, eggs were placed on moist filter paper among barley seedlings grown in test tubes on 3% aqueous agar [Fig. 5C(b)]. Eggs were incubated at 25°C and the vials and test tubes were laid on the sides. Mature eggs which failed to hatch eventually turned black indicating death of the embryo. When eggs were hatched on filter paper among barley seedlings, the first instars crawled onto seedlings thus eliminating hazards associated with transferring newly hatched nymphs to host plants. Nymphs raised to adults under aseptic conditions were transferred to barley seedlings cultivated in large test tubes [Fig. 6A). Hatching of eggs by placing them directly on aqueous agar in vials was also attempted but was abandoned because of a high mortality of newly hatched nymphs in the moist film on the agar.

FIG. 5:

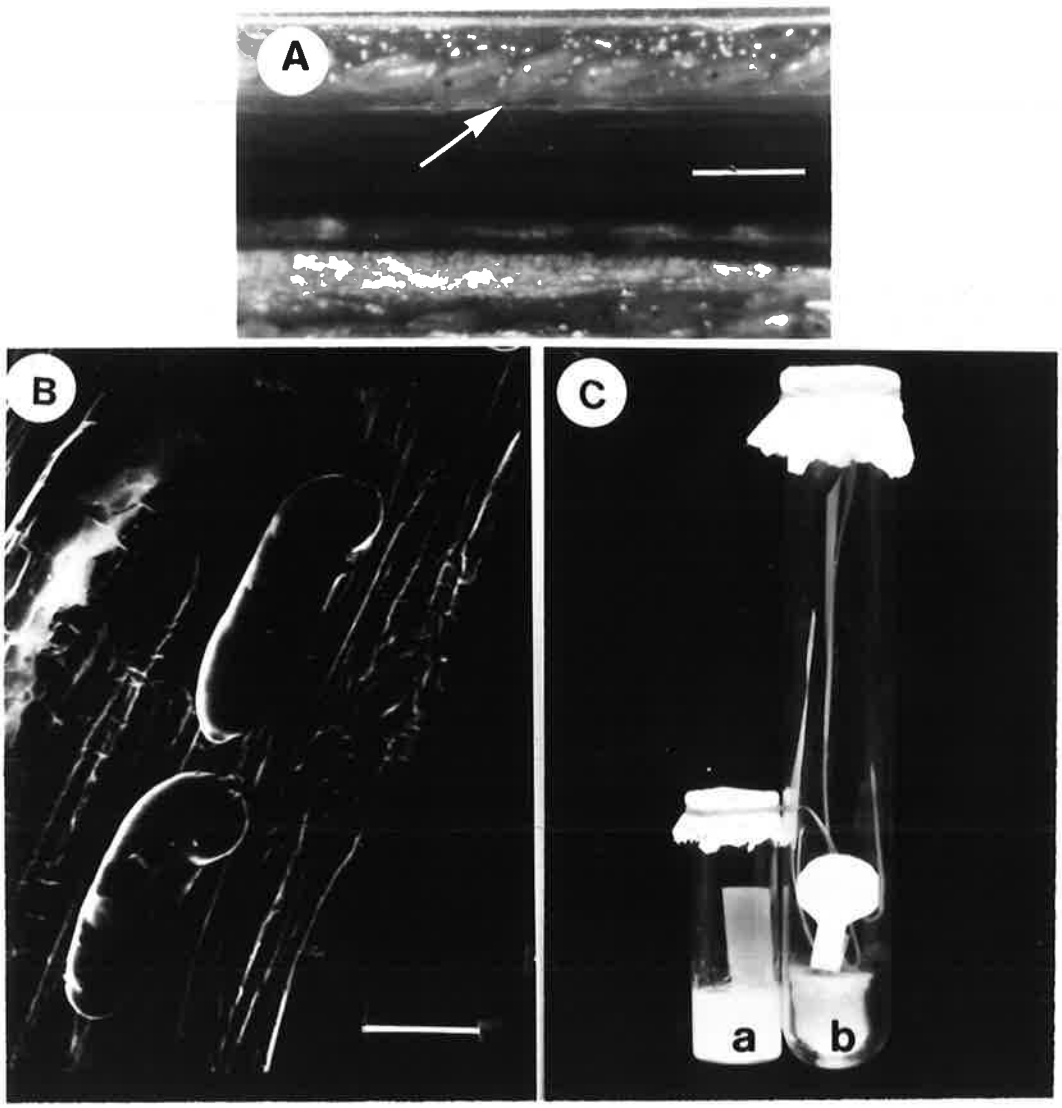
A. Epidermis and part of the mesophyll layer removed to expose *C. bimaculata* eggs lodged along the mid-rib of a maize leaf.
Bar represents 1 mm.

B. Mature eggs of *C. bimaculata* lodged in leaf tissue as revealed by scanning electron microscopy.
Bar represents 250 µm.

C. Two different methods for hatching eggs of *C. bimaculata*.

a) Small tube (2.5 cm x 7 cm) for hatching of a single egg. (After hatching the first instars were transferred to host plants).

b) Large tubes (3.5 cm x 20 cm) for bulk hatching of eggs. (After hatching the first instars crawled onto barley seedlings grown in the tubes on aqueous agar).


A
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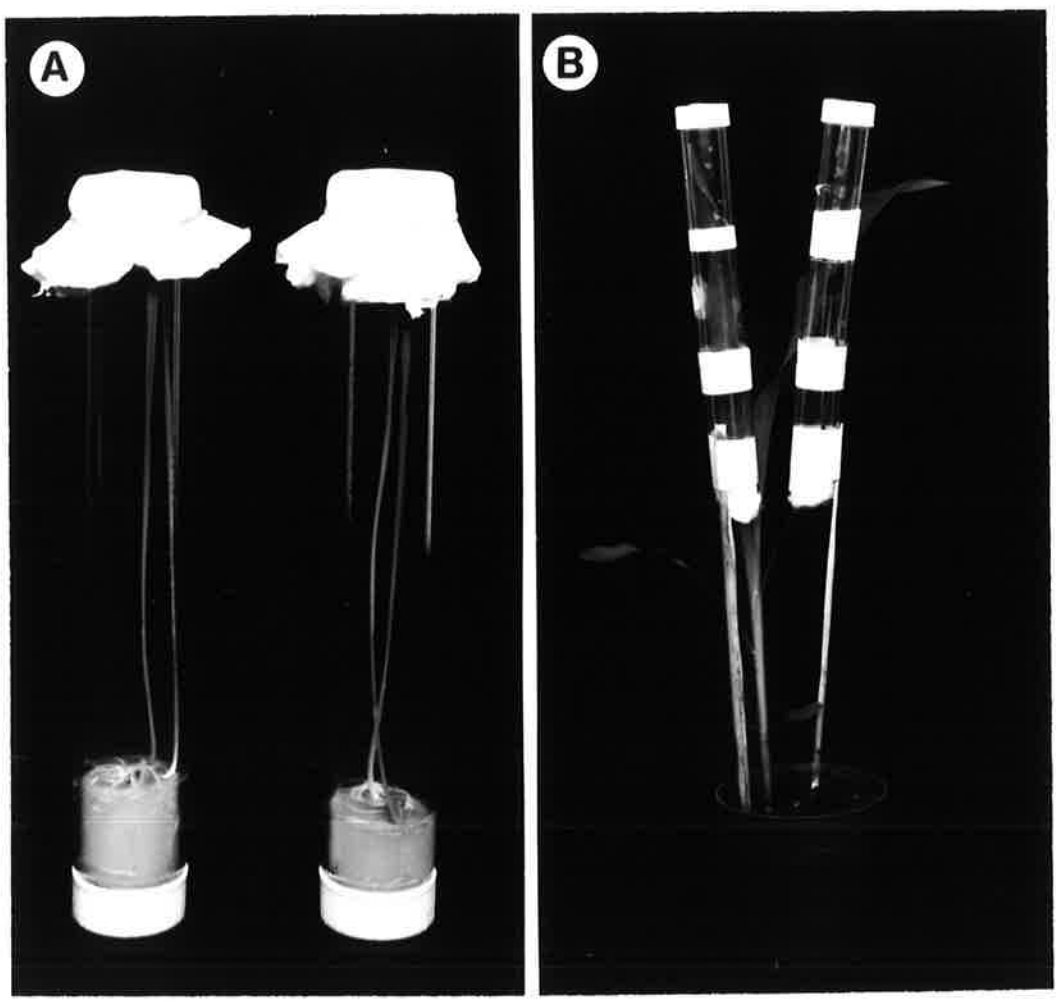
C
a
b

FIG. 6:

A. Tubes used for rearing *C. bimaculata* on barley seedldings under aseptic condition.

B. Tube assemblies used for caging ovipositing *C. bimaculata* on single leaves of maize seedling for egg collection.


A
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(d) *Collection of first instar nymphs of C. bimaculata:*

Groups of 10-20 impregnated females caged on a single leaf of maize plant grown in pots were removed after 24-48 hours [Fig. 6B]. The plants were maintained at 25-35°C in a glasshouse compartment away from other insect colonies. First instar nymphs emerged 10-12 days later. Nymphs that emerged on each day were collected into test tubes by inserting the leaves with the nymphs into the tube and brushing off the nymphs from the leaf surface with a camel hair brush. Pieces of maize leaf were put in the test tubes for the nymphs to feed on. The tubes were plugged with cotton wool wrapped in Kleenex paper tissue and maintained at 25°C under humid conditions. The mymphs were either transferred in bulk onto maize plants in pots at the 2nd instar stage or raised singly on barley seedlings grown in test tubes on agar medium.

(e) *Collection of virgin females and newly emerged males:*

Nymphs during their last instar which had wing pads were raised singly in vials containing pieces of maize leaf and kept at 25°C. In order to prevent early drying of the leaf tissue the vials were put in plastic bags containing wet cotton wool. The vials were examined each day and the emerged adults were sexed and males and females caged separately on maize seedlings grown in 15 cm pots.

(f) *Identification of insects at various stages of development and their sexing:*

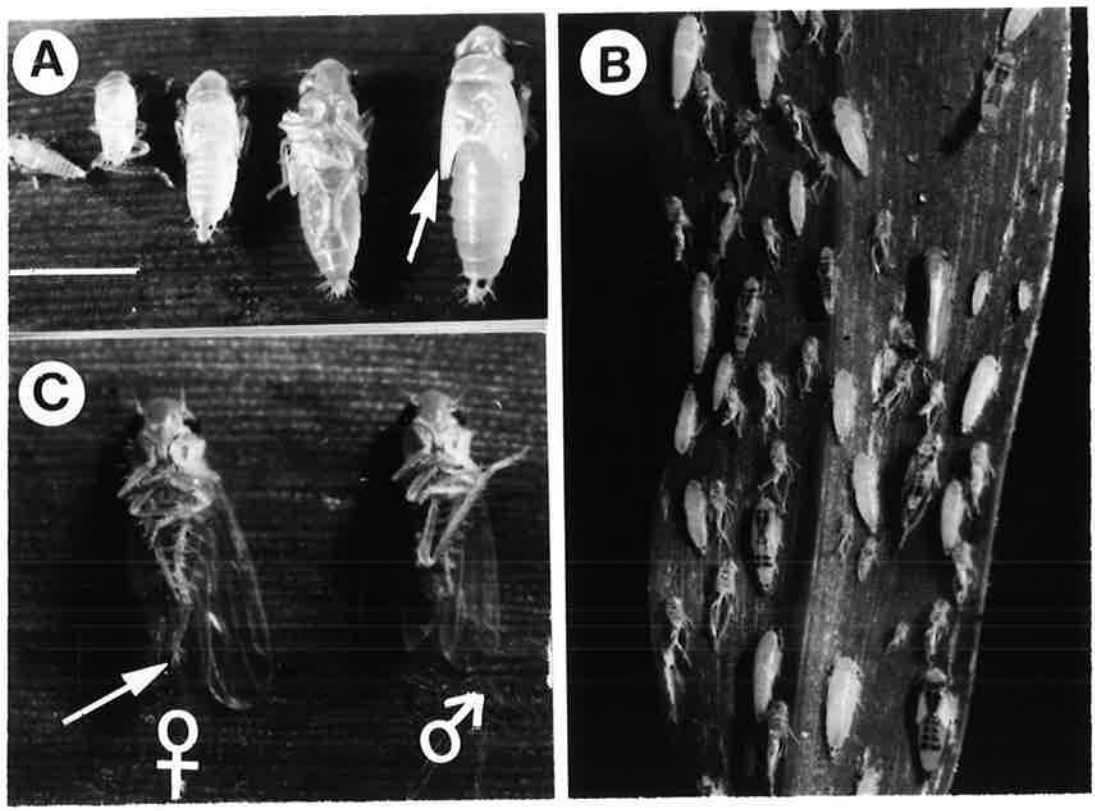
C. bimaculata went through five nymphal stages before emerging as adults [Fig. 7A]. The nymphs were mostly found on the under surfaces of maize leaves [Fig. 7B]. Fifth instar nymphs were easily recognised by the presence of wing pads. Newly emerged

FIG. 7:

A. The five nymphal stages of *C. bimaculata* showing from left to right 1st, 2nd, 3rd, 4th and 5th instar nymphs (5th instar nymph has wing pads shown by arrow). Bar represents 1 mm.

B. Infestation of a maize leaf by *C. bimaculata*.

C. Ventral view of a male and female adult *C. bimaculata*. (Female ovipositor arrowed).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adults had limited flight ability until the wings were fully expanded. *C. bimaculata* could not be sexed until they were adults when the male and female genitalia could be identified under a binocular dissecting microscope. Sex prediction was however done when nymphs were in their fifth instar from the body size and the curvature of the distal end of the abdomen. Individuals with smaller bodies usually emerged as adult males and those with bigger bodies as females. Male nymphs usually had a more pointed last abdominal segment than female nymphs.

The main distinguishing characteristic employed in separating adult females from males was the presence or absence of the ovipositor in adult insects [Fig. 7C, see also Fig. 8]. The ovipositor of newly emerged female has a hyaline appearance and heavily sclerotised tip. The male pygofer terminates in four pointed spines (Evans, 1940).

(g) *Feeding of C. bimaculata and N. pallida on a synthetic diet containing LAV:*

Second and third instar nymphs of both species of leafhoppers from LAV-free colonies were fed on partially purified LAV preparations in 5% sucrose. The diet was sandwiched between two layers of parafilm membrane secured at the top of a feeding chamber constructed from plastic tube 15 mm in diameter [Fig. 9]. Nymphs were immobilized by cooling and transferred to filter paper and the open end of the feeding chamber was placed over the insects. The feeding insects were maintained at 25°C and continuous illumination of 3,800-4,300 lumens/m^2. Insects were allowed 48 hours feeding time and were either all transferred onto caged maize seedling or raised singly on barley seedlings grown on aqueous agar medium in test-tubes.

FIG. 8: Male and female genitalia as revealed by scanning electron microscopy showing:

A. Female ovipositor extending over the entire length of the last abdominal segment.
Bar represents 100 µm.

B. Ventral view of the abdomen of a male insect showing two of the four spines at the end of the pygofer.
Bar represents 100 µm.

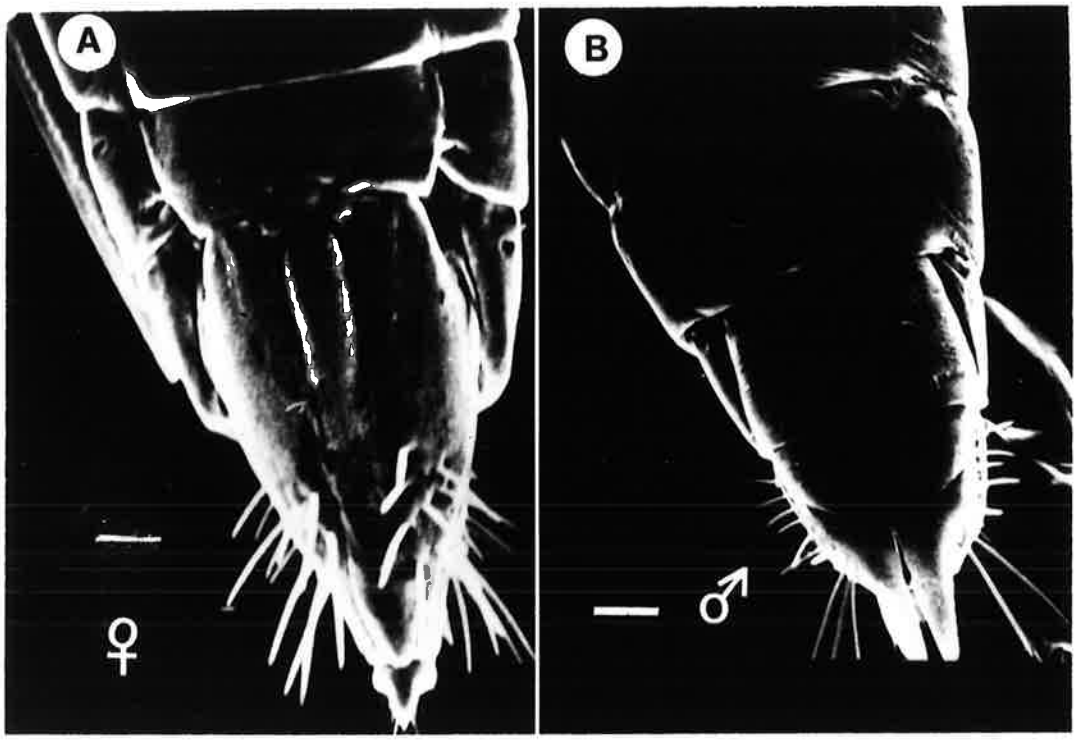
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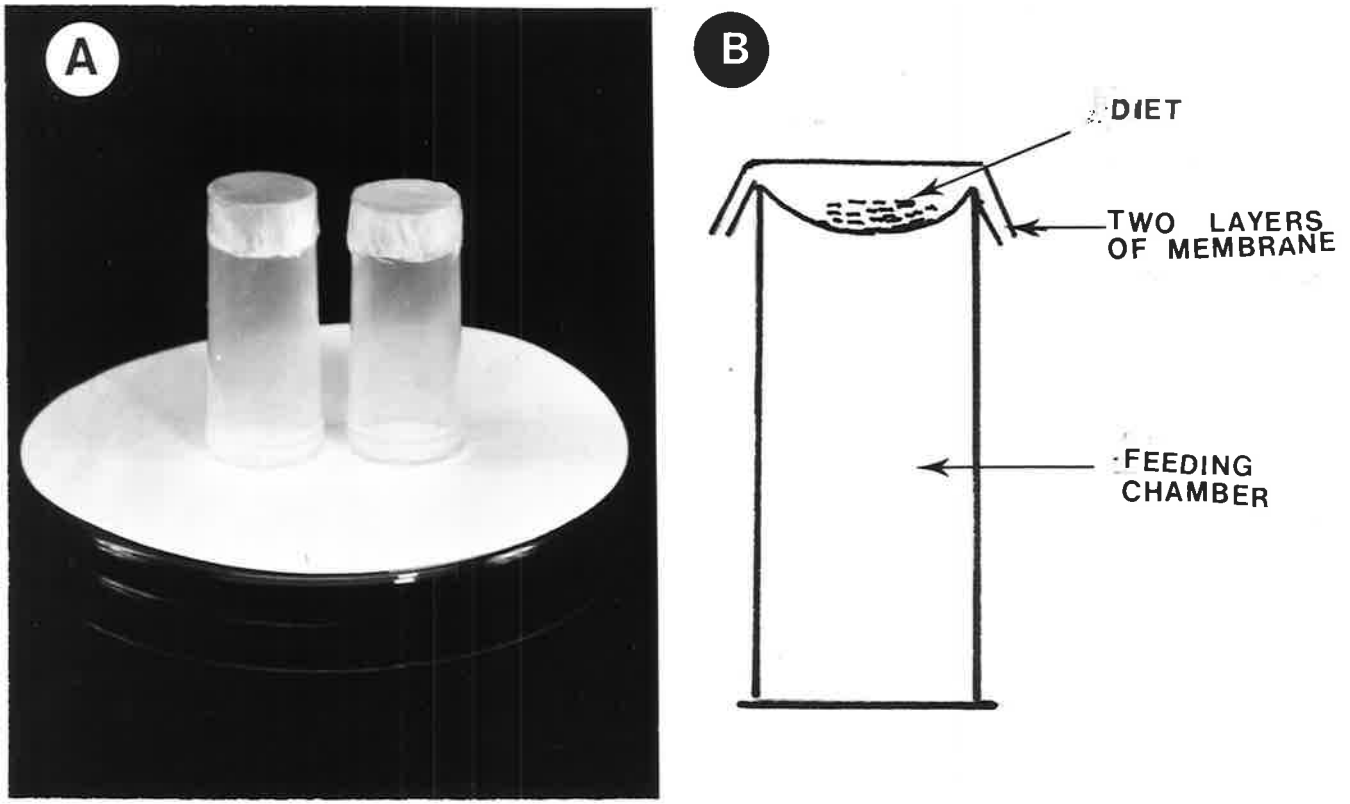
FIG. 9:

A. Chamber for feeding leafhopper nymphs on synthetic diets containing LAV.

B. Diagramatic representation of Chamber.


A
B
DIET
TWO LAYERS OF MEMBRANE
FEEDING CHAMBER

4. Virological Techniques

4.1 *DETECTION OF LAV BY IMMUNOELECTRON MICROSCOPY (IEM)*

The method for detecting LAV was similar to the Derrick (1973) particle trapping technique. This was done with extracts from single or groups of 10-20 adult insects.

(a) *Preparation of insect extract:*

All operations were done at 0-4°C in 0.16M phosphate buffer, pH 7.6 containing 5mM ethlenediaminetetraacetate (EDTA) [PE buffer]. Adult inscts killed by freezing at -20°C were either stored at that temperature or were immediately used for LAV extraction. Single insects were ground in 20-30 µl of the PE buffer on microscope slides with the scratched surface of a pestle constructed from a glass rod. Extracts from single insects were not clarified. Batches of 10-20 insects were ground in 100-150 µl of PE buffer with pestle and mortar and the extract transferred into 1.5 ml Eppendorf tubes and clarified by centrifugation at 2,500g for 5 min.

(b) *Coating of grids with antiserum:*

Specimen grids (400 mesh) coated with formvar and carbon were ionised by glow discharge and floated on drops of anti-LAV serum (Boccardo *et al.*,1980) diluted 1:100 with 10 mM phosphate buffer, pH 7.0. After 5 min, excess liquid was removed from the grids with filter paper and they were rinsed twice with distilled water and the excess water removed with filter paper.

(c) *Attachment of LAV particles to antiserum coated grids:*

Drops of extract were placed on a parafilm membrane in a Petri

dish containing moist filter paper. The antiserum coated grids were floated face down on the drops of extract for 20-30 mins at 25°C. Excess extract was removed from the grids with filter paper and the grids were rinsed twice with distilled water and dried with filter paper. They were negatively stained with a drop of aqueous 2% uranyl acetate for 30s and the excess stain was removed with filter paper. After drying the grids were examined in a JEM 100CX electron microscope.

4.2 *PURIFICATION OF LAV*

(a) *Partial purification of LAV from insects extract:*

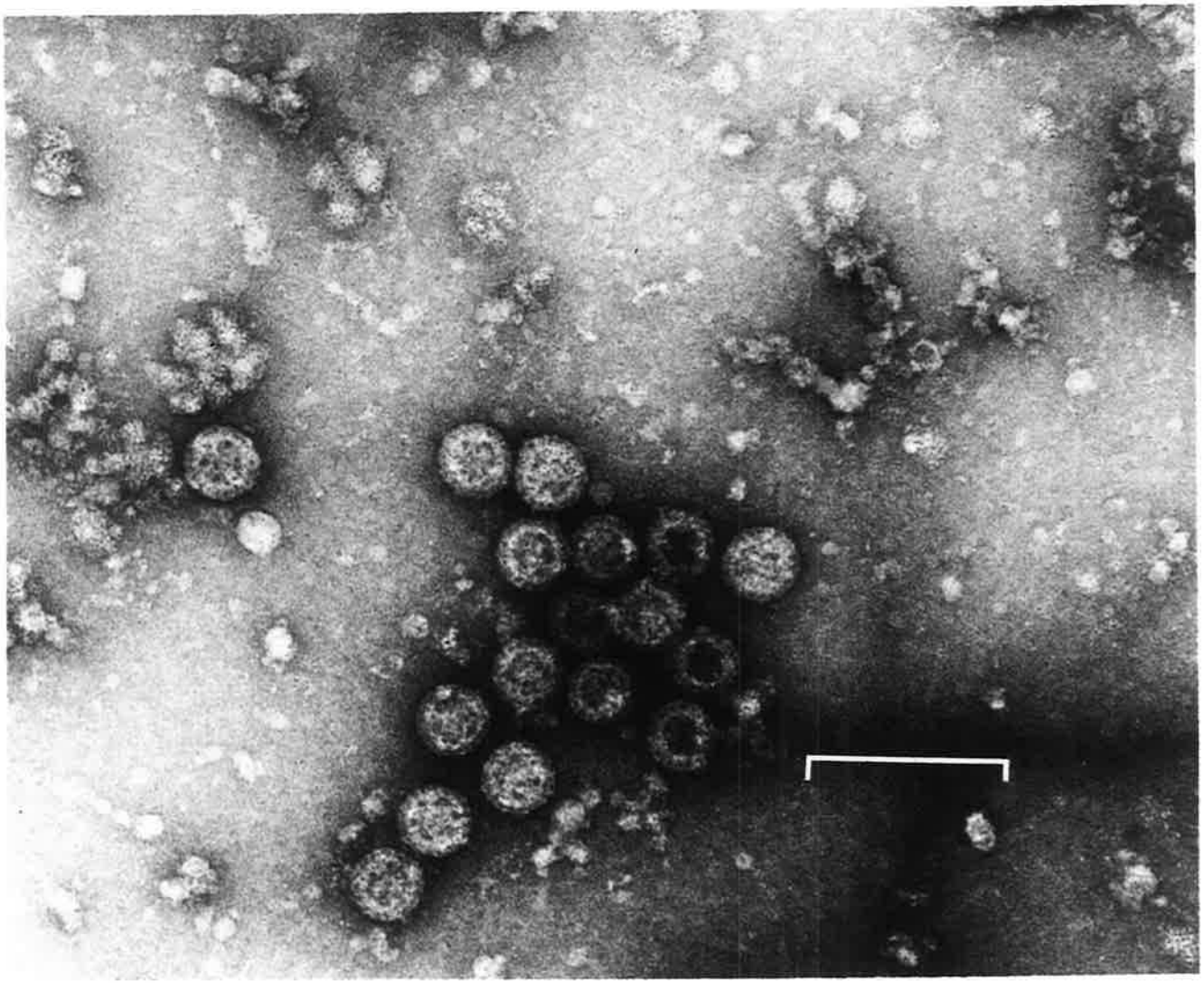
This was done by the method of Boccardo *et al.* (1980). Frozen or freshly collected insects (0.5 - 5g) were ground with pestle and mortar in 8-10 volumes (w/v) of PE Buffer and strained through a double layer of cheesecloth. Nonidet P40 was added dropwise to a final concentration of 3% while stirring and kept for 30 min at 4°C. The extract was centrifuged at 4,000g for 10 min. and the supernatant was layered over 2.5 ml of 30% sucrose cushion in PE buffer in a 12 ml plastic tube and centrifuged for 30 min. at 250,000g. The pellet was resuspended in PE buffer and the preparation centrifuged at 4,000g for 10 min. The supernatant was again centrifuged through a 2.5 ml sucrose cushion to yield a preparation of partially purified LAV particles [Fig. 10].

(b) *Detection of LAV after partial purification from insects extract:*

The pellet formed after the second centrifugation at 250,000g was resuspended in 20 µl of PE buffer and the LAV particles trapped in the same manner as described in Section 4.1(c).

FIG. 10: Intact particles (IPs) of LAV partially purified from insects (*C. bimaculata*) extract.

Bar represents 200 nm.



CHAPTER III

INDUCTION OF MAIZE WALLABY EAR DISEASE (MWED) BY *CICADULINA BIMACULATA* FREE OF LAV

Grylls (1975) selected a colony of *C. bimaculata* which could not induce wallaby ear disease symptoms in maize plants and the colony did not carry the reovirus-like particles detected in colonies which induced the disease. The absence of the virus in the non-symptom inducing colony was taken as evidence that wallaby ear disease was caused by a virus transmitted by the leafhopper *C. bimaculata*. In experiments described below a different approach was adopted to determine whether the reovirus-like particles (LAV) detected in *C. bimaculata* was involved in the etiology of wallaby ear disease.

Experimental

Five LAV-free clones were established as described in Section 2(b) (Chapter II). Concentrated extracts of insects from all five clones were periodically assayed for virus. About 1-2g of insects from each clone was used in every assay and each of the five clones was assayed thrice over a period of 1 yr. Insects from all five clones induced wallaby ear disease in maize plants on which they were maintained but no LAV particles were detected in concentrated extracts from the insects from any of the clones. However, numerous LAV particles were detected in all control preparations from LAV-infected glasshouse population of *C. bimaculata*.

Insects of all five LAV-free clones reproduced at a slower rate than the LAV-infected *C. bimaculata* maintained on maize in a glasshouse. However, an interbred colony established from insects from two of the most vigorous of the five clones reproduced as fast as the LAV-infected insects

from the glasshouse population of leafhoppers. The interbred colony which also induced disease symptoms in maize plants was maintained throughout the period of this project. Concentrated extracts of insects from this colony were periodically assayed and shown to be free of LAV. The colony was used in all subsequent experiments as a source of virus-free insects.

In another series of experiments, impregnated females from the LAV-infected colony maintained on maize in a glasshouse were each caged on a maize seedling grown in a separate pot. The plants were maintained at 25°C and continuous illumination of 3800-4300 lumens/m^2. The plants were observed for disease development and the emergence of nymphs. The experiment was terminated after 2 months when all progeny insects were collected from each plant and counted. Extracts from all the insects collected from each plant were concentrated and assayed by immunoelectron microscopy for LAV.

TABLE 1: Induction of wallaby ear disease and the presence of LAV in *C. bimaculata* colonies derived from single impregnated females from a LAV-infected colony.

Number of Colonies	Number of Colonies with LAV	Time to emergence of first nymphs (days)	Time to appearance of symptoms on maize plants (days)	Number of insects in colony after 2 months
Expt. 1				
5	5	13.8±0.20*	22.0±0.44	28.2±6.32
5	0	13.8±0.37	22.6±0.40	29.4±1.08
Expt. 2				
5	5	11.4±0.24	15.0±2.14	26.0±2.77
5	0	11.8±0.40	15.0±2.14	33.2±6.62

*Mean ± Standard error (S.E.)

Data from two such experiments are summarised in Table 1. Half of the insect colonies derived from individual females were LAV-free whereas

virus was readily detected in the other colonies. Although nymphs emerged sooner and maize wallaby ear symptoms appeared earlier in Expt. 2 than in Expt. 1, in both experiments LAV-free insects induced symptom as quickly as those infected with LAV. There was no significant difference in the fecundity of LAV-infected and LAV-free insects.

Conclusion

It was concluded from the results of the experiments described above that the ability to induce wallaby ear disease was an inherent characteristic of *C. bimaculata* independent of their LAV-infection. The symptoms observed in maize seedlings were probably a response of the plants to toxic secretions of *C. bimaculata*.

CHAPTER IV

DEVELOPMENT OF WALLABY EAR DISEASE IN MAIZE SEEDLINGS COLONISED BY *CICADULINA BIMACULATA*

Carter (1973) suggested helpful guidelines in distinguishing phytotoxemia from plant virus infection, already mentioned in the introduction. In this chapter are reported experiments which were done to determine whether maize wallaby ear disease had attributes of a phytotoxemia.

EXPERIMENTAL

1. Effect of *C. bimaculata* density of infestation on growth of maize seedling

Severe stunting of maize plants is one of the characteristic symptoms of maize wallaby ear disease. In order to determine the effect of the numbers of insects feeding on the growth of maize plants, three separate but similar experiments described below were done. Various numbers (1-20) of impregnated female insects collected from a LAV-infected colony of *C. bimaculata* maintained on maize in a glasshouse were caged on single maize seedling. All seedlings were selected for uniformity and each was grown in a separate pot.

In the first experiment, treatments comprised of 5,10,15 and 20 insects per seedling. Treatments in the second experiment were 1,2,3,4 and 5 insects per seedling. In the third experiment, treatments of 2,4,6,8 and 12 insects per seedling were used. Control plants (free of any insects) were included in all experiments and were all done under similar conditions. The seedlings were kept in a growth chamber maintained at 25°C and 17 hours illumination of 52,000 lumen/m^2. A Latin square design was used in all three experiments. In order to eliminate the possibility of any of the insects reproducing, all the experiments were terminated

after 8 days. The eight days allowed was sufficient for young seedlings to develop wallaby ear disease symptoms. In the first experiment, seedlings were harvested and the dry weight determined after they had been oven-dried at 60°C for 3 days.

Results of the experiments summarised in Fig.11, show that stunting of the maize seedlings correlated well with the reduction of dry matter produced. There was also a progressive reduction in plant height as insect numbers per seedling were increased from one through to five. However, increases beyond 5 insects per seedling did not cause a proportionate reduction in plant height. About 50% reduction in plant height was induced by 5 insects while 20 insects induced 60% reduction (Fig. 12, see also Fig. 13).

2. Recovery of maize plants from wallaby ear disease after removal of infesting *C. bimaculata*

Boccardo *et al.* (1980) observed that wallaby ear disease symptoms in maize seedlings were arrested and the plants recovered after removing the infesting *C. bimaculata*. The experiments described below were done to determine the effect of duration of exposure of maize seedlings to *C. bimaculata* on their recovery.

Young maize seedlings selected for uniformity (three in each 15 cm pot) were exposed to a glasshouse population of *C. bimaculata* infected with LAV. The pots were placed at random among plants heavily infested with leafhoppers. After different periods, groups of plants were removed from the insect colony, sprayed with pyrethrum insecticide and transferred to an insect-free glasshouse. The first group of plants were transferred when maize wallaby ear disease symptoms first developed. The initial insecticidal spray of all plants removed from the insect colony

FIG. 11: Effect of density of infestation by *C. bimaculata* on the height and dry matter weight of maize seedling. Measurements were taken 8 days after infestation.

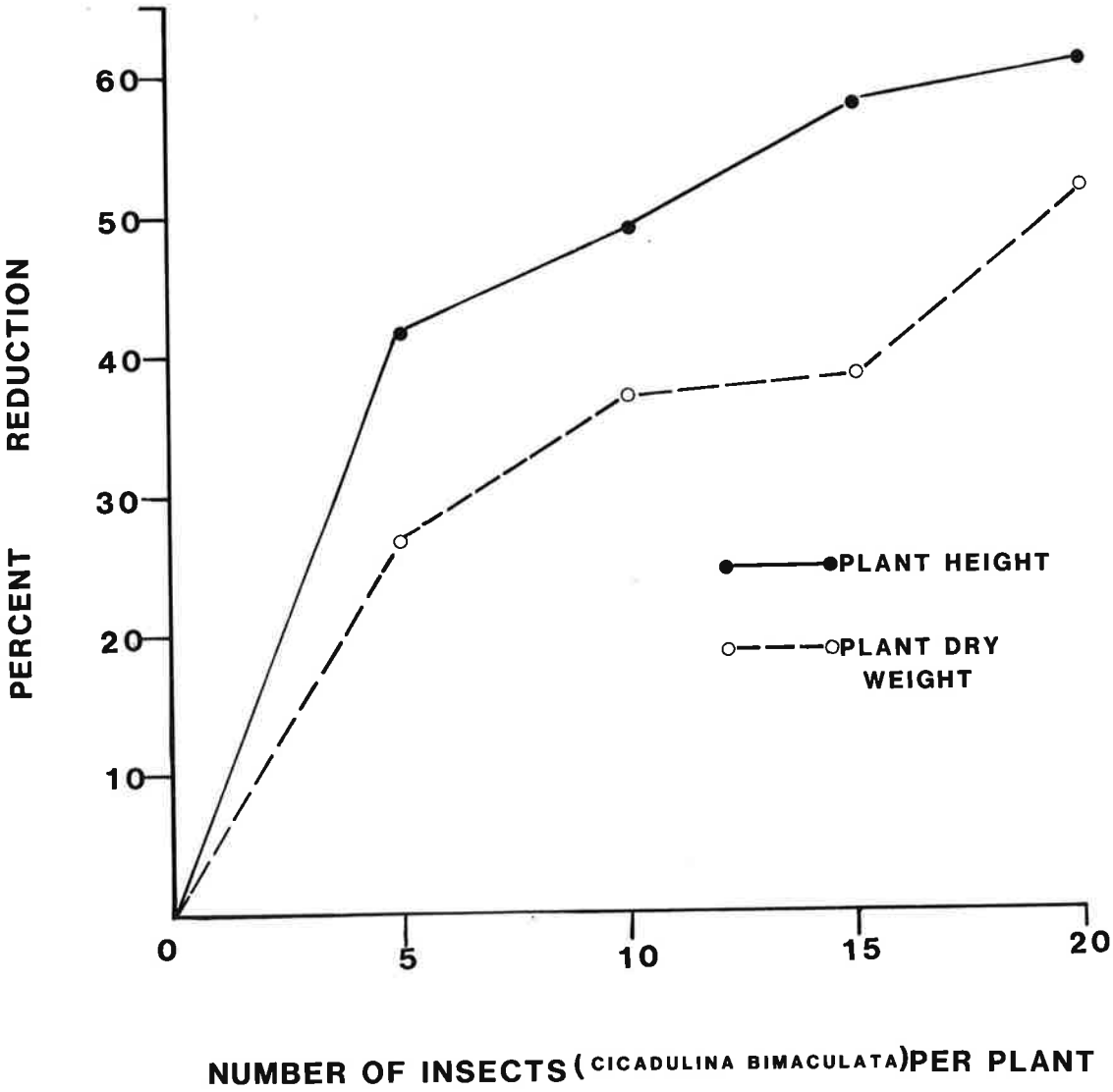

PERCENT REDUCTION
60
50
40
30
20
10
0
5
10
15
20
PLANT HEIGHT
PLANT DRY WEIGHT
NUMBER OF INSECTS (CICADULINA BIMACULATA) PER PLANT

FIG. 12: Effect of number of insects *(C. bimaculata)* infesting each maize seedling on plant growth.
Measurements were made after 8 days of infestation.

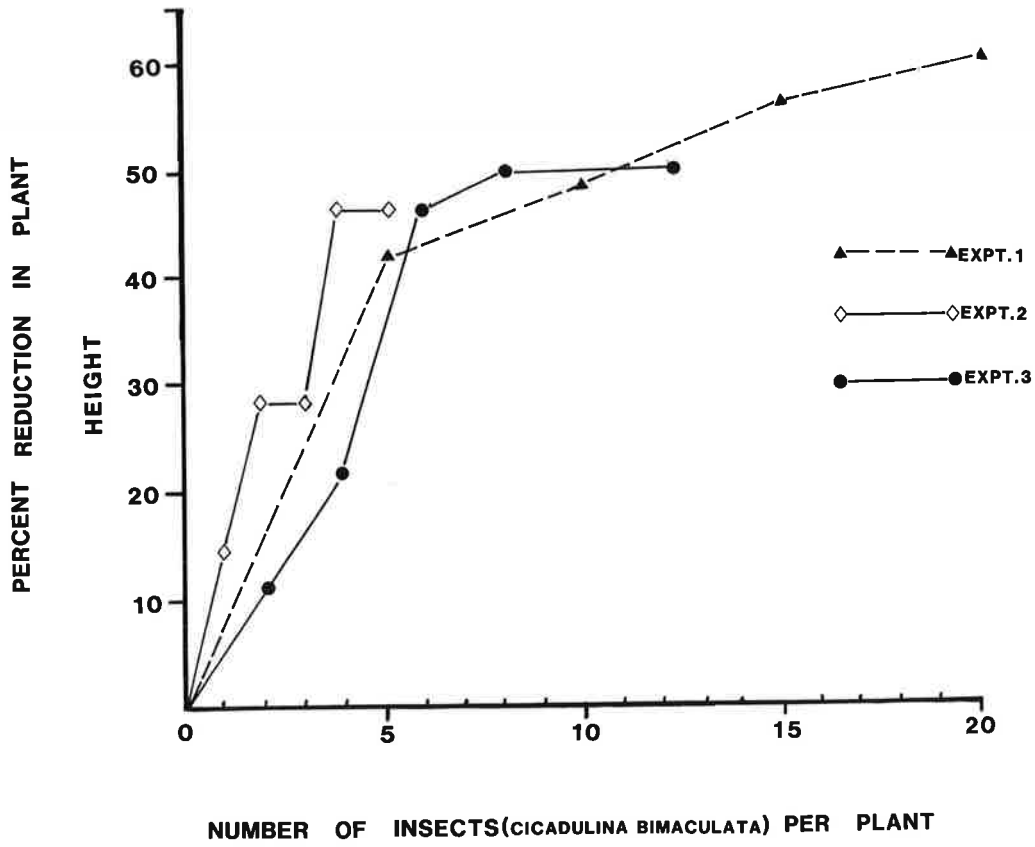

PERCENT REDUCTION IN PLANT HEIGHT
60
50
40
30
20
10
0
5
10
15
20
EXPT.1
EXPT.2
EXPT.3
NUMBER OF INSECTS(CICADULINA BIMACULATA) PER PLANT

FIG. 13: Representative maize seedlings after eight days exposure to various numbers of female impregnated *C. bimaculata* from a LAV-infected colony.

A - Control seedling. B - 5 insects per seedling. C - 10 insects per seedling. C - 15 insects per sedling, and E - 20 insects per seedling.


A
B
C
D
E

was followed by periodic ones as a measure against newly hatched nymphs.

Results of two separate experiments summarised in Table 2 indicate that damage caused by *C. bimaculata* was arrested by removal of infesting insects after which plants grew normally and produced symptom-free young leaves. However, damage sustained by prolonged exposure to the insects became irreversible and killed the plants (Fig. 14, see also Fig. 15). The time required by plants to recover from the disease also depended on the duration of exposure to insect infestation.

TABLE 2: Recovery of maize plants from wallaby ear symptoms following removal of *C. bimaculata*.

Time of exposure of plants to *C.bimaculata* (days)*	Number of plants killed	Number of plants recovered	Time required for plant recovery (days)
Expt.1			
6	0/9	9/9	8-10
12	0/9	9/9	10-14
18	4/9	2/9	17-23
24	7/9	1/9	29
Expt.2			
8	0/6	6/6	10-14
12	0/6	5/6	10-18
16	5/6	0/6	-
20	5/6	0/6	-
24	0/6	0/6	-
28	0/6	0/6	-

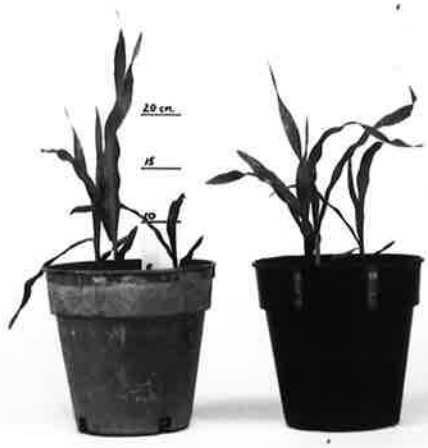
*After the indicated times, insects were killed by insecticide and the plants transferred to an insect free glasshouse.

FIG. 14:

A. Maize seedlings showing mild wallaby ear disease symptoms after 1 week exposure to LAV-infected *C. bimaculata*.

B. Recovery of seedlings from A, 15 days after removing insects from the seedlings and periodically spraying plants with insecticide (Pyrethrum).

Ⓐ


20 cm.
15
10

Ⓑ


20 cm.
15
10

FIG. 15:

A. Maize seedlings showing severe wallaby ear disease symptoms after 4 weeks exposure to LAV-infected *C. bimaculata*.

B. Failure of the maize seedlings from A to recover from wallaby ear disease symptom after removing the insects from the plants and periodically spraying with insecticide (Pyrethrum).

The photograph was taken 3 weeks after removal of the insects.

Ⓐ


20 cm.
15
10
5

Ⓑ


20 cm.
15
10
5

3. Effect of plant age on severity of maize wallaby ear disease

To determine the effect of age of maize seedling at the time of *C. bimaculata* infestation on the severity of disease, the experiment described below was done.

Groups of maize seedlings at different times after germination (from 3-26 days) were selected so that each group of nine plants was uniform in size and appearance. The plants were exposed to LAV-infected *C. bimaculata* maintained on maize in a glasshouse. The plants were arranged at random among plants heavily infested with insects and were observed for 24 days. The results of the experiment summarised in Table 3 demonstrate that young seedlings developed maize wallaby ear disease symptoms earlier than older plants and that mortality was much higher in younger plants than older ones. All seedlings which were exposed to insects 3 days after germination died within 20 days of exposure whereas those of the oldest group (exposed 26 days after germination) all survived.

TABLE 3: Effect of age of maize seedlings exposed to *C. bimaculata* on the severity of maize wallaby ear disease

Plant age when exposed to insects (days)	Time taken for plants to develop symptom (days)	Number of plants killed by disease	Time taken to kill plants
3	6-8	9/9	16-20
11	6-8	4/9	18-20
16	6-10	2/9	21
21	11-15	1/9	21
26	13-15	0/9	-

Each age group consisted of 9 seedlings (3 per pot). The experiment was terminated 24 days after initial exposure of seedlings to insects.

4. Comparison of severity in stunting induced by similar numbers of various forms of *C. bimaculata* in maize seedlings.

It has been shown in a previous experiment that severity of stunting induced in maize seedlings by impregnated females of *C. bimaculata* from a LAV-infected colony depended on the density of infestation of the leafhoppers. The experiments described below, were done to determine whether similar numbers of virgin females, impregnated females and males from a LAV-infected colony would induce similar levels of stunting in maize seedlings.

In two separate trials, groups of five virgin females, impregnated females and males were caged on single maize seedling grown in separate pots to determine the effect of the insects on the gain in plant height. Seedlings were selected for uniformity in both experiments which were done under similar conditions. The caged seedlings with insects were kept in a growth chamber maintained at 25°C and 17 hours daylight of 52,000 lumens/m^2. Each treatment including the control was replicated five times in both experiments and the pots were arranged in a randomised complete block design. Both experiments were terminated after eight days when all surviving insects from each plant were retrieved and counted. Each seedling was measured and the gain in height determined.

Results of the two trials summarised in Table 4 show that all three forms of *C. bimaculata* induced stunting in maize seedlings. There were differences in the severity of stunting induced by impregnated females and that caused by males and virgin females in both trials. However, the difference was highly significant in Trial 1 while in Trial 2 there was no significant difference in stunting induced by any of the three forms of *C. bimaculata*. Results of the two trials suggest that groups of similar numbers of *C. bimaculata* may induce different levels of stunting

in maize seedlings. Since groups of insects may differ in the ability to induce stunting, it is also possible that individuals within a group may also differ in their ability to induce stunting in maize seedlings.

TABLE 4: Comparison of the severity of stunting in maize seedlings induced by virgin females, impregnated females and males of *C. bimaculata*.

Treatment	Mean gain in plant height (cm)	Number of insects that survived	Mean gain in plant height (cm)	Number of insects that survived
Control	32.2	-	41.3	-
Seedlings with virgin females	29.4	17	33.5	20
Seedlings with impregnated females	16.1	19	27.5	19
Seedlings with males	29.1	17	33.0	17
	LSD P=0.01, 9.98; P=0.05, 7.17		LSD P=0.01,10.26; P=0.05,7.32	

CONCLUSION

It was concluded from the results of the experiments described in this chapter that the severity of wallaby ear disease in maize depended on the density of infestation by *C. bimaculata*, the age of seedlings at the time of infestation and the duration of exposure of the seedlings to the leaf-hoppers.

CHAPTER V

INSUSCEPTIBILITY OF *N. PALLIDA* TO LAV AND ITS INABILITY TO INDUCE WALLABY EAR DISEASE

Grylls (1975) claimed that *N. pallida* when injected with fractionated extract from wallaby ear diseased maize plants was able to induce the disease. The objectives of experiments described in this section were to determine if *N. pallida* could acquire the ability to induce wallaby ear disease and if it was susceptible to infection by LAV.

EXPERIMENTAL

1. Attempts to transmit LAV and the ability to induce WED from *C. bimaculata* to *N. pallida* when breeding as a mixed colony

C. bimaculata multiplies well on maize and *N. pallida* on Rhodes grass *(Chloris gayana)* and colonies of these insects were routinely maintained on these plants. However, attempts to establish a *C. bimaculata* colony on Rhodes grass and that of *N. pallida* on maize failed. The insects always died within two weeks of being confined on the plants. Thus to maintain a mixed colony of the two insect species, another host plant was required. Barley (*Horedeum vulgare*) was found to be a satisfactory host for both species of leafhopper and a mixed colony was established, and was maintained for a period of four months.

Ten young adult insects were caged on each pair of barley seedlings growing in 10 cm pots. The source of insects was as follows, each combination being replicated five times and all pots having been selected so that the barley seedlings were uniform in size.

(1) Five male and five female *N. pallida* from a mixed colony growing on barley.

(2) Five male and five female *C. bimaculata* from a mixed colony

growing on barley.

(3) Five male and five female *N. pallida* from a colony growing on *Chloris gayana*.

(4) Five male and five female *C. bimaculata* from a colony growing on maize.

(5) Plants were caged in the absence of any insects (control plants).

The pots were arranged in a latin square design in a growth chamber maintained at 25°C with artificial lighting of 52,000 lumens/m^2 for 12 hr. days. After 5 weeks the experiment was terminated. Insects from each cage were collected, counted and assayed for LAV by immunoelectron microscopy in extrcts from each group. The heights of the plants were measured and the plants were inspected for the presence of galls characteristic of wallaby ear disease.

Results of the experiment summarised in Table 5 show that *N. pallida* bred together with *C. bimaculata* did not induce wallaby ear disease in the barley plants and failed to be infected by LAV. However, the *C. bimaculata* both from the pure culture bred on maize and the mixed culture bred on barley induced wallaby ear disease on barley seedlings and carried LAV.

The results (Table 5) also show that the fecundity of *C. bimaculata* on barley seedlings was similar irrespective of whether the insects had been previously bred on maize or on barley. However, the fecundity of *N. pallida* on barley was much more prolific when the insects had been previously bred on Rhodes grass than on barley.

In spite of the lack of wallaby ear disease symptoms on barley infested by *N. pallida* from the pure and mixed colonies, the insects had a significant inhibitory effect on the growth of plants (Table 5). The effect appeared to be proportional to the number of *N. pallida* infesting barley seedlings.

TABLE 5: Failure of *N. pallida* to acquire LAV and the ability to induce wallaby ear disease

Species and source of insects cultured on barley seedlings	Number of plants developing wallaby ear disease symptoms	Number of colonies infected with LAV	Mean number of insects progeny per set of two seedlings	Mean gain in plant height (cm)
Five male and five female *N. pallida* from a mixed colony growing on barley	0/10	0/5	26 ± 10.7	31.8 ± 1.56
Five male and five female *C. bimaculata* from a mixed colony growing on barley	5/10	4/5	42 ± 4.2	21.8 ± 1.84
Five male and five female *N. pallida* from a colony growing on *Chloris gayana*	0/10	0/5	114 ± 14.7	21.8 ± 1.17
Five male and five female *C. bimaculata* from a colony growing on maize	5/10	3/5	45 ± 5.8	21.2 ± 3.18
				Control 41.69 ± 0.91

L.S.D. P=0.01=9.98
P=0.05=7.01

2. Attempts to transmit LAV to *N. pallida* by membrane feeding on partially purified virus

The experiment described below was done to determine whether *C. bimaculata* and *N. pallida* could acquire LAV which Grylls (1975) claimed could infect both species of leafhopper.

Twenty-five second and third instar nymphs from both LAV-free *C. bimaculata* maintained on maize and *N. pallida* supported on Rhodes grass were fed on a LAV preparation in 5% sucrose for 48 hours. Nymphs of both species of leafhoppers were transferred and caged together on barley seedlings grown in 15 cm pots followed by further weekly transfers onto new barley seedlings. The surviving adults were collected and each was assayed separately for LAV 30 days after the nymphs were fed. Results of the experiment is summarised in Table 6 which show that, no LAV particles were detected in any of the adult *N. pallida*, but about half the adult *C. bimaculata* that survived were infected with LAV.

TABLE 6: Failure of *N. pallida* to acquire LAV by feeding through a membrane on a diet containing LAV

Insect species	Number of nymphs fed on LAV preparation	Proportion of adults that survived after 30 days	Proportions of adults infected with LAV
C. bimaculata	25	13/25	6/13
N. pallida	25	15/25	0/15

CONCLUSION

From the data presented it is concluded that, unlike *C. bimaculata*, *N. pallida* is immune to infection by LAV presented in a synthetic diet. Furthermore *N. pallida* appears to be incapable of acquiring either LAV or the ability to induce wallaby ear disease while co-habiting the same plants with LAV-infected *C. bimaculata*. It is also concluded that, gall formation on plants should be a prime diagnostic character of wallaby ear disease because *N. pallida* induced stunting in plants but no leaf galls.

CHAPTER VI

DETERMINATION OF MODE OF SPREAD OF LAV

All virus hosts have limited lifespans and hence for virus survival an efficient mode(s) of tansmission from one host to another is essential. Efficient virus spread can be either horizontal or vertical, or both. (Matthews,1981). The experiments decribed in this chapter were done to determine the mode(s) of spread of LAV from one host to another.

EXPERIMENTAL

1. Test for vertical transmission of LAV in *C.bimaculata*

In three separate trials, fertilized female insects collected from a LAV-infected colony were each caged on a maize seedling leaf for 48 hrs and allowed to oviposit. The mother insects were then removed and assayed for LAV-infection. Samples of eggs laid by the insects were collected from the infested leaves ten days later, surface sterilized and allowed to hatch on filter paper strips dipped in 3% aqueous agar under asceptic conditions. After hatching the nymphs were transferred to maize seedlings and after a further 35 days each adult was assayed for LAV by immunoelectron microscopy. Results of three trials (Table 7) show that 24% of ovipositing *C. bimaculata* and 19% of the progeny insects were infected with LAV.

Another experiment was done to determine if there was a vertical transmission through sperm. Individual virgin females from a LAV-free colony were each mated with a single male from a LAV-infected colony. Each pair of insects was kept on a barley seedling grown in a large test tube for 14 days. The males were then removed and each insect was assayed for LAV. The females were maintained for a further 20 days when

TABLE 7: Trans-ovarial transmission of LAV by *C. bimaculata*.

Experiment number	Proportion of mother insects LAV-infected	Proportion of eggs hatched	Proportion of progeny insects LAV-infected
1	4/20	13/90	3/13
2	3/15	16/60	2/16
3	5/15	18/60	2/18
Total	12/50 (24%)	47/210 (22%)	7/37 (19%)

they too were assayed for virus. The plants were retained, allowing any eggs to hatch and the emerged insects to develop. Twenty days after the appearance of progeny adults, the insects derived from each female *C. bimaculata* were pooled and assayed for LAV. Results of two independent trials (Table 8) show that more than half the males, but none of the female parent insects, were infected with LAV. Furthermore none of the progeny insects were infected with LAV. The results also show that LAV was not transmitted through sperm or by contact between insects colonizing the same plant.

TABLE 8: Lack of LAV transmission in *C. bimaculata* through sperm and by insect-to-insect contact.

Experiment number	Proportion of insect pairs surviving	Proportion of LAV-infected	Proportion of LAV-infected	Proportion of progeny insect colonies LAV-infected*
1	14/20	5/14	0/5	0/5
2	19/40	14/10	0/14	0/14
Total	33/60 (55%	19/33 (58%)	0/19 (0%)	0/19 (0%)

*Colonies derived from mother insects which were tested for infection with LAV.

2. Horizontal transmission of LAV in *C. bimaculata*

The results of the previous experiments (Table 8) suggest that LAV could not be transmitted horizontally in *C. bimaculata* through contact between LAV-infected insects and ones free of the virus. Nevertheless, other experiments were done to determine other possible ways of horizontal transmission of LAV in *C. bimaculata*.

In two separate trials, partially purified virus preparations from LAV-infected insects (1-2g) were smeared on leaves of maize plants on which second instar nymphs from the LAV-free colony of *C. bimaculata* were caged. After 34 days the surviving adults were recovered and each was assayed separately for LAV-infection by immunoelectron microscopy and all were found to be virus-free (Table 9, Experiments 1 and 2). However, when similar virus preparations were presented to nymphs from the LAV-free colony in feeding chambers behind parafilm membranes, nearly half of the insects acquired LAV (Table 9, Experiments 3-5).

TABLE 9: Tests for acquisition of LAV from contaminated leaf surfaces and a synthetic diet.

Experiment number	Method of presenting LAV to insects	Proportion of insects surviving	Proportion of insects LAV-infected
1	Contamination of leaf surfaces	13/20	0/13
2		18/25	0/18
	Total	31/45 (69%)	0/31 (0%)
3	Feeding through membrane	19/25	8/19
4		30/35	14/30
5		28/35	13/28
	Total	77/95 (81%)	35/77 (45%)

Experiments were also done to determine if *C. bimaculata* could acquire LAV by feeding on plants infested with LAV-infected insects. Groups of insects from the LAV-free colony of *C. bimaculata* were either allowed to feed on maize plants simultaneously with LAV-infected ones or were allowed to do so subsequently to infestation by LAV-infected *C. bimaculata*. When LAV-free insects were to be fed at the same time as non-infected ones, LAV-free nymphs were first introduced and confined in cages assembled on insect-free maize plants (Fig. 16). First and second instar nymphs from the LAV-free colony were used. The plants were then exposed to LAV-infected *C. bimaculata* in large cages containing heavily infested maize plants. On the other hand, when LAV-free insects were to be allowed to feed on maize plants subsequently to infestation by LAV-infected insects, empty cages were assembled on insect-free maize plants and the plants were then exposed to LAV-infected insects. LAV-free insects were introduced into the leaf cages after removing all insects outside the cages at the end of the exposure period.

In three separate trials LAV-free insects were allowed to feed simultaneously with LAV-infected ones for 7 days. After 7 days the cages and the leaf tissue in them were removed by excision and the nymphs were transferred from the cages onto insect free maize seedlings. After a further 28 days the surviving adults were each assayed for LAV by immuno-electron microscopy.

Results of the three trials are summarised in Table 10 (Experiments 1,2a and 3a). Over 20% of insects from the LAV-free colony became infected with LAV when fed simultaneously with LAV-infected ones.

In two similar experiments LAV-free insects were allowed to feed on maize plants subsequently to infestation by LAV-infected insects. In one experiment, first and second instar nymphs from the LAV-free colony of

FIG. 16: Tube assembly used for caging LAV-free *C. bimaculata* on a maize plant exposed to infestation by LAV-infected insects outside the caged areas.

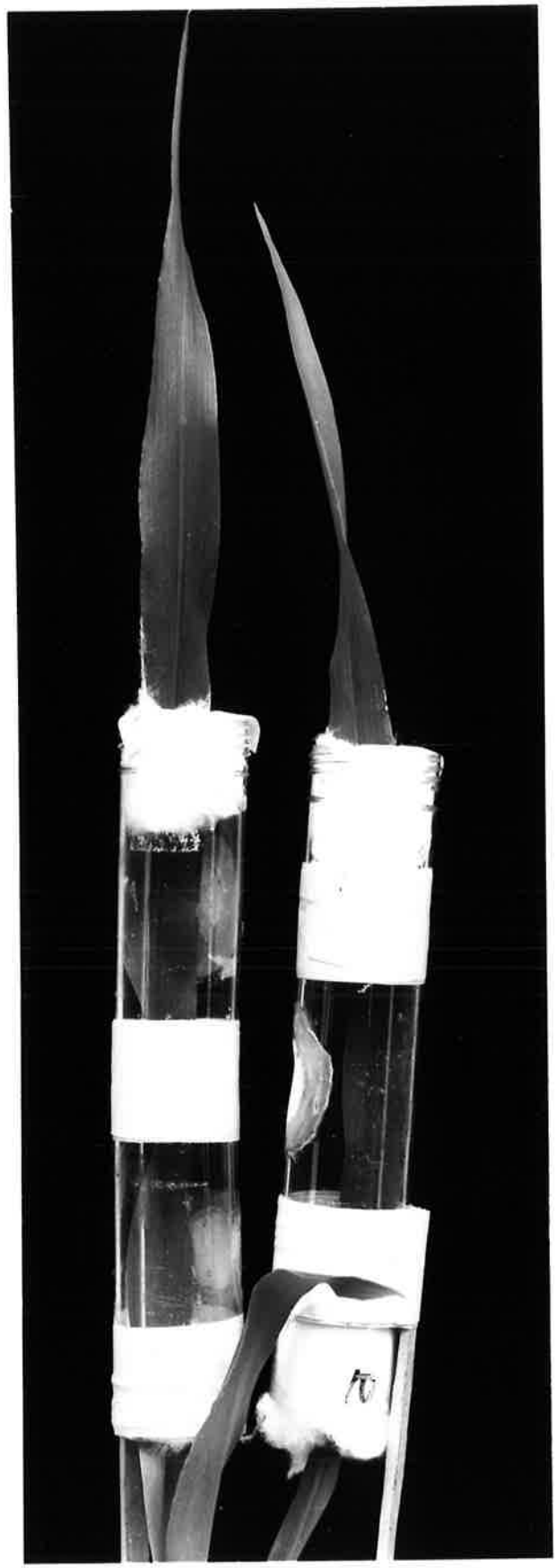


TABLE 10: Acquisition of LAV by caged virus-free *C. bimaculata* from maize plants colonized by virus-infected insects

Experiment Number	Time of caging virus-free insects	Number of insects infected with LAV relative to that surviving during the experiment		
		Individual cages*	Total	%
1	During infestation by infected insects	0/6, 0/3. 3/5, 1/3, 0/1, 1/4, 1/4, 0/5, 2/7, 1/4	9/42	21
2(a)	During infestation by infected insects	6/15, 1/5, 2/10, 1/7, 2/13, 1/6	13/56	23
(b)	Immediately after removal of infected insects	1/13, 0/7, 1/15, 0/8, 3/10, 2/17	7/70	10
3(a)	During infestation by infected insects	3/7, 2/12, 4/12, 4/11, 2/8, 1/10	16/60	27
(b)	9 days after removal of infected insects	0/8, 0/10, 0/11, 0/10, 0/8, 0/11	0/58	-

*Ten nymphs were introduced into each cage in Experiment 1 and 20 nymphs in Experiments 2 and 3.

C. bimaculata were allowed on the maize plants immediately after the LAV-infected insects were removed. Insects that emerged later outside the cages were removed manually. Surviving adults from each cage were each assayed separately for LAV infection after 34 days. Ten percent of adults that survived were infected with LAV (Table 10, Experiment 2(b)). However, when LAV-free nymphs were caged on maize plants that had been exposed to LAV-infected *C. bimaculata* but were removed 9 days before caging, none of the surving adult insects carried the virus when assayed after 34 days (Table 1, Experiment 3(b)).

CONCLUSION

The following were concluded from the results of the experiments.

(1) LAV was vertically transmitted through eggs (transovarially) but there was no transmission through sperm.

(2) *C. bimaculata* was infected horizontally by feeding on virus-contaminated diet through a membrane and also by feeding on plants co-infested with LAV-infected insects. However, the LAV inoculum in plants depended on co-infestation by virus-infected insects and once these were removed, the virus did not persist in the plants.

(3) LAV was not transmitted horizontally through contact between insects and the virus could not be acquired as a surface contaminant by *C. bimaculata*.

CHAPTER VII

EFFECTS OF LAV INFECTION ON *C. BIMACULATA*

Boccardo *et al.* (1980) observed that wallaby ear disease symptoms were arrested when plants were freed of *C. bimaculata* infestation. They also found no evidence to suggest that LAV multiplied in maize plants. Results of earlier experiments in this thesis (Chapter III) suggest that the ability of *C. bimaculata* to induce wallaby ear disease was an inherent characteristic of the leafhopper and independent of their LAV-infection. It has also been demonstrated that LAV particles injected into maize plants during infestation by LAV-infected leafhoppers did not persist in maize plants. These observations all support the view that LAV is an insect virus infecting only the leafhopper *C. bimaculata* and not able to replicate in maize plants. Experiments described below were done to determine whether LAV infection affected the biology of *C. bimaculata* in any way. Biological aspects investigated were: (i) the effect of LAV infection on the fecundity of the insects and (ii) the effect of LAV infection on nymphal development and life span of insects.

EXPERIMENTAL

1. Effect of LAV on fecundity of *C. bimaculata*

Each of forty-six virgin females from a LAV-infected insect colony was paired with a newly emerged male from a LAV-free colony and each insect pair was caged on a single leaf of maize plants grown in pots. The plants were maintained at 25°C and continuous illumination of 3,800-4,300 lumens/m^2. The insect pairs were retrieved 15 days later and the males and females assayed for LAV-infection by immunoelectron microscopy.

Another experiment similar to the one described above was done to determine whether LAV infection had any effect on the fecundity of *C. bimaculata* during later stages of the adult life of the insects. Twenty virgin females from a LAV-infected colony and thirty newly emerged males from a LAV-free one were pooled together and caged on a maize seedling grown in 15 cm pots. The plant was maintained under similar conditions as the first experiment. The female insects were retrieved 15 days later and each was caged separately on a single maize leaf as in the previous experiment and maintained under similar conditions.

TABLE 11: Effect of LAV infection on the fecundity of *C. bimaculata*.

	Virus-free ♀	LAV-infected ♀
Experiment 1:		
Number surviving for 15 days after mating	32/46	14/46
Range in number of eggs laid per insect during 15 days after mating	8-150	19-115
Number of eggs laid per insect during 15 days after mating	73 ± 11.12	72 ± 5.08
Number of nymphs emerged per insect during 15 days after mating	3.1 ± 1.25	3.4 ± 0.92
Experiment 2:		
Number surviving for 30 days after mating	3/20	5/20
Range in number of eggs laid per insect between 15 and 30 days after mating	39-85	46-85
Number of eggs laid per insect between 15 and 30 days after mating	62 ± 12.3	67 ± 7.5
Number of nymphs emerged per insect between 15 and 30 days after mating	4.1 ± 0.91	4.0 ± 0.95

The results of the two experiments are summarised in Table 11. In both experiments LAV had no effect on the fecundity of *C. bimaculata* and the mean number of nymphs per female that emerged during the experimental

period was the same for both LAV-infected and virus-free insects.

2. Effect of LAV on nymphal development and life span of *C. bimaculata*

One hundred first instar nymphs (progeny of females from a LAV-infected colony) were each raised separately on barley seedlings grown in test-tubes on 3% aqueous agar medium in Hoaglands nutrient solution. The nymphs were maintained at 25°C and continuous illumination of 3800-4300 lumens/m^2.

Emerged adults were further maintained on the barley seedlings and were transferred onto new barley seedlings when the plants became old. The insects were examined twice daily and data on emergence of each adult as well as the sex were recorded. Dead insects were retrieved and each was either immediately assayed for LAV infection or stored at -20°C for assay later. Results of the experiment are summarised in Table 12. The results suggest that LAV infection had no effect on the nymphal development of *C. bimaculata*; however, the adult life span was shorter in LAV-infected insects than virus-free ones.

TABLE 12: The effect of LAV on nymphal development and longevity of *C. bimaculata*

	Free of LAV		LAV-infected	
	♂	♀	♂	♀
Number of insects	31	26	17	26
Time of nymphal development (days)	20 ± 0.5	20 ± 0.4	22 ± 0.7	21 ± 0.5
Adult life-span (days)	57 ± 5.2	57 ± 4.6	37 ± 5.1	47 ± 4.3
	57 ± 4.0		43 ± 3.0	

CONCLUSION

It was concluded from the results of the experiments that LAV had no effect on the fecundity of *C. bimaculata* at both early and late stages of the life of insects and there was also no effect of the virus on the nymphal development. Both LAV infected males and females, as well as LAV non-infected ones, have a similar nymphal development period; however, virus-infection shortened the life span of the insects.

CHAPTER VIII

GENERAL DISCUSSION

1. Etiology of maize wallaby ear disease

Data presented in this thesis support the conclusion that wallaby ear disease (WED) in maize is not the result of infection by LAV, a reovirus, as claimed by Grylls (1975). The conclusion is based on the following evidence.

(1) LAV-infected and LAV-free *C. bimaculata* were equally efficient in inducing wallaby ear disease in maize.

(2) Diseased plants recovered after the removal of infesting insects.

These data favour the view that wallaby ear disease in maize is caused by an insect toxin (1973).

This conclusion is contrary to that reached by Grylls (1975) and it seems important to analyse the basis of disagreement. Grylls (1975) approached the problem by breeding selectively for a *C. bimaculata* colony unable to induce WED in maize whereas initially, I addressed myself solely to the question of whether insects free of LAV could induce the disease in maize plants. Much effort was exerted in raising LAV-free *C. bimaculata* colonies from surface-sterilised eggs and the resulting colonies were used to demonstrate that LAV-free insects could induce WED in maize. Subsequently the ability of LAV-free insects to induce the disease was also demonstrated by a much less laborious method in which colonies were established from single impregnated females taken from a LAV-infected colony. In these experiments about half of the female insects were LAV-free and gave rise to LAV-free progeny which, however, were able to induce WED in maize plants just as efficiently as those in-

fected with the virus. None of the insect colonies established in my experiments failed to induce the disease.

It seems that the selective breeding approach used by Grylls (1975) was more likely to provide an answer to a genetically controlled characateristic of the insect species than to give a clue as to whether a virus was responsible for the wallaby ear disease. In one of his trials he observed that induction of galls in maize seedlings by the offspring of non-disease inducing parents fell to less than 1% over nine generations. This suggests that the ability of insects to induce disease is under genetic control.

Other evidence used by Grylls (1975) to support his claim that WED was caused by a virus was the failure of some progeny from disease-inducing parents to cause the disease. He interpreted this as indicating that the disease was induced by a virus which was transmitted only in some individuals. However, the observation can also be interpreted that the ability of *C. bimaculata* to induce disease was controlled by a recessive gene. One could expect toxin production by toxicogenic insect species to be such a genetically controlled character (Kanervo *et al.*, 1957). However, Nuorteva (1958, 1962) was of the opinion that the differences in the ability of the leafhopper *Calligypong* (Delphacodes) *pellucida* F. from two different localities to induce toxicosis in oat reported by Kanervo *et al.* (1957) was due to difference in diet rather than their genetic characters. In my work I was unsuccessful in selecting insects unable to induce WED.

In his work, Grylls (1975) did not assay the various generations of insects for the presence of LAV. However, after completing his breeding experiments he maintained two colonies of *C. bimaculata*, one in which the insects were capable of inducing wallaby ear disease and another in which the insects were unable to cause the disease. The colonies were examined

for the presence of virus and reovirus-like particles were detected in the former but not the latter, which was taken to support the notion that LAV is the causal agent of WED. The correlation between the ability to induce disease and presence of LAV in insects from the two colonies was confirmed by Boccardo *et al* (1980). Although they provided evidence that LAV was not the causal agent of WED they did make the erronous suggestion that toxin production by *C. bimaculata* was dependent on infection by LAV.

If the assumption that wallaby ear disease inducing ability of *C. bimaculata* is a genetic character is valid and because 100% transovarial passage of a virus is unlikely; selective breeding could produce any one of the following colonies of leafhoppers:

(i) Virus-free and non-disease inducing.

(ii) Virus-infected and non-disease inducing.

(iii) Virus-free and disease inducing, and

(iv) Virus-infected and disease inducing.

The colonies with characteristics of (iii) and (iv) used in my experiments argue against any suggestion that LAV causes WED but does not provide a conclusive evidence that the ability of insects to induce it is genetically controlled.

Grylls (1975) concluded that WED in maize may occur in either a mild form from which there is an apparent recovery or as a severe infection from which there is no recovery. This observation does not support his claim that WED in maize is due to a virus infection. Results of my experiments established that the severity of WED was dependent on the number of insects colonising each plant, the duration of infestation of plants by the insects and the age of plants at the time of insect infestation. It was also demonstrated that the disease could reach a stage after which

plants could no longer recover. These observations argue against any suggestions that virus infection is involved in the disease development. Furthermore they conform to the guide lines suggested by Carter (1973) for distinguishing phytotoxemia from plant virus infections.

Even though Grylls (1975) detected some LAV particles in partially purified extracts of diseased maize plants, he did not give any experimental details as to the condition of plants used for the virus extractions. If the maize plants were harvested when still infested with insects or soon afterwards, some LAV particles would have been present in the plant system as shown by Boccardo *et al.* (1980); probably due to those particles injected into the plant tissue by LAV-infected leafhoppers.

Data presented in Chapter VI of this thesis demonstrates that LAV-free insects failed to acquire the virus from plants which were freed from LAV-infected ones about 1 week prior to infestation by LAV-free test insects. This observation supports the view that LAV cannot persist in maize plants and hence argues convincingly against any possibility that the virus replicates in the maize plant.

In the light of evidence presented in this thesis on the etiology of maize wallaby ear disease it is suggested that some other plant diseases reported in the literature as being caused by virus infection should be re-evaluated. In this regard I have no hesitation in suggesting that wallaby ear disease of sugarcane reported as a plant virus infection by Ryan *et al.* (1980) is almost certainly a toxicosis similar to wallaby ear disease in maize. The disease was associated with infestation by *C. bimaculata* and Ryan *et al.* (1980) actually observed that the ratoon crop from previously diseased sugarcane appeared healthy.

Ahlawat and Raychaudhuri (1976) described a disease in maize with vein enation symptom and concluded that it was caused by a virus transmitted by *Cicadulina mbila* Naude. However, their evidence for a viral etiology is not well founded. Test insects which were collected from the field were not assayed for the putative virus. They also failed to give any indication whether virus particles were detected in purified extracts of diseased host plants. It seems the viral nature of the disease was assumed on the basis of symptomatology which could be misleading. According to Maramorosch (1961), H.H. Storey reported of a non-persistent galling in maize caused by *C. mbila* and a related species *C. zaae* China in Africa.

A disease in white clover (*Trifolium repens* L.) reported from Italy was attributed to virus infection on the basis of a reccurrance of the symptoms (leaf enation) in vegetatively propagated plants (Bos *et al.*, 1961). It is suggested that this attribute of the disease does not establish that virus infection is the cause of the disease. Reappearance of symptoms is known to occur in toxicosis when recovered plants are exposed to re-infestation by toxicogenic insect species. No insect vector was identified and attempts to transmit the disease mechanically also failed. However transmission of the supposed virus was achieved through side-cleft grafting. No mention was made of precautions taken to exclude infestation of grafted plants by insects. Furthermore no structures associated with virus replication were observed in thin sections of gall tissue from diseased plants. It is suggested that until such time that the supposed virus is purified and characterised, the etiology of the disease in white clover should be considered unresolved.

2. LAV in relation to *C. bimaculata*

It has been demonstrated that LAV was not involved in the etiology of maize wallaby ear disease, however, some members of the Reoviridae to which LAV must belong by virtue of its particle morphology (Matthews, 1982) are reported to affect their insect hosts in various ways, (Fukushi, 1969; Harpaz, 1972; Maramorosch, 1975; Harris, 1979). Evidence presented in this thesis demonstrate that LAV had no effect on the fecundity or the nymphal development of the leafhopper *C. bimaculata* and the virus only shortened the leaf span of insects, suggesting a long co-existence between LAV and *C. bimaculata*.

It may be pointed out that in determining the effect of LAV on the lifespan of insects certain unnatural steps were taken. Each insect was confined to a barley seedling grown in a test-tube and natural factors such as ovipositing in females and mating in males which were likely to cause varying levels of stress in the insects, were eliminated. Insects used in the tests were either virgin females or unmated males and the experiment was conducted at a constant temperature of 25°C. It is likely that the elimination of stresses which are likely to affect insects under natural conditions prolonged the lifespan of the insects in my experiments. Nevertheless, under the experimental conditions LAV-infected *C. bimaculata* died sooner than LAV-free insects. Perhaps an investigation into the effect of the virus in combination with natural stresses on the life-span of the insects should be undertaken in the future.

It has been shown that LAV can spread vertically through eggs which has also been reported with some leafhopper-borne plant viruses (Fukushi, 1933, 1939, 1940; Black, 1948, 1950, 1953; Black and Brakke, 1952; Maramorosch, 1952), as well as horizontally through maize plants.

The experiments demonstrating transovarial tansmission of LAV in *C. bimaculata* indicate that the proportion of mother and progeny insects infected with the virus were similar. This, and the apparent lack of a detrimental effect of the virus infection on insects fecundity, suggest that the maintenance of LAV in succeeding generation of leafhoppers can probably be accounted for by the vertical transmission of LAV. Although the horizontal transmission of the virus through maize was also demonstrated, its significance to virus spread under natural conditions is difficult to assess.

Horizontal transmission of LAV was dependent on the co-infestation of LAV-infected and LAV-free insects on the maize plants. The results of experiments presented in this thesis suggest that the probability of insects acquiring the virus from a maize plant was one in about five chances. If this assumption is correct then it seems that the immature forms of the insects which are more sedentary feeders compared to the adult insects, have a better chance of picking the virus from maize plants. Nevertheless, the efficiency of the horizontal spread of the virus will also depend on the ratio of LAV-infected insects and LAV-free ones as well as the population density of LAV-infected ones co-infesting the maize plants. Horizontal transmission for LAV through maize plants demonstrates that a Fijivirus-like particle can be transmitted through maize without infecting the plant. This suggests some Fijiviruses which infect plants may be able to be transmitted through non-host plants and this could have a consequence on the survival of some viruses in insects which do not have access to host plants.

At the moment LAV cannot be included in the genus Fijivirus with which it shares many common properties (Boccardo *et al.*, 1980) because it has not been shown to have a plant host. The only presently existing

genus within the family Reoviridae which includes viruses confined to insects is the cytoplasmic polyhedrosis virus group (Matthews, 1982). However, LAV by virtue of its structure should not be included in this taxon (Hatta and Francki, 1982).

APPENDIX

Publication

OFORI, F.A. and FRANCKI, R.I.B. (1983). Evidence that maize wallaby ear disease is caused by an insect toxin. *Ann. appl. Biol.* 103: 185-189.

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