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Identification, purification and some properties of a mosaic virus of okra (*Hibiscus esculentus*)

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SUMMARY

In the Ivory Coast, an apparently undescribed virus was isolated from okra (*Hibiscus esculentus*) in which it caused mosaic and leaf vein banding. The virus was sap transmissible to a wide range of plants and had a thermal inactivation point of 80 °C. It was named okra mosaic virus (OMV). A purification procedure was developed. Electron microscopy and analytical and density gradient centrifugation showed that OMV was an isometric virus accompanied by empty shells (top component). Serological tests showed OMV to be a member of the tymovirus group.

INTRODUCTION

In 1969, symptoms of chlorosis, mosaic and vein banding were noticed on the leaves of okra (*Hibiscus esculentus* L.) at the Station d'Expérimentation Agricole in Bouaké (Ivory Coast, West Africa), and also on okra plants grown in African villages, in the vicinity of Abidjan.

The disease was mechanically transmissible to okra seedlings grown in a greenhouse.

This paper presents the host range, symptomatology, purification and some physico-chemical and serological properties of the virus, which appears to be newly described and has been named okra mosaic virus (OMV).

MATERIALS AND METHODS

Virus and plant sources. The virus was found to occur naturally in the field. It was multiplied in the two okra cultivars Clemson spineless and Court, which were kept in a screen-house.

Virus purification. The method eventually developed for purification of the virus is shown in Table 1. Good results were also obtained using diethyl-ether instead of *n*-butanol for clarification. The extraction and purification were carried out in a cold room (4 °C). Virus preparations were highly infectious.

Table 1. *Purification of OMV*

Discarded	Retained	Treatment
	Diseased leaves	Frozen or fresh leaves homogenized in a Waring blender with 1·5 volume of 0·01 M sodium phosphate buffer, pH 7·0, sodium bisulphite 0·5 %, bentonite 1 %. Filtration through cheesecloth
Residue	Filtrate	Centrifuged 20 min at 12000 *g**
Pellet	Supernatant	Emulsified 5 min with 0·5 volume of *n*-butanol. Incubated 1 h at 4 °C. Centrifuged 5 min at 1500 *g*
Interphase and organic solvent phase	Aqueous phase	Bubbled with nitrogen and centrifuged 10 min at 8000 *g*
Pellet	Supernatant	Centrifuged 120 min at 100000 *g*†
Supernatant	Pellet	Resuspended overnight in 0·1 M sodium phosphate (pH 7·3) containing 0·01 M EDTA. Centrifuged 20 min at 12000 *g*
Pellet	Supernatant	Centrifuged 120 min at 100000 *g*
Supernatant	Pellet	Resuspended overnight in 0·1 M sodium phosphate (pH 7·3) containing 0·01 M EDTA. Centrifuged 20 min at 12000 *g*
Pellet	Supernatant	Centrifuged 120 min at 100000 *g*
Supernatant	Pellet	Resuspended overnight in 0·01 M phosphate buffer pH 7·0 using 0·01 of the initial volume. Centrifuged 20 min at 12000 *g*
Pellet	Supernatant	

* Sorvall RC2-B automatic superspeed refrigerated centrifuge.

† Beckman Model L preparative ultracentrifuge.

Methods for studying in vitro *properties*. Sap for these studies was obtained from the leaves of *H. esculentus* plants which had been inoculated at the cotyledonary stage. Twenty days later the three leaves on each plant were ground with 0·01 M sodium phosphate buffer pH 7·0 (1 g tissue to 1 ml buffer) and sterilized sand in a chilled mortar. The sap was pressed through four layers of cheese cloth. Relative virus concentrations were estimated by the infectivity index method of Raymer & Diener (1969) using five *H. esculentus* indicator plants for each dilution.

Determinations of the dilution end-point were made using serial dilutions of the sap in 0·01 M sodium phosphate buffer pH 7·0 at 4 °C.

The thermal inactivation point was tested by immersing 2 ml quantities of sap contained in 5 ml Pyrex tubes in a water-bath at various temperatures for 10 min and then placing them in an ice-bath.

To test ageing *in vitro*, the infectivity of the sap kept at 24 °C was determined every second day for 2 wk and resistance to air drying was determined by weekly tests of the infectivity of extracts of diseased okra leaves hung in the laboratory.

Density gradient centrifugation. The supernatant obtained at the end of the purification schedule (see Table 1) was layered on a 10–50 % sucrose density gradient in 0·01 M sodium phosphate buffer pH 7·0 containing 0·01 M EDTA and the tubes centrifuged for 6 h in the SW 25.1 rotor of the Spinco ultracentrifuge. The tubes were pierced at the bottom and the gradient allowed to drip out into collecting tubes.

Electron microscopy. Fractions from density gradients were dialysed against 0·05 M acetate buffer pH 5·8 for 24 h. A droplet of virus suspension was placed on a formvar-coated grid. One per cent uranyl acetate and 0·5 % sodium ethylene diamine tetra acetate (EDTA) pH 7·0 (Mellena, Van Bruggen & Gruber, 1968) were added and the excess of liquid withdrawn after a few seconds. The preparation was examined in a Siemens Elmiskop 1 A electron microscope at a magnification of 40000.

Absorbancy. The different fractions of density gradients were dialysed against 0·01 M sodium phosphate buffer pH 7·0 for 24 h. Ultraviolet absorption spectra were determined with a Zeiss PMQ II Spectrophotometer in a 1 cm cell.

Analytical ultracentrifugation. Unfractionated preparations of OMV were examined in a Spinco Model E ultracentrifuge equipped with Schlieren optics.

Antiserum production. A rabbit was injected with freshly prepared virus obtained from 150 g of leaves by the method described in Table 1. This material at a 10^{-3} dilution had an optical density at 260 nm of 1·50. Six, weekly intramuscular injections of 1 ml of virus preparation emulsified in 1 ml of Freund's incomplete adjuvant (Difco) were given using alternately left and right thigh muscles.

Two days before the third injection, a blood sample was taken from the ear to determine the antiserum titre. This operation was repeated every week. One week after the last injection, the rabbit was exsanguinated. The antiserum (titre 1/2048) was stored at −20 °C.

Serological tests. Double-diffusion tests (Ouchterlony, 1958; van Regenmortel, 1966) were performed in flat bottom Petri dishes, 14 cm diam., containing 80 ml of 0·7 % agar (Special Agar Noble Difco), 0·9 % NaCl and 0·1 % NaN_3 and kept at 4 °C. Wells 6 mm diam. were cut in an octagonal pattern with a central well 7·5 mm diam. into which antiserum was placed and which was 6 mm from the surrounding wells which contained antigen.

RESULTS

Transmissions

Mechanical transmission of OMV with infective *H. esculentus* sap to *H. esculentus* was always successful. All of the transmissions reported in this paper were made by inoculation with *H. esculentus* sap.

Transmissions by dodder (*Cuscuta subinclusa* Durr & Hilg.) were attempted from *H. esculentus* to *H. esculentus*, *Cucumis sativus* to *H. esculentus*, *Chenopodium quinoa* to *H. esculentus*, *C. quinoa* to *C. album* and *Arachis hypogaea* to *H. esculentus*.

None was successful.

113 seedlings were raised in the greenhouse from seeds from diseased okra plants. After one month none of the seedlings showed symptoms nor was any virus recovered from them.

Host range

The virus was mechanically inoculated to 118 species and cultivars in eleven families (Table 2) and induced symptoms in seventy-one of them from all of which the virus was subsequently retransmitted to okra.

Symptomatology

The description of the symptoms is based on the criteria proposed by Bos (1964).

Malvaceae

H. esculentus. Plants naturally infected in the field showed symptoms similar to those experimentally inoculated; the fruits sometimes bore chlorotic blotches. Symptoms appeared on the first leaf 7–8 days after inoculating the cotyledons. Generally the first diseased leaf showed a green mosaic or a regular vein chlorosis (Pl. 1, figs. *b*, *c*, *d*). Exceptionally, the chlorotic leaf exhibited a mosaic characterized by a mixture of irregularly shaped dark and light green or whitish areas on the lamina. On the second and third diseased leaf, one to three of the principal veins were bordered by large chlorotic bands of varying width (Pl. 1, figs. *e*, *f*).

Every infected *Hibiscus* species presented similar symptoms but in these and all other species of Malvaceae the symptoms disappeared after having been visible on the first, second or third leaf which fell off, and they did not appear on subsequent leaves.

Sida species showed a coarse chlorotic mosaic or a vein banding and *Gossypium* species had a light green mosaic or irregular chlorotic areas on the leaves.

Affected plants belonging to other genera of Malvaceae (*Malva*, *Malope*, *Wissadula*) bore light green blotches delineated by veinlets or irregular chlorotic or yellow bands along the main veins.

Symptoms (which persisted) on other plants were as follows:

Amaranthaceae

Gomphrena species: irregular spotting.

Table 2. *Results of mechanical inoculation of different species of plants*

Plant species	Number of plants inoculated	Number of plants showing symptoms	Persistence of symptoms
Amaranthaceae			
Amaranthus caudatus	88	0	.
A. spinosus	60	0	.
Gomphrena celosioïdes	18	15	+
G. globosa	15	13	+
Chenopodiaceae			
Beta vulgaris	8	0	.
Chenopodium album	39	17	+
C. amaranticolor	30	30	+
C. ambrosioïdes	29	29	+
C. botrys	27	27	+
C. ficifolium	32	10	+
C. foetidum	37	36	+
C. hybridum	10	7	+
C. multifidum	3	3	+
C. polyspermum	34	14	+
C. quinoa	20	19	+
C. rubrum	20	10	+
Kochia scoparia	19	4	+
Spinacia oleracea	60	37	+
Compositae			
Calendula officinalis	30	0	.
Zinnia elegans	15	0	.
Convolvulaceae			
Calystegia sepium	10	2	+
Convolvulus elongatus	32	15	+
C. pantapetaloïdes	23	3	+
C. siculus	10	6	+
Ipomea purpurea var. *caerulea*	10	5	+
Pharbitis purpurea	15	7	+
Cruciferae			
Brassica oleracea	20	0	.
Cucurbitaceae			
Bryonia dioïca	7	2	+
Citrullus colocynthis	4	2	+
C. lanatus var. *citroïdes*	14	0	.
Cucumis melo ssp. *agrestis*	14	0	.
C. sativus cv. Blanc très gros de Bonneuil	25	25	+
Cucurbita ficifolia	10	0	.
C. maxima	6	0	.
C. mixta	12	0	.
C. pepo var. *pyriformis*	17	0	.
Luffa aegyptiaca	17	0	.
Euphorbiaceae			
Euphorbia dentata	15	14	+
E. foliata	22	6	+
E. lathyris	22	6	+
E. martini	20	0	.
E. myrsinites	3	0	.
E. terracina	30	15	+
E. wulfenii	24	0	.
Manihot flabellifolia	11	0	.
Ricinus communis var. *sanguineus*	3	0	.

Table 2 (*cont.*)

Plant species	Number of plants inoculated	Number of plants showing symptoms	Persistence of symptoms
Leguminosae			
Arachis hypogaea	24	20	+
Glycine max cv. 227 CNS	7	0	.
Mucuna pruriens	14	0	.
Phaseolus lathyroïdes	10	0	.
P. vulgaris cvs. Bountiful	34	0	.
Bush bean Bupee's stringless green pod	35	0	.
The Prince	29	0	.
Flageolet rouge	6	0	.
Triomphe de Farcy	5	0	.
Pisum sativum hybrid INRA 257	21	0	.
Vicia faba	9	0	.
Vigna sinensis	60	30	+
Malvaceae			
Abutilon arboreum	12	10	—
A. avicennae	27	24	—
A. indicum	6	5	—
A. molle sweet	24	23	—
Althea rosea	20	3	—
A. sinensis	22	3	—
Gossypium arboreum	20	20	—
G. barbadense	20	15	—
G. hirsutum	25	25	—
Hibiscus asper	15	15	—
H. cannabinus	18	18	—
H. esculentus	20	20	—
H. gossypinus	24	23	—
H. manihot	11	10	—
H. micranthus	9	7	—
H. moscheutos	2	2	—
H. pedunculata	3	3	—
H. sabdariffa	10	10	—
H. syriacus	3	2	—
H. trionum	12	12	—
Lavatera cretica	16	4	—
L. thuringiaca	10	10	—
L. trimestris	24	21	—
Malope trifida	17	17	—
Malva alcea	20	19	—
M. crispa	30	25	—
M. moschata	24	24	—
M. neglecta	21	11	—
M. pusilla	19	19	—
M. rotundifolia	12	10	—
M. sylvestris	13	9	—
M. verticillata	21	11	—
Malvastrum coromandelianum	6	6	—
Sida cordifolia	7	4	—
S. rhombifolia	41	4	—
S. stipulata	12	7	—
S. urens	8	5	—
Thespesia populnea	2	1	—
Urena lobata	5	3	—
Wissadula cretica	16	4	—

Table 2 (*cont.*)

Plant species	Number of plants inoculated	Number of plants showing symptoms	Persistence of symptoms
Polygonaceae			
Polygonum persicaria	3	0	.
Rumex alpinus	18	0	.
R. arifolius	2	0	.
R. crispus	19	0	.
R. scutatus	12	0	.
Solanaceae			
Atropa belladonna	15	0	.
Capsicum annuum	20	0	.
C. frutescens	4	0	.
Datura metel	4	0	
D. stramonium	17	0	.
Lycopersicon esculentum	20	0	.
Nicotiana clevelandii	18	18	+
N. glutinosa	18	0	.
N. tabacum cvs. Samsun	12	0	.
White Burley	10	0	.
Xanthi	25	0	
Petunia hybrida	20	0	.
Physalis floridana	12	0	.
Solanum melongenum	12	0	.
S. nigrum	20	20	+

Chenopodiaceae

Chenopodium album L.: light spotting; *C. amaranticolor* Coste et Reyn.: dotting; *C. ambrosioïdes* L.: a few large chlorotic local lesions developed 3–4 days after inoculation and were followed by a spotting; *C. botrys* L. dark green and chlorotic mosaic; *C. ficifolium* Sm.: spotting and irregular vein banding; *C. foetidum* Schrad.: numerous chlorotic small local lesions 3 days after inoculation, then chlorotic specking and general chlorosis; *C. polyspermum* L.: rare chlorotic ring spotting; *Spinacia oleracea* L.: coarse chlorotic mosaic.

Convolvulaceae

Vein clearing (*Calystegia sepium, Bryonia dioïca*); light green blotches or irregular vein banding (*Convolvulus*).

Cucurbitaceae

Cucumis sativus L. cv. Blanc très gros de Bonneuil: large chlorotic local lesions on cotyledons 7 days after inoculation and fine vein chlorosis on the succeeding leaves.

Leguminosae

Vigna sinensis Savi: regular and fine vein chlorosis; *Arachis hypogaea* L.: dark and light green variegation.

Solanaceae

Nicotiana clevelandii A. Gray: dark and light green and whitish mosaic, dwarfing; *Solanum nigrum* L.: mosaic and stunting.

In vitro *properties*

Dilution end point. Seven days after inoculating five okra plants with a 10^{-6} dilution of the crude sap, symptoms appeared on one or two plants; no symptoms appeared on plants inoculated with sap after dilution to 10^{-7} or 10^{-8}.

Thermal inactivation point. Sap heated to 50 °C for 10 min was as infective as untreated sap but when heated at 72 °C it lost 66 % of its infectivity calculated from the infectivity index. 8 % of the infectivity remained at 78 °C and 2 % at 80 °C. At 82 °C the sap was non-infective.

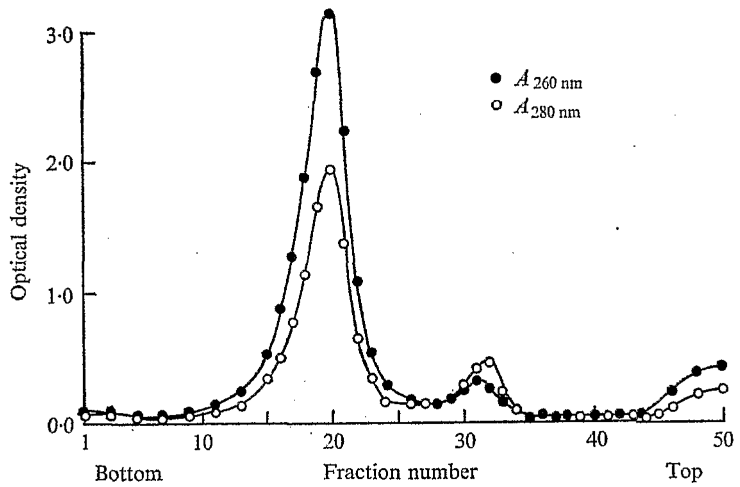
Ageing in vitro. The infectivity of sap was greatly reduced after 7–9 days and was lost after 10 days.

Resistance to air drying. Infectivity was lost after 76 days of drying.

Effect of freezing. Ground infected okra leaves (harvested 17 days after inoculation) frozen and thawed every day for 28 days remained infective.

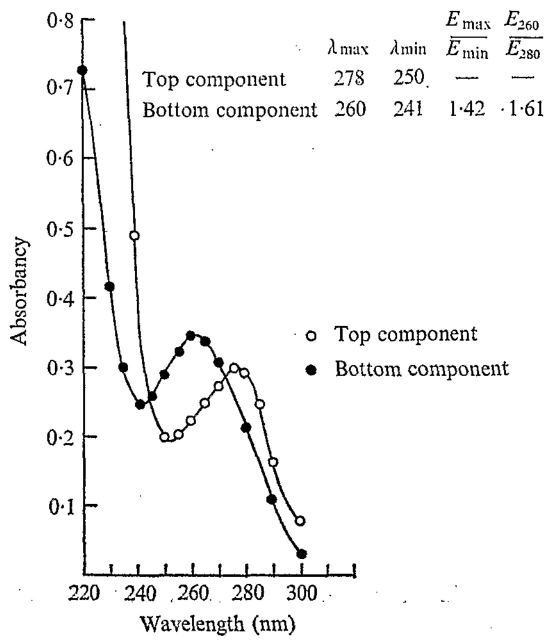
Physico-chemical properties

Electron microscopy. Electron micrographs (Pl. 2, fig. 1) of OMV revealed the presence of isometric particles *c.* 28·5 nm diam. (± 2·5 nm). The preparation showed numerous empty particles. As the staining procedure may give rise to empty particles, the occurrence of empty particles in the purified preparation was tested by density gradient centrifugation.



Text-fig. 1. The ultra-violet absorbancy profile at 260 nm (●–●) and 280 nm (o–o) after separation of top and bottom components of OMV by sucrose density gradient centrifugation. 2 ml of virus preparation ($OD_{260\,nm}$ 20) was layered on a gradient of 10–50 % sucrose (27 ml total) in 0·1 M sodium phosphate buffer pH 7·0 containing 0·01 M EDTA and centrifuged for 6 h at 25000 rev/min in the SW 25.1 rotor.

Density gradient centrifugation. The optical densities of the two components obtained from the purified preparation by sucrose density gradient centrifugation are shown in Text-fig. 1. The u.v. absorption characteristics of the top and bottom components of OMV are shown in Text-fig. 2. The absorbance obtained with the bottom component was typical of a nucleoprotein with a maximum at 260 nm and a minimum at 241 nm. The 260 : 280 absorption ratio was 1·62. The absorbancy profile obtained with top component was typical of a protein with a maximum at 278 and a minimum at 250 nm.



Text-fig. 2. Ultra-violet absorption of top (o–o) and bottom (●–●) components of OMV separated by sucrose density gradient centrifugation.

Analytical ultracentrifugation. Pl. 2, fig. 2, shows the Schlieren pattern of an unfractionated preparation of OMV. The two components had sedimentation coefficients of 106 S (bottom component) and 42 S (top component).

Type of nucleic acid. Preliminary experiments on the comparative action of ribonuclease and deoxyribonuclease on the nucleic acid and tests with the Mejbaum (1939) and Dische (1930) reactions showed that the nucleic acid of OMV was a ribonucleic acid.

Experiments by J. P. Bouley and L. Givord (unpublished) have demonstrated that the base composition of the RNA was U 25·5, C 39·8, G 17·2, A 17·5.

Molecular weight of protein subunits. Preliminary experiments by means of polyacrylamide gel electrophoresis have shown the molecular weight of the OMV protein subunits to be *c.* 20000.

Serological properties. In gel diffusion serological tests, the purified OMV reacted with homologous antiserum up to a dilution of 1/2048. Certain properties of OMV

Table 3. *Differences between OMV and viruses occurring in Malvaceous plants or infecting* Hibiscus esculentus

Virus	References	Symptoms	Transmissions*	*In vitro* properties	Morphology
Abutilon mosaic virus 1 Baur	1	.	.	‡	‡
Infectious chlorosis of Malvaceae	2	.	‡	.	.
Leonurus sibiricus mosaic	3	.	‡	.	.
Cotton anthocyanosis	4	.	‡	.	.
Cotton Leaf curl	5	‡	.	.	.
Cotton Leaf crumple	6	‡	.	.	.
Hibiscus esculentus yellow vein mosaic virus	7	.	‡	.	.
H. esculentus vein thickening	8	‡	.	.	.
H. ternifolius witches' Broom and phyllodie	9	‡	‡	.	.
H. manihot mosaic disease	10	.	‡	.	.
H. rosa-sinensis leaf curl	11	‡	.	.	.
H. rosa-sinensis line pattern	12	‡	‡	.	.
Malva yellow vein mosaic	13	.	‡	.	.
Malva virus 1 Ryzhkov	14	.	‡	‡	.
Malva yellow virus	15	.	‡	.	.
Malva vein clearing	16	.	.	.	‡
Wissadula amplissima mosaic virus	17	.	‡	.	.
Jute mosaic virus	18	.	‡	.	.
Rhyncosia minima mosaic	19	.	‡	.	.
Watermelon mosaic virus	20	.	.	.	‡
Beet curly top virus	21	.	‡	.	.
Tobacco ringspot virus	22	.	‡	‡	.
Tobacco broad ringspot	23	.	.	‡	.
Tobacco streak virus	24	.	‡	‡	.

* Including host range and mode of transmission.

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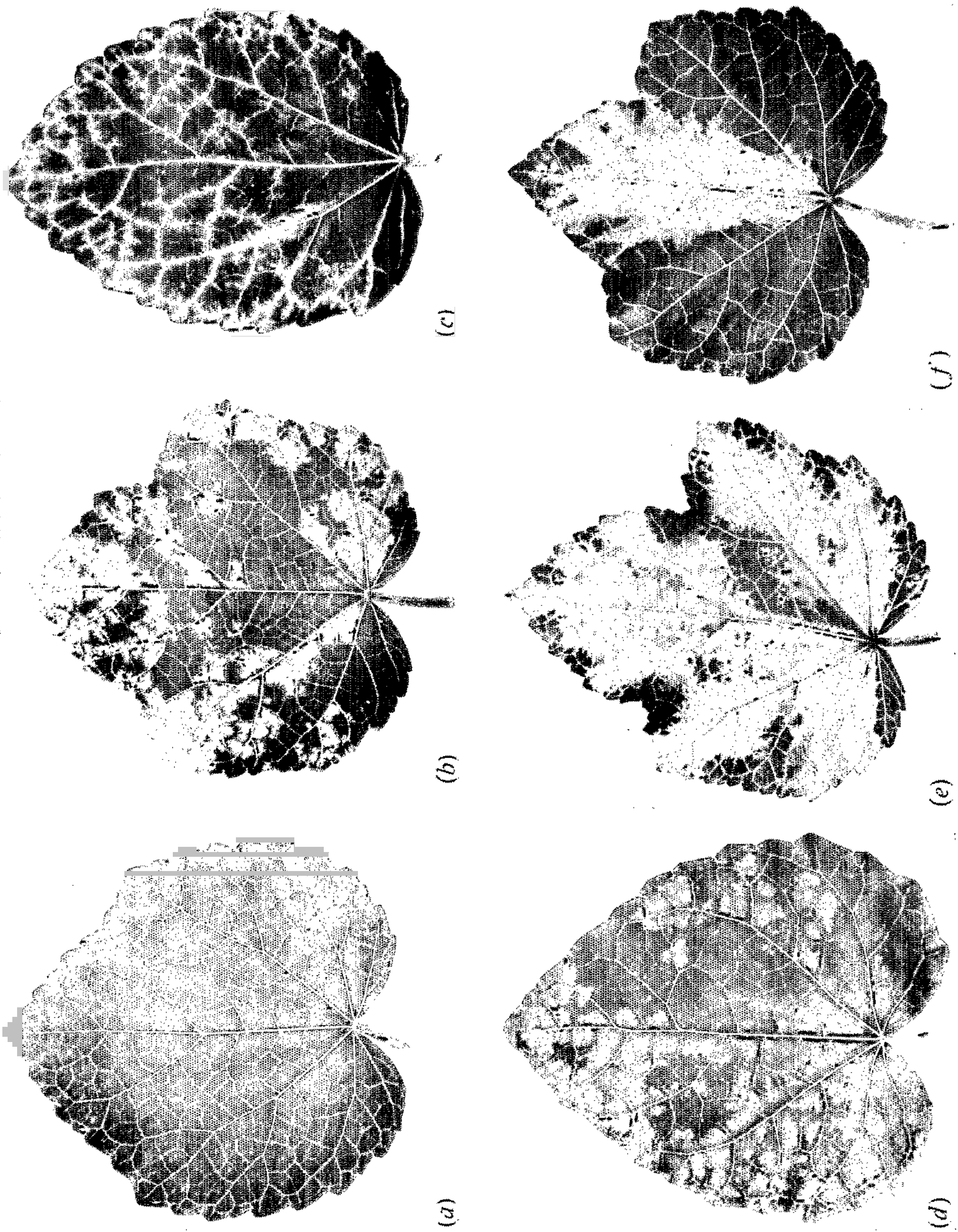
(a)
(b)
(c)
(d)
(e)
(f)

Fig. 1

Fig. 2

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(40% cytosine in the RNA, thermal inactivation point of 80 °C, presence of a top component) suggested that the virus was a member of the turnip yellow mosaic virus group (Gibbs, 1969). When OMV was tested against a TYMV antiserum with a homologous titre of 1/8000, it reacted up to a serum dilution of 1/32.

DISCUSSION

Numerous virus diseases have been described on *H. esculentus* and other Malvaceous plants in many different countries. Table 3 shows the virus diseases that have been reported in Malvaceae and the reasons for considering that OMV is probably not the cause of any of these diseases.

The following properties: base composition of the nucleic acid, the presence of empty protein shells, the high thermal inactivation point, and the positive serological relationships to TYMV, indicate that OMV is a member of the turnip yellow mosaic virus group or 'Tymovirus' group (Harrison *et al.* 1971). Further experiments are in progress to establish in detail the serological relationships between OMV and other members of 'Tymovirus' group.

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EXPLANATION OF PLATES

Plate 1

Symptoms of OMV in *Hibiscus esculentus* L. cv. Clemson spineless and cv. Court mechanically inoculated at the cotyledonary stage. (*a*) Healthy first leaf of Clemson spineless. (*b*) Mosaic on first leaf of Clemson spineless. (*c*) Veinal chlorosis on first leaf of Clemson spineless. (*d*) Mosaic on first leaf of cv. Court. Generally Court displayed symptoms only on the first leaf. (*e*) Large bands along the main veins of the second leaf of Clemson spineless. (*f*) Third leaf of Clemson spineless bearing a single large band.

PLATE 2

Fig. 1. Electron micrograph of a purified suspension of OMV.

Fig. 2. Schlieren pattern of unfractionated preparation of OMV in 0·01 M sodium phosphate buffer pH 7·0. Photograph taken 8 min after reaching a speed of 42040 rev/min, temperature of run 22·7 °C, bar angle 55°, sedimentation from left to right. The peaks from left to right: top component (42 S), bottom component (106 S).