IDENTIFICATION AND CHARACTERIZATION OF CUCUMBER MOSAIC AND BEAN YELLOW MOSAIC VIRUSES AFFECTING CHICKPEA (CICER ARIETINUM L.) IN INDIA

Thesis submitted to the
Andhra Pradesh Agricultural University
in part fulfilment of the requirements
for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

Shri T. Venkata Chalam has satisfactorily prosecuted the course of research and that the thesis entitled "Identification and characterization of cucumber mosaic and bean yellow mosaic viruses affecting chickpea (*Cicer arietinum* L.) in India" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

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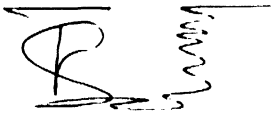

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Department of Plant Pathology
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Andhra Pradesh
India

CERTIFICATE

This is to certify that the thesis entitled "Identification and characterization of cucumber mosaic and bean yellow mosaic viruses affecting chickpea (*Cicer arietinum* L.) in India" submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Agriculture, in the major subject of Plant Pathology of the Andhra Pradesh Agricultural University, Hyderabad, is a record of the *bona fide* research work carried out by Mr. T. Venkata Chalam under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma.

All assistance and help received during the course of the investigations have been duly acknowledged by him.

Y.L. NENE
Co-Chairman
Advisory Committee

J. SUBBAYYA
Chairman
Advisory Committee

The thesis approved by the Advisory Committee.

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Member (A. NARAYANAN)

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CONTENTS

List of Illustrations

List of Tables

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The fellowship and facilities provided by ICRISAT during the course of this investigation are gratefully acknowledged.

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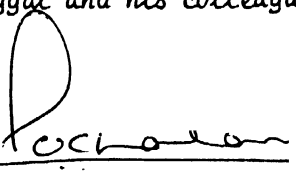

November 24, 1982

T. VENKATA CHALAM

ABSTRACT

Author	:	T. Venkata Chalam
Title of the thesis	:	Identification and characterization of cucumber mosaic and bean yellow mosaic viruses affecting chickpea (*Cicer arietinum* L.) in India
Degree	:	Ph.D.
Faculty	:	Plant Pathology
Guides	:	Y.L. Nene (ICRISAT) J. Subbayya (APAU)
University	:	Andhra Pradesh Agricultural University
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Two mechanically transmissible viruses; viz., cucumber mosaic virus (CMV) and bean yellow mosaic virus (BYMV) were found to naturally infect chickpeas. These were identified by studying their host range, biophysical properties, insect transmission, electron microscopy and serology. Also elite chickpea germplasm accessions were screened for resistance to these viruses.

Both the viruses initially produced twisting of terminal bud in chickpea. Subsequently, CMV either produced wilting and death of plants or proliferation and bushiness of branches bearing very small green leaves and very small pods, whereas BYMV produced proliferation of branches with narrow or filiform leaves, bearing very small pods.

The CMV could infect 13 plant species belonging to five families, whereas the host range of BYMV was restricted to Leguminosae only. The results on *in vitro* properties indicated that CMV was more stable than BYMV. Both the viruses could be transmitted by two aphid species; viz., *Aphis craccivora* and *Myzus persicae* in a non-persistent manner.

An efficient purification procedure developed for CMV yielded 80-100 mg of virus per kg of plant tissue. The CMV particles were

spherical and measured about 30 nm in diameter. The purified CMV had a buoyant density and sedimentation coefficient of 1.325 g/ml and 104S, respectively. Using the polyacrylamide gel electrophoresis (PAGE), the molecular weight of CMV coat protein was determined to be 25,000 daltons. The PAGE of CMV RNAs revealed the presence of four RNA species. The present strain of CMV was relatively more closely related to CMV-C pool, followed by CMV-Ix and M-CMV. The antiserum produced to CMV had a titre of 1/1024 as determined by agar double-diffusion test.

A satisfactory purification procedure was developed for BYMV. The particles of BYMV measured 750 nm in length and 15 nm in width. Pinwheels and laminate aggregates typical of potyviruses were observed in the cytoplasm of BYMV-infected chickpea cells. Since the virus preparation free of host components could not be obtained, the physico-chemical properties of BYMV were not determined.

Elite chickpea germplasm accessions were screened for resistance to both CMV and BYMV, and sources of resistance were identified for both the viruses.

List of symbols and abbreviations

A		absorbance
A_{260}		absorbance at 260 nm
A_{280}		absorbance at 280 nm
A max		absorbance maximum in a absorption spectrum
A min		absorbance minimum in a absorption spectrum
APAU		Andhra Pradesh Agricultural University
BYMV		bean yellow mosaic virus
cm		centimeter(s)
CMV		cucumber mosaic virus
cv		cultivar
DEP		dilution end point
E		an alternative symbol for A
$E_{260}{}^{0.1\%}_{10\ mm}$		specific extinction coefficient = the absorbance value of a 0.1% solution in a cuvette with an optical path length of the light of 10 mm
$E_{280}{}^{0.1\%}_{10\ mm}$	:	idem, but at a wave length of 280 nm
EDTA	:	ethylenediaminetetraacetic acid, sodium salt
ELISA	:	enzyme-linked immunosorbent assay (serological test)
g	:	gram(s) (a unit symbol of mass)
g	:	initial acceleration in free fall. Values in ultracentrifugation are given in this gravity unit
HCl	:	hydrochloric acid
hr	:	hour(s)
ICARDA	:	International Center for Agricultural Research in the Dry Areas
ICC	:	ICRISAT chickpea
ICRISAT	:	International Crops Research Institute for the Semi-Arid Tropics
KCl	:	potassium chloride
kg	:	kilogram(s)
KH_2PO_4	:	potassium phosphate, mono basic

LIV	longevity *in vitro*
M	molar concentration in grams per litre
mM	milli molar concentration = 0.001M
mg	milligram = 0.001 g
mA	milli ampere
min	minute(s)
ml	milliliter = 0.001 litre
mm	millimeter = 0.001 meter
mol.wt.	molecular weight
N	normality
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium phosphate, monobasic
Na_2HPO_4	sodium phosphate, dibasic
NaOH	sodium hydroxide
nm	nanometre = 10^{-9}m (formerly mμ was used)
OD	alternative symbol for A
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pH	hydrogen ion exponent (a unit symbol for the degree of alkalinity or acidity)
PO_4	phosphate
psi	per square inch
PSV	peanut stunt virus
PVP	polyvinyl pyrrolidone
RNA	ribonucleic acid
RNAs	ribonucleic acids
RNase	ribonuclease
rpm	revolutions per minute (centrifugation)
S	svedberg units, 10^{-13} sec. Values of sedimentation coefficients
$S_{20}{}^{W}$	: sedimentation coefficient at 20°C in water
SDS	: sodium dodecyl sulfate

TAV		tomato aspermy virus
TEMED		N,N,N',N' tetramethyl ethylene diamine
TIP		thermal inactivation point
Tris		tris (Hydroxymethyl) Aminomethane
Tween-20		polyoxyethylene sorbitan monolaurate
UV		ultraviolet
var		variety
v/v		volume/volume
w/v		weight/volume
w/w		weight/weight
µg		microgram = 10^{-6} g
µl		microlitre = 10^{-6} litre
Δx		difference in the distance travelled between any two time intervals
Δ^t	:	difference between any two time intervals
ω	:	rotor's angular velocity
$\overline{X}$	:	mean of the distances travelled

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important grain legume crop of dry-land agriculture in Asia, Africa and Central and South America. The total cultivated area of chickpea in the world is about 9.3 million hectares with annual production of about 4.8 million tonnes (FAO, 1980) The average yields per hectare are estimated to be around 700 kg. Chickpea is known by other names such as Bengal gram, gram, Egyptian pea, Spanish pea, Chestnut bean (all English), *Pois chiche* (French), *chana*, (Hindi), *homos* (Arabic), *grao-de-bico* (Portuguese), *garbanzo* or *garavance* (Spanish), etc.

About 50 pathogens have so far been reported on chickpea from different parts of the world (Nene, 1980). While many reports are mere records of occurrence, several diseases are widespread and a few are devastating. A survey of the literature reveals that only a few diseases have been investigated in detail (Nene *et al.*, 1978b).

Among chickpea diseases, wilt and root rots are considered to be of major importance. Considerable confusion existed in the past in diagnosing various chickpea disorders, particularly wilts and root rots (Nene *et al.*, 1978a). At ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), a project was initiated in 1974 on 'wilt complex'. During the course of investigations into the etiology of so-called wilt complex, two viruses; viz., alfalfa mosaic virus and pea leaf roll virus were identified and found to be the components of wilt complex (Nene *et al.*, 1978a). Chickpea plants affected by these two viruses often die prematurely and hence were wrongly considered to be affected by "wilt".

Present studies were initiated to know if other viruses

affected chickpeas under natural conditions in India, and their contribution, if any, to the "wilt complex".

With the exception of brief reports, very litte has been published on the identity of viruses affecting chickpeas in nature. Viral diseases of chickpea may have escaped detection in many countries since the macroscopic symptoms produced in such plants are often not conspicuous. Because of premature death of these plants, symptoms are frequently confused with those of wilt or root rots. It is therefore, necessary to observe the suspected plants carefully and the viral nature needs to be confirmed by specific procedures (Kaiser and Danesh, 1971a).

The present investigations were undertaken with the objective of isolating viruses affecting chickpeas in nature. Two mechanically transmissible viruses; viz., cucumber mosaic virus (CMV) and bean yellow mosaic virus (BYMV) were isolated and their identity was confirmed by procedures such as host range, biophysical properties, insect transmission, serology, and electron microscopy. In addition, production of antiserum and screening of germplasm lines for disease resistance were also attempted.

REVIEW OF LITERATURE

I. CHICKPEA VIRUSES

Six viruses have been reported to infect chickpea from different parts of the world (Nene, 1980). These were: alfalfa mosaic virus from Algeria, India, Iran, Morocco and U.S.A.; bean yellow mosaic virus from Iran and U.S.A.; cucumber mosaic virus from Colombia, India, Iran and USSR; lettuce necrotic yellows virus from Australia; pea leaf roll virus from Algeria, Bangladesh, Ethiopia, India, Iran, Lebanon, Morocco, New Zealand, Pakistan, Sudan, Syria, Tunisia, and Turkey; pea enation mosaic virus from U.S.A.

In India, four viruses have been reported to naturally occur on chickpea; viz., alfalfa mosaic virus (Nene *et al.*, 1978a), pea leaf roll virus (Nene and Reddy, 1976; Nene *et al.*, 1978a; ICRISAT, 1980), cucumber mosaic virus (Dhingra *et al.*, 1979) and an unidentified virus causing leaf distortion and mosaic (Mali and Vyanjane, 1980).

II. CUCUMBER MOSAIC VIRUS (CMV)

A. First Description

The virus was first described by Doolittle (1916) and Jagger (1916) in cucumber. The natural occurrence of CMV on chickpea was first reported by Kaiser *et al.* (1968). In India its natural occurrence on chickpea was reported by Dhingra *et al.* (1979).

B. Symptoms on Chickpea

Kaiser and Danesh (1971a) reported that CMV caused bushiness and shortening of internodes in chickpea. Dhingra *et al.* (1979) also

observed diseased plants both infected naturally and experimentally, developed (in 3-4 weeks after infection) excessive axillary shoots bearing very small, narrow, slightly chlorotic, and deeply dentate leaves. Affected plants remained stunted giving a bushy appearance. Phloem of roots and collar region stem developed slight necrosis in severe cases of infection. Diseased plants bore fewer flowers and pods than that of the healthy, and plants infected at a very early stage of growth died prematurely. Similar symptoms were observed in plants artificially inoculated with the causal virus.

C. Host Range

Host range of CMV as reported by Price (1940), Komuro (1958, 1973), Smith (1972), and Douine *et al.* (1979) is given below:

AIZOACEAE : *Mesembryanthum crystallinum* L., *Tetragonia expansa* Murr.

AMARANTHACEAE : *Amaranthus caudatus* L., *Amaranthus retroflexus* L. *A. tricolor* L., *Celosia argentia* L.

APOCYNACEAE : *Vinca minor* L., *V. rosea* L.

ASCLEPIADACEAE : *Asclepias syriaca* L.

BALSAMINACEAE : *Impatiens balsamina* L.

BORAGINACEAE : *Anchus* sp., *Cynoglossum amabile* Stapf and Drummond., *Heliotropium corymbosum* Ruiz and Pav., *H. peruvianum* L., *Myosotis sylvatica* Hoffm.

BROMELIACEAE : *Ananas comosus* Merr.

CAMPANULACEAE : *Lobelia cardinalis* L.

CAPPARIDACEAE : *Cleome spinosa* L., *Polanisia trachysperma* Torr and Gray.

CARRYOPHYLLACEAE : *Lychnis alba* Mill., *L. chalcedonica* L., *L. viscaria* L.

CHENOPODIACEAE : *Beta vulgaris* L., *Chenopodium album* L., *C. murale* L., *Spinacia oleracea* L.

COMMELINACEAE : *Commelina communis* L., *C. errecta* L., *C. nudiflora* L., *Tradescantia* sp., *Zebrina pendula* Schnizl.

COMPOSITAE : *Ambrosia elatior* L., *Calendula officinalis* L., *Callistephus chinensis* Nees., *Centaurea moschata* L., *Chrysanthemum* sp., *Cichorium endivia* L., *Emilia sagittata* (vahl)Dc., *Helianthus annuus* L., *H. debilis* Nutt., *Helichrysum bracteatum* Andr., *Lactuca sativa* L., *Tagetes erecta* L., *T. patula* L., *Zinnia elegans* Jacq.

CONVOLVULACEAE : *Cuscuta subinclusa* Durr., *C. californica* Hook & Arn., *Ipomoea batatus* Lam., *I. purpurea* Lam.

CRUCIFERAE : *Brassica rapa* L., *Lobularia maritima* Desv., *Nasturtium officinale* R. Br.

CUCURBITACEAE : *Benincasa cerifera* Cogn., *B. hispida* Cogn., *Bryonia alba* L., *B. dioica* L., *Bryonopsis laciniosa* Naudin., *Chayota edulis* Jacq., *Citrullus vulgaris* Schrad., *Cucumis anguria* L., *C. ficifolia* Bouche., *C. flexuosus* Naudin., *C. grossulariaeformis* Hort., *C. melo* L., *C. metuliferus* May., *C. odoratissimus* Moensch., *C. sativus* L., *C. utilissima* Roxbg. *Cucurbita maxima* Duchesne., *C. moschata* Duchesne., *C. pepo* L., *Ecballium elaterium* A. Rich., *Kedrostis foetidissima* Cogn., *Lagenaria leucantha* Rusby., *L. vulgaris* Sch., *Luffa acutangula* Roxb., *L. cylindrica* Roem., *Melothria scabra* Naudin., *Micrampelis lobata* (Mich.) Greene., *Memordica balsamina* L., *M. charantia* L., *M. involucrata* E. Meyer., *Physedra barteri* Cogn., *Sicyos angulatus* L., *Trichosanthes anguina* L.

DIPSACEAE : *Dipsacus* sp., *Scabiosa atropurpurea* L., *S. japonica* Miq.

EUPHORBIACEAE : *Euphorbia splendens* Bojer.

GERANIACEAE : *Geranium carolinianum* L., *Pelargonium hortorum* Bailey.

GRAMINEAE : *Euchlaena mexicana* Schrad., *Holcus sorghum* L., *Secale cereale* L., *Triticum aestivum* L., *Zea mays* L.

HYDROPHYLLACEAE : *Phacelia tanacetifolia* Benth., *P. whitlavia* Gray.

LABIATAE : *Coleus blumeri* Benth., *Nepeta cataria* L., *Salvia splendens* Ker.

LEGUMINOSAE : *Cicer arietinum* L., *Crotalaria intermedia* Kotschy., *Lupinus angustifolius* L., *L. leteus* L., *Phaseolus aureus* Roxb., *P. lunatus* L., *P. vulgaris* L., *Pisum sativum* L., *Trifolium incarnatum* L., *Vicia faba* L., *Vigna sinensis* Endl.

LILIACEAE : *Allium cepa* L., *Hyacinthus orientalis* L., *Lilium auratum* Lindl., *L. candidum*., *L. formosanum* Stapf., *L. croceum* Chaix., *L. longiflorum* Thunb., *L. testaceum* Lindl., *L. harrisii* Carr., *L. trigrinum* Ker-Gawl., *Tulipa gesneriana* L.

LOBELIACEAE : *Lobelia cardinalis* L., *L. erinus* L., *L. tenuitor* R. Br.

LOGANIACEAE : *Buddleia* sp.

LYTHRACEAE : *Lythrum salicaria* L.

MALVACEAE : *Hibiscus manihot* L., *Lavatera trimestris* L.

MUSACEAE : *Musa cavendishii* Lamb. *M. paradisiaca* Kuntze., *M. sapientum* Cowan., *M. textilis* Nees.

PASSIFLORACEAE : *Passiflora edulis* Sims.

PHYTOLACCACEAE : *Phytolacca decandra* L.

POLEMONIACEAE : *Gilia capitata* Dougl., *G. liniflora* Benth., *Phlox drummondii* Hook.

POLYGONACEAE : *Fagopyrum esculentum* Gaertn., *Rheum rhaponticum* L., *Rumex crispus* L.,

PORTULACACEAE : *Portulaca grandiflora* Hook.

RANUNCULACEAE : *Aquilegia canadensis* L., *Delphinium consolida* L.

SCROPULARIACEAE : *Antirrhinum majus* L., *Cymbalaria muralis* Gaertn., Mey and Scherb, *Mimulus moschatus* Dougl.,

Nemesia strumosa Benth., *Pentstemon* sp., *Verbascum phoeniceum* L., *Veronica longifolia* L., *Zaluzianskya villosa* Schmdt.

SOLANACEAE : *Atropa belladonna* L., *Browallia speciosa* Hook., *Capsicum annuum* L., *C. frutescens* L., *Datura metaloides* Dunal., *D. metal* L., *D. stramonium* L., *Hyoscyamus niger* L., *Lycopersicon esculentum* Mill., *Nicandra physaloides* (L.) Pers., *Nicotiana glauca* Grah., *N. glutinosa* L., *N. bigelovii* Wats., *N. rustica* L., *N. sanderae* Sander, *N. sylvestris* Spegaz and Comes, *N. tabacum* L., *Petunia hybrida* Vilm., *P. violacea* Lindl., *Physalis alkakengi* L., *P. angulata* L., *P. heterophylla* Nees., *P. lagascae* R. and S., *P. peruviana* L., *P. pubescens* L., *P. subglabrata* MacKenzie and Bush., *Salpiglossis sinuata* Ruiz and Pav., *Schizanthus wisetonensis* Low., *Solanum aviculare* Frost., *S. carolinense* L., *S. melongena* L., *S. nigrum* L., *S. nodiflorum* Desv., *S. tuberosum* L.

THYMELAEACEAE : *Daphna mezereum* L.

TROPAEOLACEAE : *Tropaeolum majus* L., *T. peregrinum* L.,

UMBELLIFERAE : *Anethum graveolens* L., *Anthriscus cerefolium* Hoffm., *Apium graveolens* L., *Daucus carota* L., *Foeniculum vulgare* Hill, *Pastinaca sativa* L., *Petroselinum hortense* Hoffm.

VIOLACAEA : *Viola cornuta* L.

D. *In vitro* Properties

The CMV has thermal inactivation point between 60° and 70°C, the dilution end point between 1:1000 and 1:10,000 and the longevity *in vitro* between 3 and 4 days (Gibbs and Harrison, 1970). However, different isolates of the virus differ in their *in vitro* properties.

The reported *in vitro* properties for different isolates/strains are given below:

TIP (°C)	DEP	LIV	Virus source	Reference
65-70	10^{-3} - 10^{-4}	-	Prunus	Willison and Weintraub (1957)
65-70	1:5000-1:10000	-	Chickpea	Dhingra *et al.* (1979)
60-65	1:10000-1:20000	3 days	Sugarbeet	Ragozzino (1973)
55-60	10^{-4}	1 day	Zinnia	Sastry *et al.* (1974)
80-90	10^{-4} - 10^{-5}	35-48 hrs	Ridge gourd	Goel and Verma (1973)
68-70	10^{-4} - 10^{-5}	19-22 days	Wild cucumber	Horvath and Szirmai (1973)
70	10^{-4}	3-5 days	Cowpea	Talens (1979)
65	10^{-4}	5 days	Gladiolus	Kanikska (1977)
68	1:2000	10-12 days	Cucumber	Topchiiska and Topchiiski (1977)
50-55	1:1000-1:5000	20-22 days	Potato	Matsunami *et al.* (1972)

TIP = Thermal inactivation point; DEP = Dilution end point;
LIV = Longevity *in vitro*.

E. Transmission

1. Mechanical

The CMV is readily sap-transmissible inducing a variety of symptoms depending on virus strain and host cultivar (Francki *et al.*, 1979).

2. Graft

Graft transmission of CMV has been documented by Schmidt and

The reported *in vitro* properties for different isolates/strains are given below:

TIP (°C)	DEP	LIV	Virus source	Reference
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1. Mechanical

The CMV is readily sap-transmissible inducing a variety of symptoms depending on virus strain and host cultivar (Francki *et al.*, 1979).

2. Graft

Graft transmission of CMV has been documented by Schmidt and

Karl (1970) in case of sann hemp, Jones (1976) in case of wineberry, and Dhingra *et al.* (1979) in case of chickpea.

3. Seed

The CMV has not been reported seed transmissible in chickpea. However, seed transmission occurs to varying degrees in 18 species (Neergaard, 1977), including some weed species. The hosts in which CMV is seed transmissible are given below with the transmission percentages in parentheses.

Benincasa hispida (1%), *Capsicum annuum* (1%), *Cerastium holosteoides* (2%), *Cucumis melo* and *Cucumis sativus* (trace), *Cucurbita moschata* (0.7%), *Cucurbita pepo* and *Echinocystis lobata* (9%), *Lamium purpureum* (4%), *Lupinus luteus* (14%), *Lycopersicon esculentum* (0.2%), *Phaseolus aureus* (5%), *P. vulgaris* (7%), *Spergula arvensis* (2%), *Stellaria media* (21-40%), *Vigna cylindrica* cv. Monarch, *V. sesquipedalis*, *V. unguiculata* (4-28%).

4. Dodder

Schmelzer (1957) obtained positive transmission of CMV through 8 species of *Cuscuta*, viz., *C. campestris*, *C. californica*, *C. subinclusa*, *C. gronovii*, *C. americana*, *C. europaea*, *C. epithymum*, and *C. lupiliformis*. In chickpea, positive transmission of the virus through *C. reflexa* was obtained by Dhingra *et al.* (1979).

5. Insect

CMV is transmissible in a non-persistent manner by a number of aphid species. The aphid species involved in the transmission are listed below:

Aphid species	Virus source	Reference
Amphorophoro rhododendri	Gladiolus	Swenson and Nelson (1959)
A. rubi	Raspberry	Harrison (1959)
A. sonchi	Gladiolus	Swenson and Nelson (1959)
Aphis cardui	Gladiolus	Swenson and Nelson (1959)
A. craccivora	*Capsicum annuum*	Szalay-Marzso and Solymossy (1961)
A. euonymi	Chickpea	Dhingra *et al.* (1979)
A. fabae	Pumpkin	Forghani *et al.* (1966)
A. gossypii	Cucumber	McClanahan and Guyer (1964)
A. nasturtii	*C. annuum*	Szalay-Marzso and Solymossy (1961)
A. pomi	Cherry	Swenson and Marsh (1967)
Brevicoryne brassicae	Cantaloups	Goudriet (1962)
Dactynotus henrichi	Cucumber	Heinze (1959)
Dysaculacorthum pseudosolani	Pumpkin	Forghani *et al.* (1966)
Dysaphis crataegi	-	Karl (1971)
Echinocystis lobata	Cucumber	McClanahan and Guyer (1964)
Hysteroneura setariae	Cantaloups	Goudriet (1962)
Macrosiphum barri	Cantaloups	Goudriet (1962)
M. californicum	Cherry	Swenson and Marsh (1967)
M. daphnidis	Cucumber	Heinze (1959)
M. euphorbiae	Cucumber	McClanahan and Guyer (1964)
M. gei	-	Vovk (1956)
M. pisi	Cantaloups	Goudriet (1962)
M. rosae	Gladiolus	Swenson and Nelson (1959)
M. solanifolii	Pumpkin	Forghani *et al.* (1966)
M. sonchifolii	Gladiolus	Swenson and Nelson (1959)
Myzocallis asclepiadis	Cucumber	McClanahan and Guyer (1964)
Myzus circumflexus	Gladiolus	Swenson and Nelson (1959)

Aphid species	Virus source	Reference
M. lythri	Cherry	Swenson and Marsh (1967)
M. persicae	Cherry	Swenson and Marsh (1967)
Pentalonia nigronervosa	Banana	Medeiros (1963)
Phorodon cannabis	Sann hemp	Schmidt and Karl (1970)
Rhophalosiphum maidis	Cantaloups	Goudriet (1962)
R. pisi	Gladiolus	Swenson and Nelson (1959)

F. Purification

Several methods for purification of CMV have been used most of which are modifications of the one devised by Scott (1963). The different methods used are summarized below:

1. The method of Scott (1963)

Infected leaves were homogenized in 0.5 M sodium citrate, pH 6.5, containing 5 mM EDTA and 0.5% thioglycollic acid. After squeezing through cheesecloth the extract was emulsified with chloroform. The aqueous phase obtained after low speed centrifugation was dialysed against 0.005M borate buffer, pH 9.0 and subjected to three cycles of differential centrifugation.

Tremaine (1968) reported more satisfactory preparation of Y strain of CMV by using Scott's method.

Takanami and Tomaru (1969) improved Scott's method in which sephadex gel filtration and EDTA were used to avoid aggregation of the virus.

2. The method of Lot *et al.* (1972)

They modified Scott's method in which virus was precipitated

by polyethylene glycol (10%) which was added to the aqueous phase, obtained after chloroform emulsification. After incubation, it was centrifuged at 12,000 g for 10 min and the sediment was resuspended in 5mM sodium borate buffer, 0.5mM EDTA, pH 9.0, to which Triton X-100 was added to 2% and stirred. It was then subjected to three cycles of differential centrifugation. The virus was further purified by centrifugation in 5-25% sucrose density gradients (Francki, 1972).

3. The method of Tomlinson *et al.* (1958)

Infected leaves were homogenized in 0.5M potassium phosphate buffer, pH 7.5, containing 0.1% thioglycollic acid. Clarification of the filtered sap was done by 8.5% n-butanol. The virus was recovered by one cycle of differential centrifugation, resuspending the high speed pellets in 0.05M potassium phsophate buffer, pH 7.5.

Tomlinson *et al.* (1959) used the same method as above, but further purified the virus by precipitation with 10% acetic acid followed by further differential centrifugation.

4. The method of Shohara and Osaki (1975)

They purified CMV by repeated precipitations with 8% PEG and 0.2M NaCl, followed by PEG solubilit gradient (0-6% PEG stabilized in 35-5% sucrose) centrifugation.

5. Other methods

Other methods for CMV purification (Morris *et al.*, 1978; Mossop *et al.*, 1976; Waterworth and Povish, 1975) involved slight modifications of one or the other of the above methods.

Virus yields varied according to the virus strain and host

plant used, but over 500 mg virus/kg of leaf material have been obtained in some instances (Francki *et al.*, 1979).

G. Properties of Virus Particles

1. Structure

The CMV particles are isometric, about 28-30 nm in diameter (Francki *et al.*, 1966, 1979; Debrot *et al.*, 1974; Betto *et al.*, 1964). Waterworth and Povish (1975) reported that a strain of CMV from Ixora plants (CMV-IX) differed in having particles of 24 nm in diameter rather than 28 nm reported for other CMV strains. Particles of a strain of CMV from Prunus measured 35 nm (Willison and Weintraub, 1957). Matsunami *et al.* (1972) observed CMV particles from potato to be 33 nm in diameter. The particles of CMV are known to disrupt when mounted unfixed in neutral phosphotungstate for electron microscopy (Francki *et al.*, 1979). However, particles remain intact and contrast well in uranyl acetate, pH 4.5 (Francki *et al.*, 1966).

2. Sedimentation coefficient ($S_{20}W$)

The particles of CMV sediment as a single component with $S_{20}W$ (Swedbergs) of 98.6 (Francki *et al.*, 1966). A slightly higher sedimentation coefficient (101S) was reported by Tomlinson *et al.* (1959).

3. Extinction coefficient ($E_{260\ 10mm}^{0.1\%}$)

It has been determined as 5.0 (Francki *et al.*, 1966).

4. A_{260}/A_{280} ratio

It has been determined as about 1.7 (Francki *et al.*, 1979).

5. Buoyant density

It has been determined as 1.361 and 1.359 g/ml for CMV-S and CMV-D, respectively (Lot and Kaper, 1976).

6. Nucleic acid

The CMV particles contain single-stranded ribonucleic acid, about 18% of particle weight (Kaper and Re, 1974). The fact that CMV contains a functionally divided genome has been revealed by Peden and Symons (1973), who detected four species of RNA.

The exact molecular weights of the CMV RNAs is a subject of debate. Values, ranging between 1.01-1.35, 0.89-1.21, 0.68-0.93 and 0.33-0.38 $x10^6$ daltons have been obtained for RNAs 1, 2, 3 and 4, respectively (Kaper and West, 1972; Peden and Symons, 1973; Marchoux *et al.*, 1973). However, electrophoretic mobilities of RNA species may differ slightly between strains (Hanada and Tochihara, 1980; Mossop, unpublished data cited by Francki *et al.*, 1979).

A small, replicating, single-stranded satellite RNA of about 1 $x10^5$ daltons has been isolated in some strains of CMV (Mossop and Francki, 1977; Gould *et al.*, 1978). One of these, called CARNA 5 (cucumber mosaic virus associated RNA 5) appeares to be responsible for lethal necrosis of tomato (Kaper and Waterworth, 1977).

7. Protein

The CMV can be readily disrupted in the presence of sodium dodecyl sulphate (SDS) (Kaper, 1975). Analysis of SDS-treated viral protein in polyacrylamide gel electrophoresis revealed that the protein consisted of a single polypeptide of 24,500 daltons (Habili and Francki, 1974) to 25,200 daltons (van Regenmortel *et al.*, 1972).

H. Serology

The immunogenicity of the virus is poor but, can be enhanced by fixation with formaldehyde (Francki and Habili, 1972; Habili and Francki, 1975). The virus precipitates on exposure to physiological salt solutions and, therefore, serological tests are usually done by agarose double diffusion tests in buffers of low molarity or in water (Francki *et al.*, 1966; Scott, 1968). Unstabilized virus may give one or two precipitin lines, one near the antigen well, caused by more or less intact virus and a second one near the antiserum well caused by degraded virus (Scott, 1968; Devergne and Cardin, 1970).

In its physical and chemical properties, CMV has affinities with tomato aspermy virus (TAV) and peanut stunt virus (PSV) (Francki *et al.*, 1979). More reliance has been placed on the results of serological tests than any other single criterion to trace the degree of relationships among the three viruses (Habili and Francki, 1975). Thorough serological analysis of CMV strains indicated the presence of at least two serogroups both of which are serologically related to some strains of TAV and PSV (Devergne and Cardin, 1975). In contrast, Habili and Francki (1975) failed to detect any serological relationship between the Q-CMV (Queensland strain of CMV) and V-TAV (Victoria strain of TAV). The lack of agreement on the serological relationship between TAV and CMV has been probably due to the use of different strains of the viruses which appear to be very variable, and to the lack of high titred antisera due to the poor immunogenicity of the viruses (Rao, 1982). However, Rao (1982) showed by agar double-diffusion test and ELISA, a distant serological relationship between six strains of CMV and two of TAV.

I. Strains

Many symptom variants of CMV are known to occur making the

virus often difficult to identify from symptoms alone. Strains well characterized by their biological properties include the Y strain which infects cowpea systemically (Price, 1934), the spinach strain (Bhargava, 1951) isolated from chlorotic and deformed leaves of spinach, which produces local lesions in several hosts, and M strain originally isolated from Price's (1934) yellow strain which induces severe chlorosis in several plant species (Mossop *et al.*, 1976). The CMV strains characterized physico-chemically include the Y strain (Kaper *et al.*, 1965), the Q strain (Francki *et al.*, 1966; Habili and Francki, 1974) and the S strain (van Regenmortel *et al.*, 1972). However, other strains reported are:

- CMV-Ixora (Waterworth and Povish, 1975) which differed from other strains in not inducing fern leaf symptoms in *Lycopersicon esculentum* cv. Rutgers. It also produced a few large chlorotic spots in *Cucumis sativus* rather than infecting systemically.

- CMV-Commelina strain (Doolittle and Webb, 1960).

- CMV-Cowpea strain (Anderson, 1955a).

- CMV-Easter lily strain (Brierley and Travis, 1958).

- CMV-Prunus strain (Willison and Weintraub, 1957)

d. Yield Losses

Kaiser *et al.* (1968) in a field inoculation trial in chickpea with CMV reported a yield loss of 52% due to infection. The yield losses depended on the stage of plant infection. The virus caused about 98% and 87% decrease in chickpea yields when plants were inoculated at 4 weeks (pre-bloom) and 9 weeks (full bloom) after planting, respectively (Kaiser and Danesh, 1971b).

K. Screening of Germplasm

There is no report in literature regarding screening of chickpea germplasm against CMV.

III. BEAN YELLOW MOSAIC VIRUS (BYMV)

A. First Description of the Virus

Pierce (1933), while working on the relative resistance of bean varieties to common bean mosaic (Bean virus 1), isolated another virus from a plant of the Red Valentine variety. Its specificity was first suspected not only from the fact that it produced symptoms distinct from that of the common bean mosaic virus, but also because it affected the varieties - Corbet Refugee, Great Northern UI No.1 and Robust, which are resistant to common mosaic. The virus was designated as bean virus 2 (Bean yellow mosaic virus). Occurrence of BYMV on chickpea which caused yellowing was first reported by Erwin (1958), and Erwin and Snyder (1958). However, they attributed the disease to other viruses also. The first authentic evidence on the natural occurrence of this virus on chickpea was provided by Kaiser and Danesh (1971a). In India, there is no report on the natural occurrence of BYMV on chickpea.

B. Symptoms on Chickpea

Kaiser and Danesh (1971a) reported two strains of BYMV on chickpea; a virulent strain causing wilting and yellowing, and a less virulent strain inducing feathery and deformed leaves.

The scattered incidence of aphid-transmitted virus yellowing has been reported by Erwin and Snyder (1958) in the causation of which

the bean yellow mosaic virus is stated to be a dominant factor, though other aphid-transmitted viruses, including pea enation mosaic and alfalfa mosaic viruses also infect this host in California.

The bright yellow color of the leaves and discolouration of the stem tissues in the phloem of plants affected with a virus-induced yellows disease was attributed to bean yellow mosaic and possibly other viruses (Erwin, 1958).

C. Host Range

BYMV, initially thought to be restricted to leguminous plants, is now known to infect several non-leguminous plants (Bos, 1970a). Host range of the virus under different families is given below.

AIZOACEAE : *Tetragonia expansa* Murr.

AMARANTHACEAE : *Gomphrena globosa* Murr.

CHENOPODIACEAE : *Chenopodium amaranticolor* Coste et Reyn., *C. quinoa* Willd.

LEGUMINOSAE : *Astragalus sinicus* Thunb., *Cassia tora* L., *Cicer arietinum* L., *Crotolaria spectabilis* Roth., *Glycine max* Merr., *Lathyrus odoratus* L., *Lens esculenta* Moench., *Lespedeza capitata* Michx., *Lupinus albus* L., *L. luteus* L., *L. pilosus* Walt., *Melilotus alba* Desr., *M. dentatus* Pers., *M. officinalis* Lam., *Phaseolus vulgaris* L., *P. acutifolius* Gray, *P. aureus* Roxb., *P. coccineus* L., *P. calcaratus* Roxb., *Pisum sativum* L., *Trifolium arvense* L., *T. compestre* Schreb., *T. hybridum* L., *T. incarnatum* L., *T. michelianum* Gaud., *T. pratense* L., *T. repens* L., *T. versiculosum* Savi., *Vicia faba* L., *V. hirsuta* Gray, *V. narbonensis* L., *V. villosa* Roth., *Vigna sesquipedalis* Wight.

PEDALIACEAE : *Sesamum indicum* L.

SOLANACEAE : *Nicotiana benthamiana* Domin.

D. *In vitro* Properties

In vitro properties depend greatly on virus source, test plant and test conditions. The thermal inactivation point is usually between 55 and 60°C though it may range between 50 and 70°C, the dilution end point usually 10^{-3} - 10^{-4}, and longevity *in vitro* normally 1-2 but some times over 7 days (Bos, 1970a).

In vitro properties reported for some of the isolates/strains of the virus are given below:

TIP(°C)	DEP	LIV	Virus source	Reference
60-70	10^{-3} - 10^{-4}	2 days	Red clover	Kume *et al.* (1970)
55-60	10^{-3} - 10^{-4}	2-3 days	White beans	Tu (1980)
60-62	10^{-2} - 10^{-3}	1 day	Broad bean	Shagrun (1973)
60-62	10^{-3}	1 day	Broad bean	El-Attar *et al.* (1971)
50-55	1:3000-1:5000		French bean	Tosso and Vidal (1976)
55-60	1:500-1:1000	1-2 days	Broad bean	Thottappilly *et al.* (1976)

TIP = Thermal inactivation point; DEP = Dilution end point;
LIV = Longevity *in vitro*.

E. Transmission

1. Mechanical

The virus is readily mechanically transmissible (Bos, 1970a)

2. Graft

No graft transmission of the virus has been reported in the literature.

3. Dodder

Schmelzer (1957) failed to transmit BYMV through eight *Cuscuta* spp., which could transmit CMV.

4. Seed

The virus is not seed-borne in French bean, but transmitted to a small percentage in *Lupinus albus, L. luteus, Melilotus alba, Pisum sativum* and *Vicia faba* (Bos, 1970a; Neergaard, 1977).

5. Insect

More than 20 aphid spp. can transmit the virus in a non-persistent manner (Kennedy *et al.*, 1962). Different aphid spp. reported to transmit the virus are given below:

Aphid species	Virus source	Reference
Acyrthosiphon pisum	Chickpea	Kaiser and Danesh (1971b)
A. sesbaniae	Chickpea	Kaiser and Danesh (1971b)
Amphorphora sp.	Broad bean	Swenson (1957)
A. sonchi	Broad bean	Swenson (1957)
Anuraphis tulipae	Broad bean	Swenson (1957)
A. bakeri	White clover	Manglitz and Kreitlow (1960)
Aphis cardui	Broad bean	Swenson (1957)
A. craccivora	Chickpea	Kaiser and Danesh (1971b)
A. fabae	Broad bean	Kaiser *et al.* (1971)
A. gossypii	Broad bean	Kaiser *et al.* (1971)

Aphid species	Virus source	Reference
A. helianthi	Broad bean	Kaiser *et al.* (1971)
A. medicaginis	Lupins	Corbett (1958)
Capitophorus fragaefolii	Broad bean	Swenson (1957)
Hyalopterus atriplicis	Broad bean	Swenson (1957)
Macrosiphum barri	Broad bean	Swenson (1957)
M. granarium	Broad bean	Swenson (1957)
M. rosae	Broad bean	Swenson (1957)
M. solanifolii	Broad bean	Swenson (1957)
Megoura viciae	Broad bean	Tapio (1964)
Myzus cerasi	Broad bean	Swenson (1957)
M. certus	Broad bean	Swenson (1957)
M. persicae	Broad bean	Swenson (1954)
M. solani	Broad bean	Swenson (1954)

Myzus persicae was found to be the most efficient vector (Swenson, 1957; Aldez, 1959), followed by *Macrosiphum pisi* and *Anuraphis tulipae* (Swenson, 1957). *Anuraphis sonchi* was found to be very inefficient vector whereas *Brevicoryne brassicae* failed to transmit this virus (Frandsen, 1952; Swenson, 1954; Swenson, 1957).

F. Purification

Viruses of the potato Y group, to which BYMV belongs, are characteristically difficult to manipulate *in vitro* because of their tendency to aggregate, both end-to-end and laterally (Shepherd and Pound, 1960). However, various methods have been followed satisfactorily for purification of BYMV which are given below:

1. The method of Uyeda *et al.* (1975)

They described purification of isolate no.30 of BYMV propagated in broad bean. Homogenization of frozen leaves was done

in 0.1M Tris-HCl buffer, pH 7.0, containing 0.05M EDTA and 1% 2-Mercaptoethanol. After passing through cheesecloth, the extract was emulsified with ½ volume of carbon tetrachloride. The virus was recovered from aqueous phase by precipitation with PEG (4%), followed by differential centrifugation. The virus pellets were suspended in 0.01M phosphate buffer. To avoid aggregation, 0.5M urea was added to the suspending medium. Further purification was achieved by 10-40% sucrose density gradient centrifugation.

2. The method of Huttinga (1973)

He purified BYMV by applying moderate centrifugal forces. Infected pea leaves were homogenized in 0.1M tris-thioglycollic acid, pH 9.0, along with carbon tetrachloride and chloroform (40 ml each for 150 ml of buffer). The virus was concentrated from aqueous phase by applying 2 cycles of differential centrifugation (1.5 hr at 26,500 g and 10 min at 8000 g). The virus was suspended in 0.1M tris-HCl buffer pH 9.0. The low centrifugal forces yielded unaggregated virus with negligible breakage. On the contrary, higher centrifugal forces produced considerable amounts of broken particles.

3. The method of Granett and Provvidenti (1975)

They purified two strains, viz., $BYMV^S$ and $BYMV^1$ by using a modification of Huttinga's method. Each gram of chilled leaves was homozenized in 7.5 ml of 0.1M tris buffer, 2 ml each of carbon tetrachloride and chloroform. The pH of the extract was adjusted with thioglycollic acid to 6.5 for $BYMV^S$ and 8.5 for $BYMV^1$. After breaking the emulsion, the virus was recovered from the aqueous phase by centrifugation at 15,000 rpm for 1 hr. The pellet was suspended in extracting buffer and clarified at 5000 rpm for 5 min. Higher centrifugal forces during either initial clarification or pelleting increased particle aggregation and breakage, and reduced virus yield.

4. The method of Uyemoto *et al.* (1972)

They purified BYMV and BCMV by the method followed by Damirdagh and Shepherd (1970) for purification of tobacco etch virus. One gram of infected tissue was homogenized with 2 ml of 0.5M potassium phosphate buffer, pH 7.0, containing 1% 2-Mercaptoethanol. After straining through cheesecloth the homogenate was stored overnight at 8°C in butanol (8 ml per 100 ml). After clarification by centrifugation at 8,500 rpm for 10 min, the virus was recovered from supernatant with 4% PEG followed by differential centrifugation (28,000 rpm for 1.5 hr; 8,500 rpm for 10 min, suspending the virus in 0.025M phosphate buffer, pH 7.0 containing 0.5M urea and 0.1% 2-Mercaptoethanol) Further purification was achieved by 10-40% sucrose density gradient centrifugation.

G. Properties of Virus Particles

1. Structure

Particles are elongated and flexuous, 750nm long and 15nm wide (Brandes and Quantz, 1955; Brandes, 1964). Particles of greater length have also been reported. Bos (1970b), using tobacco mosaic virus as an internal size standard, found particles of 660 nm length. Taylor and Smith (1968) and Bos (1970b) found that particles from *Chenopodium amaranticolor* were 50-60 nm longer than those from legumes. Contrarily, no influence of host on particle morphology could be noticed by Moghal and Francki (1981).

2. Sedimentation coefficient ($S_{20}W$)

The sedimentation coefficient of 5 isolates of BYMV ranged between 140 and 145S (Huttinga, 1975).

3. Extinction coefficient ($E_{260}\ ^{0.1\%}_{10mm}$)

It was determined as 2.4 (Uyeda *et al.*, 1975).

4. A_{260}/A_{280} ratio

The ratio for several preparations, corrected for light scattering varied from 1.24 to 1.31 with an average of 1.28 (Uyeda *et al.*, 1975).

5. Nucleic acid

BYMV has a single molecule of single-stranded RNA (Matthews, 1979) with a presumed molecular weight of about 3.0×10^6 as for turnip mosaic, tobacco etch and maize dwarf mosaic viruses (Hill and Benner, 1976), and pea mosaic virus (Abu-Samah and Randles, 1981). Lisa *et al.* (1981) estimated the molecular weight of RNA of a potyvirus, which caused mosaic on *Cucurbita pepo* and which showed slight serological relation with BYMV, to be 2.93×10^6 daltons.

6. Protein

Huttinga (1975) determined the molecular weight of coat protein of 5 isolates of BYMV as 34,000 daltons. Moghal and Francki (1976) reported a molecular weight of 33,000 daltons for 3 isolates of BYMV. A slightly higher molecular weight of 35,000 daltons has been reported for the BYMV isolate I of Dr. D.J. Hagedorn, University of Wisconsin (Uyemoto *et al.*, 1972). Yilmaz (1979) determined the molecular weight of coat protein of BYMV from broad bean as 33,350 - 33,820 daltons.

H. Serology

Antisera to BYMV with a titre of 1/2048 have been produced (Bos, 1970a). These could be further increased by chemically concentrating the globulin fraction of the sera giving titres up to 1/256,000 (Bercks, 1960; Bercks, 1961), but this is exceptional. Uyeda *et al.* (1975) obtained antisera to 3 strains of BYMV with titres ranging from 1024-2048.

Microprecipitin and precipitin tube tests are mostly used because intact virus particles do not diffuse in agar gels. However, the diffusion rate of filamentous viruses through agar could be increased by various detergents (Hamilton, 1964; Gooding and Bing, 1970; Purciful and Batchelor, 1977) or physical treatments (Tomlinson and Walkey, 1967) which allowed degradation of virus particles into readily diffusable units.

Granett and Provvidenti (1975) found that 0.3% (w/v) of SDS is good for degradation of BYMV into diffusable units than pyrrolidone or ethanolamine.

BYMV is serologically distantly related to bean common mosaic virus (Beemster and van der Want, 1951; Moghal and Francki, 1976), soyabean mosaic, sugar beet mosaic, watermelon mosaic viruses, and potato virus Y (Bercks, 1960).

I. Strains

Various strains differing in the symptoms they produce in certain plant species have been recognised. They are:

- Pea mosaic strain: Pea mosaic virus (Doolittle and Jones, 1925) was for long considered a separate virus,

differing from BYMV by the bright yellow symptoms it causes in pea and broad bean, and by its inability to infect most varieties of *Phaseolus vulgaris* (Schroeder and Provvidenti, 1966; Cousin, 1969). However, based on their close serological relationship, it was concluded that pea mosaic virus was a strain of BYMV (Taylor and Smith, 1968).

Cowpea strain of Anderson (1955b) and Brierly and Smith (1962), isolated from and produces systemic symptoms in *Vigna sinensis*, which is not infected by common strain of the virus. The cowpea strain gives severe systemic yellow spotting in *Chenopodium amaranticolor*.

Red clover necrosis strain of Zaumeyer and Goth (1963), produced necrosis in red clover and pea, local and systemic necrosis in *C. amaranticolor*, death of *Trifolium incarnatum*, *T. subterraneum* and *Vicia faba-minor*.

Pod distorting strain of Grogan and Walker (1948), isolated from Idaho Refugee bean and other varieties resistant to common bean mosaic virus.

Black root or χ-disease strain of McWhorter and Boyle (1946) which produces purpling of the leaf bases of the lower leaves. This purpling is accompanied by dying of the tissues within and often leads to premature death of the plant.

Necrotic lesion strain: A local lesion-producing virus isolated from infected bean pods by Zaumeyer and Fisher (1953). Top necrosis, produced by the pod distorting and X-disease strains seldom develops with this strain: *Phaseolus lunatus*, *Vigna sesquipedalis* and *V. sinensis*

are susceptible to the necrotic lesion strain, but are resistant to other strains of BYMV.

Severe yellow mosaic strain of Rex and Zaumeyer (1953) which causes strong epinasty, chlorosis and veinal necrosis of inoculated primary leaves of *P. vulgaris*. These symptoms are followed by top necrosis and death of certain bean varieties and severe yellow mosaic in others. No infection was obtained outside the Leguminosae.

Other strains of BYMV reported are: BYMV-Q from *Canna* sp. in Queensland (Moghal and Francki, 1976), BYMV-G isolated from *Gladiolus communis* in South Australia (Lee *et al.*, 1979) and BYMV-S from *Vicia faba* in South Australia (Randles *et al.*, 1980).

J. Yield Losses

Kaiser *et al.*, (1968), in a field inoculation trials with BYMV reported a yield loss of 77-92% due to infection in chickpeas.

Kaiser and Danesh (1971b) reported about 92-100% and 84-85% decrease in chickpea yields when plants were inoculated at 4 weeks (pre-bloom) and 9 weeks (full bloom) after planting, respectively.

K. Screening of Germplasm

There is no report in the literature on the screening of chickpea germplasm for resistance to BYMV.

MATERIALS AND METHODS

I. GENERAL

A. Virus Source

Cucumber mosaic virus (CMV) was isolated from fields of chickpea genetic resources unit, ICRISAT, from the plants showing tip necrosis. Bean yellow mosaic virus (BYMV) was isolated from fields of the Agricultural College Farm, Parbhani, Maharashtra from plants showing the narrow leaf symptoms.

B. Maintenance of Viruses

Both the viruses were maintained in the glasshouse on chickpea by periodic mechanical inoculations. A kabuli cultivar obtained from the local market and a kabuli germplasm accession, ICC-162, were used for the maintenance of CMV and BYMV, respectively. Besides chickpea, CMV was maintained on *Nicotiana rustica* var. White pathar, obtained from the Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India, and BYMV on *Phaseolus vulgaris* var. Dwarf obtained from the local market.

C. Plants

All chickpea and other test plants were raised in a mixture of Alfisol and farmyard manure (1:4, w/w) in 15 cm pots in the glasshouse (temperature range 25-30^{o}C).

D. Inoculum and Inoculations

The inoculum was prepared by grinding the infected chickpea leaves with a pre-chilled pestle and mortar in 0.05 M potassium

phosphate buffer, pH 7.5, containing 0.01M sodium sulphite and 0.01M sodium diethyl dithiocarbamate (4 ml per gram tissue). For determining physical properties of the viruses, the standard extract was prepared by grinding the infected chickpea leaves in the same buffer as above (1:1, w/v) but without any additives. Celite (1%, w/v) was added to the extract and rubbed onto the leaves of test plants with the broad end of the pestle. Inoculated plants were thoroughly rinsed with tap water. All chickpea plants were inoculated when 10 days old, whereas other plants were inoculated when young.

II. IDENTIFICATION OF VIRUSES BY SEROLOGY

The viruses under study were identified by serology employing double-diffusion test in agar (Ball, 1974) and enzyme-linked immunosorbent assay (ELISA) (Lister, 1978; Reddy *et al.*, 1981).

A. Double-Diffusion in Agar

Agarose 0.5% (Sigma chemicals, U.S.A.) was prepared in 0.01M potassium phosphate buffer, pH 7.0, to which 0.15M sodium chloride was added. It was then autoclaved at 15 lb psi for 15 minutes and 0.05% Merthiolate (Ethyl mercurithiosalicylic acid) was added to arrest microbial contamination. Eight ml of agar were poured into plastic Petri plates (5.3 cm diameter). After solidification, wells of 7 mm diameter were cut with a cork borer. The pattern consisted of 4 peripheral wells around a central well (5 mm distance from central well). Antisera to CMV and BYMV were obtained from Dr. H.A. Scott, University of Arkansas, U.S.A., and Dr. N. Conti, Laboratorio Fitovirologia, Torino, Italy, respectively. Purified virus and sap from infected chickpea were used as antigens. Healthy sap and buffer were kept as controls. The plates were incubated in a moist chamber (desiccator with moist

cotton in the bottom) at room temperature (25-28^{o}C) and observed daily for reaction.

In the case of BYMV, the virus was treated with 0.3% sodium dodecyl sulfate before incorporating into wells, to break the virus into diffusable units (Purcifull and Batchelor, 1977).

B. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Extraction of γ-globulins

One ml of antiserum was diluted with 2 ml of phosphate buffered saline (PBS) pH 7.4 (0.8% NaCl, 0.005M Na_2HPO_4, 0.001M KH_2PO_4, 0.02% KCl, 0.02% osdium azide) and mixed gently.

To the above, 3 ml of 36% sodium sulfate were added, mixed gently and centrifuged at 5000 rpm for 15 min.

The pellet was dissolved in 4 ml of 18% sodium sulfate, and centrifuged at 5000 rpm for 15 minutes.

The pellet was resuspended in 2 ml of PBS.

Dialysis was done against PBS in refrigerator at about 4^{o}C giving two changes of 1 litre each (first dialysis was for 4 hours and second overnight).

The γ-globulin solution was remove from the dialysis bag. The concentration of γ-globulins was adjusted to 1 mg/ml, assuming an extinction coefficient of 1.6 OD ($A_{280}^{0.1\%}$ = 1.6).

2. Conjugation of γ-globulins with alkaline phosphatase enzyme

The enzyme alkaline phosphatase (Sigma P-4502, type VII,

5 mg/ml) was precipitated from enzyme solution by centrifuging at 5000 rpm for 15 minutes, and dissolved in γ-globulin solution (1.5 mg of enzyme per 1 ml of 1 mg/ml γ-globulin solution).

Dialysis of conjugated γ-globulins was done against PBS with two changes of 1 litre each (first dialysis for 4 hours and second overnight).

Conjugated γ-globulins were treated with glutaraldehyde by dialysing against PBS containing 0.06% glutaraldehyde for 1 hour at room temperature.

Dialysis bags were transferred to PBS and dialysed overnight, at $4^{o}C$, giving two changes of 1 litre each.

Conjugated γ-globulins were removed and stored in glass tubes at $4^{o}C$.

3. Preparation of plates

Microtitre plates (Dynatech labs., England) were demarcated according to the various dilutions of test solutions, leaving out peripheral wells.

γ-globulin dilutions were made in coating buffer (0.02M Na_2CO_3, 0.02M $NaHCO_3$, 0.02% sodium azide, pH 9.6) and 0.2 ml was added to each well of the plate. Water was added to the peripheral wells and the plates were incubated at $37^{o}C$ for 2½ hours.

γ-globulins were poured off and the wells were washed thrice with PBS containing 0.05% Tween-20 (PBS-Tween) leaving the buffer in wells for 3 minutes for each washing.

Dilutions of test samples (healthy and infected) were made by grinding the tissue in antigen extraction buffer (PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) of mol. wt. 40,000) and straining the extract through cheesecloth.

Test samples (0.2 ml) were added to each well and water to peripheral wells. Plates were incubated overnight at $4^{o}C$.

Test samples were poured off and the wells were washed as earlier in PBS-Tween.

Dilutions of conjugated γ-globulins were made in conjugate buffer (PBS-Tween containing 2% PVP; 0.2% ovalbumin).

Conjugated γ-globulins (0.2 ml) were added to each well and water to peripheral wells. Plates were incubated at $37^{o}C$ for 3 hours.

γ-globulins were poured off and the wells were washed in PBS-Tween as earlier.

The substrate (0.2 ml), p-nitrophenyl phosphate (1 mg/ml), prepared in substrate buffer (9.7% aqueous diethanolamine, pH 9.8) was added to each well. Plates were incubated for 30-45 minutes at room temperature.

One drop of 3M NaOH was added to each well to stop further reaction. A positive reaction was detected by the development of yellow colour in the substrate.

III. HOST RANGE AND SYMPTOMS

Host range studies were carried out for CMV and BYMV, by mechanical inoculation of different plant species belonging to the

families: Aizoaceae, Amaranthaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Leguminosae and Solanaceae raised under insect-free glasshouse conditions. Four plants were inoculated with each virus and an equal number of plants were kept as controls. Plants were observed daily and symptoms recorded as and when they appeared.

IV. PHYSICAL PROPERTIES IN CRUDE SAP

A. Cucumber Mosaic Virus

Physical properties of CMV were determined in the standard extract following the procedure described by Bos *et al.* (1960). *Chenopodium amaranticolor* was used as an assay host.

1. Thermal inactivation point

Five ml samples of the standard extract in test tubes were subjected separately for 10 minutes to heat treatments starting from 45° and 90°C with an interval of 5°C. Immediately after heat treatment, the sap was cooled by passing tap water over the outer surface of the tube. To this was added celite (1%) and rubbed on the leaves of the assay host. Ten *C. amaranticolor* plants, each with 6 leaves were used replicating each treatment 6 times (Table 1). Observations were recorded 3 days after inoculation, when the local lesions were conspicuous in control treatment.

2. Dilution end point

The standard extract was diluted to get dilutions varying from 10^{-1} through 10^{-7}. After adding celite to each, they were separately rubbed on the leaves of the assay host. Eight *C. amaranticolor* plants, each with 6 leaves were used and each treatment was replicated 6 times (Table 2). Observations were recorded 3 days after inoculation.

TABLE 1. Randomization of treatments to determine thermal inactivation point (TIP)

Leaf number from top to bottom	Plant No. 1	2	3	4	5	6	7	8	9	10
1	a	g	c	i	e	j	f	b	h	d
2	b	h	d	j	f	a	g	c	i	e
3	c	i	e	a	g	b	h	d	j	f
4	d	j	f	b	h	c	i	e	a	g
5	e	a	g	c	i	d	j	f	b	h
6	f	b	h	d	j	e	a	g	c	i

a = control; b = 45°C; c = 50°C; d = 55°C; e = 60°C; f = 65°C; g = 70°C; h = 75°C; i = 80°C; j = 85°C.

TABLE 2. Randomization of treatments to determine dilution end point (DEP)

Leaf number from top to bottom	Plant No. 1	2	3	4	5	6	7	8
1	a	g	e	c	h	f	d	b
2	b	h	f	d	a	g	e	c
3	c	a	g	e	b	h	f	d
4	d	b	h	f	c	a	g	e
5	e	c	a	g	d	b	h	f
6	f	d	b	h	e	c	a	g

a = control (undiluted); b = 10^{-1}; c = 10^{-2}; d = 10^{-3}; e = 10^{-4}; f = 10^{-5}; g = 10^{-6}; h = 10^{-7}.

3. Longevity *in vitro*

The standard extract was stored in a 50 ml beaker at room temperature (22^{o}- 24^{o}C) and was inoculated daily on assay host, until the sap was confirmed to be non-infectious.

B. Bean Yellow Mosaic Virus

The preparation of the standard extract and the treatments were similar as described for CMV. Since no local lesion host could be found for this virus, the assay was done on kabuli chickpea (ICC-162), taking 10 seedlings for each treatment.

V. INSECT TRANSMISSION

Two aphid species, viz., *Aphis craccivora* and *Myzus persicae*, were obtained from field grown pigeonpea and tobacco, respectively and healthy colonies were raised before using them in transmission studies.

Aphids were fasted in Petri plates for an hour and then allowed an aquisition feeding period of one minute on virus-infected chickpea leaves. Thereafter they were transferred onto the healthy test plants for inoculation feeding period of 12-14 hours. Thereafter, the insects were killed by spraying Rogor (0.02%). Five to 10 aphids were used per plant and 10-day-old healthy chickpea seedlings (10) were used as test plants.

VI. PURIFICATION OF VIRUSES

A. Cucumber Mosaic Virus

Chickpea was used as a source plant since it supported a

high virus concentration. Plants were harvested 7-10 days after inoculation, when they showed terminal drooping. The entire plant, except roots, was used for purification. The purification procedure developed by Tomlinson *et al.* (1958) was used with modifications which is given below. All the steps of purification procedure were carried out at about 4°C by using pre-chilled materials and keeping the preparations in an ice bucket.

Infected chickpea plants were homogenized using a pre-chilled Waring blendor in 0.5M potassium phosphate buffer, pH 7.5 containing 0.1% sodium thioglycollic acid and 1mM EDTA (4 ml/g tissue). The sap was strained through 2 layers of cheesecloth. To the filtrate 8.5% (v/v) of n-butanol was added dropwise with constant stirring. Stirring was done for another 15 minutes. Then the mixture was centrifuged at 5000 rpm for 10 minutes in SS-34 rotor in Sorvall centrifuge. The supernatant was saved to which 8% polyethylene glycol (PEG) mol. wt. 6000 (Carbowax, Fisher Scientific Company, U.S.A.) and 0.2M sodium chloride were added and dissolved for 30 minutes and incubated for 90 minutes at 4°C. It was then centrifuged at 10,000 rpm for 10 minutes and the resulting pellet was resuspended in half the original volume of 0.025M potassium phosphate buffer pH 7.5, containing 1mM EDTA (PE). The pellet was thoroughly dissolved using a vortex mixer and centrifuged at 5000 rpm for 10 mintes. The supernatant was saved and centrifuged at 30,000 rpm for 2 hours in R-35 rotor in a Beckman preparative ultracentrifuge (model L5-50) at 4°C. The pellet was resuspended in PE and centrifuged at 5000 rpm for 5 minutes. The supernatant was collected and designated as partially purified virus.

Further purification of the virus was achieved by rate-zonal density gradient centrifugation in sucrose. Sucrose gradients were prepared by layering 9, 9, 9 and 6 ml of 40, 30, 20 and 10% sucrose prepared in PE, respectively. The gradients were kept at 4°C for 16 hours before use. Four ml of the partially purified virus preparation were layered over the gradients and centrifuged in SW-27 rotor for

90 minutes at 24,000 rpm. A light scattering zone was observed 2.4 cm below the meniscus when a narrow beam of light was passed through the top of the tube in a dark room. The zone was collected by an ISCO density gradient fractionator (model 185) with UA-5 absorption monitor.

It was diluted with an equal volume of PE and centrifuged for 2 hours at 30,000 rpm in R-35 rotor or 1 hour at 45,000 rpm in R-50 rotor. The pellet was dissolved in 0.5 to 1 ml of PE and designated as purified virus. At different steps of purification, the infectivity of the virus was assayed by mechanical inoculation on *C. amaranticolor*.

From another gradient, one-ml fractions were collected by the fractionator and each fraction was separately assayed on *C. amaranticolor* after diluting 10 times, to correlate the UV-absorption component with infectivity. Three leaves were used for each fraction and the average number of local lesions was obtained.

For studying biochemical properties of the virus, further purification was attempted by quasi-equilibrium density gradient centrifugation. The virus zone collected after the rate-zonal density gradient centrifugation was diluted with PE (1:1) and layered over 60-30% sucrose gradients, prepared by layering 12, 7, 7, and 7 ml of 60, 50, 40, and 30% sucrose in PE, respectively. After centrifugation at 24,000 rpm for 2 hours in SW-27 rotor the light scattering zone was withdrawn by a bent needle attached to a syringe. It was diluted, pelleted and dissolved in PE as described in the rate-zonal density gradient centrifugation.

B. Bean Yellow Mosaic Virus

Several methods (Uyeda *et al.*, 1975; Huttinga, 1973; and Uyemoto *et al.*, 1972) were used for BYMV purification and the one

described below was found to be suitable. All the steps of purification procedure were carried out at about 4°C as in the case of CMV.

BYMV infected chickpea tissue was homogenized in 0.1M Tris-PO_4 buffer, pH 8.5 containing 0.1% thioglycollic acid and 0.01M EDTA (1:3, w/v). Carbon tetra chloride (¼ volume of the buffer) was added during homogenization in a Waring blendor. After straining through two-layered cheesecloth, it was centrifuged at 5000 rpm for 10 minutes. The aqueous phase was recovered to which 4% PEG was added and incubated overnight at 4°C. Then it was centrifuged at 10,000 rpm for 10 minutes. The resultant pellet was dissolved in half the original volume of 0.01M Tris-PO_4 buffer, pH 8.5 containing 0.2M urea (TPU) and centrifuged at 5000 rpm for 5 min. Twenty-five ml of supernatant were layered over 13 ml of a sucrose pad (30% sucrose in TPU containing 4% PEG) and centrifuged at 24,000 rpm for 2 hours. The pellet was dissolved in TPU and centrifuged at 5000 rpm for 5 minutes.

Four ml of the supernatant (partially purified virus) were layered over the preformed sucrose density gradient column prepared in TPU as in case of CMV and centrifuged at 24,000 rpm for 90 min. An improminent, diffused virus zone formed just above the mid point was collected with a bent needle attached to a syringe. It was diluted with TPU and centrifuged at 24,000 rpm for 90 min. The pelle was dissolved in a small amount of 0.01M Tris-PO_4 buffer (purified virus).

VII. ELECTRON MICROSCOPY

Electron microscopy of purified preparations of CMV and BYMV was done. In addition, electron microscopy of dip preparations and ultrathin sections of BYMV-infected chickpea leaves was also done.

A. CMV-Purified Preparation

A drop of purified virus was kept on a parafilm on which a formvar and carbon-coated copper grid (300 mesh) was floated for 2 min. The grid was removed with fine forceps, excess solution wiped off by the pointed edge of filter paper and then floated on a drop of 1% uranyl acetate for one minute. Thereafter, it was taken out, excess stain wiped off, dried and observed under a Philips 201C electron microscope. From negative electron photo micrographs, the average diameter of the virus particles was calculated by measuring 100 particles, using a Bousch and Lomb magnifier.

B. BYMV-Purified Preparation

The procedure was similar to that described for CMV, except that neutral phosphotungstic acid was used for negative staining.

C. Leaf Dip Preparation

The dip preparation was done with BYMV-infected chickpea leaves since it is necessary in case of rod-shaped viruses to measure the particles in crude sap. A BYMV-infected chickpea leaf was chopped in a drop of water on a parafilm. The water drop was removed with the help of a pasteur pipette and used for the preparation of a grid for the electron microscopy. The length of 100 particles was measured, a length distribution histogram was plotted and a modal length calculated.

D. Ultrathin Sections of BYMV-Infected Chickpea Leaves

Ultrathin sections of BYMV-infected chickpea leaves were prepared to observe inclusion bodies induced due to infection.

Leaves were cut into small pieces of 1 mm x 1.5 mm. They were fixed in 3% glutaraldehyde for 2 hours, washed for ½ hr in 0.1M phosphate buffer pH 7.3, containing 0.5% sucrose. They were stored overnight in refrigerator in the same buffer. Next day, they were fixed in 2% osmium tetroxide made in 0.1M phosphate buffer pH 7.3 containing 5% sucrose for 2.5 hrs. They were washed in distilled water for 20 minutes and then dehydrated in 50, 70 and 90% acetone (20 min each). The final dehydration was done in 100% acetone thrice, each time for 20 minutes. Acetone dehydrated tissues were transferred to 1:1 mixture of 100% acetone and epoxy embedding resin (Ladd Inc., U.S.A.) and shaken for 1 hr. The tissues were drained on a paper towel and soaked in fresh resin for 4 hrs with a change of resin every hour. The tissues were drained once again and transferred to fresh resin in silicon rubber blocks which were kept at 65°C for 48 hours. Sections were cut (50-60 nm thick) with a 'Reichert-Jung Ultracut', stained in saturated uranyl acetate for 3 min and in 5% lead citrate for 3 min, washed, dried and observed under the electron microscope.

VIII. PHYSICO-CHEMICAL PROPERTIES OF PURIFIED CMV

A. UV-Absorption

The UV-absorption spectra of the purified CMV were recorded in Beckman D.B.G.T. spectrophotometer at wave lengths ranging from 200 to 300 mm. Ratios of 260/280, 280/260, and A max/A min were calculated. The yield of the virus was estimated using an extinction coefficient of 5.0 OD ($A^{0.1\%}_{260}{}_{10mm}$ = 5.0) (Gibbs and Harrison, 1970).

B. Sedimentation Coefficient

Sedimentation coefficient of the purified CMV was determined in a Spinco model E analytical ultracentrifuge. The purified virus suspended in 0.025M potassium phosphate buffer was run at 30,000 rpm.

After reaching the desired speed, the Schleiren pattern was photographed at 3 min intervals. The sedimentation coefficient was determined by using the following formula:

$$S_{20}W = \frac{\Delta X}{\Delta^t \times \omega^2 \times \bar{X}} \times 10^{13}$$

C. Buoyant Density

One ml of the purified CMV was layered over 4 ml of 40% cesium chloride (density = 1.42 g/ml) in 5 ml polyallmoer tubes, and centrifuged in SW-50 rotor at 40,000 rpm for 18-20 hrs at 4°C. The gradient column was fractionated by puncturing the bottom of the tube and collecting 0.2 ml fractions. These fractions were monitored in the Beckman Spectrophotometer at 260 nm, and the densities of the fractions were determined by their refractive indices measured with an Abbe refractometer.

D. Polyacrylamide Gel Electrophoresis (PAGE) of CMV Coat Protein

The PAGE of CMV coat protein was done following the method of Laemmli (1970).

Stock solutions

The stock solutions were prepared as follows:

Solution A (3M Tris-HCl buffer, pH 8.9)

Tris	:	36.6 g
1N HCl	:	48.0 ml
TEMED	:	0.23 ml

Made up to 100 ml with distilled water and adjusted pH to 8.9 with 1N HCl

Solution B (1M Tris-HCl buffer, pH 6.8)

Tris	:	5.98 g
1N HCl	:	48.0 ml
TEMED	:	0.46 ml

Made up to 100 ml with distilled water and adjusted pH to 6.8 with 1N HCl

Solution C (Acrylamide : Bis)

Acrylamide	:	30.0 g
Bis	:	0.735 g

Made up to 100 ml with distilled water.

Solution D (Acrylamide : Bis)

Acrylamide	:	10.0 g
Bis	:	2.5 g

Made up to 100 ml with distilled water.

Preparation of the Gels

Resolving (running) gel (10%)

Solution A	:	2.0 ml
Solution C	:	5.2 ml
1% Sodium dodecyl sulfate (SDS)	:	1.6 ml
10% Urea	:	4.8 ml
Distilled water	:	1.4 ml
1% Ammonium persulfate	:	1.0 ml

Stacking (spacer) gel (3.6%)

Solution B	:	0.5 ml
Solution D	:	1.4 ml
1% SDS	:	0.4 ml
10% Urea	:	1.2 ml
Ammonium persulfate	:	0.25 ml

With the help of a long-beaked Pasteur pipette, the resolving gel was poured into Bio-rod gel tubes up to a length of 6 cms and allowed to polymerize for 15 minutes. Over this stacking gel was poured up to a length of 1 cm and allowed to polymerize for 30 min. After polymerization, the tubes were inserted in the electrophoretic apparatus and filled with electrophoresis buffer (0.05M Tris, 0.384M glycine, 0.1% SDS, pH 8.3).

The virus was dissociated in 0.0625M Tris-HCl buffer, pH 6.8 containing 1% 2-Mercaptoethanol, 1% SDS and 6M urea. It was then incubated at 100°C for 2 min (in boiling water). Ten to 100 µl of the dissociated virus sample was layered on each gel. A mixture of six marker proteins (Bio-Rod Chemicals, U.S.A.) containing 20-100 µg of each polypeptide was also layered separately as well as mixed with virus proteins (co-electrophoresis). After electrophoresis at 30 volts for 6 hours at room temperature, the gels were taken out of the tubes by lubricating the inner walls of the tubes with water, using a syringe with a blunt needle.

The gels were stained for ½ hr in 0.2% coomassie brilliant blue prepared in 20% methanol in 7% acetic acid. Destaining of the gels was done overnight with 20% methanol in 7% acetic acid. The gels were than scanned at 540 nm in a Gillford spectrophotometer.

The molecular weight of CMV coat protein was determined by the linear relationship between the migration distance of marker proteins and the logarithm of their known molecular weights.

E. Polyacrylamide Gel Electrophoresis of CMV RNAs

The PAGE of CMV RNAs was performed using the method as described by Adesnik (1970). A combination of 2.5% acrylamide and 0.5% agarose was used.

Stock solutions

The stock solutions were prepared as follows:

Solution A (15% acrylamide)

Acrylamide	:	7.1 g
Bis	:	0.4 g

Dissolved in distilled water and made up to 50 ml.

Solution B (15 x buffer, pH 7.5)

Tris	:	0.4M
NaH_2PO_4	:	0.3M
EDTA	:	0.01M

Solution C

10% ammonium persulfate (prepared just before use)

Preparation of gels

Mix

Solution A	5.0 ml
15 x buffer	2.0 ml
Distilled water	7.7 ml
TEMED	0.03 ml
1% agarose	15.0 ml
10% ammonium persulfate	0.3 ml (added at the end)

The gels were immediately cast into the Bio-rod gel tubes with the help of a long-beaked Pasteur pipette. Two drops of distilled water were layered over the gel surface with the help of a syringe and needle to obtain a sharp meniscus. The gel tubes were inserted into an electrophoretic apparatus and filled with electrophoresis buffer (1 x buffer) to which 0.05% diethyl pyrocarbonate was

added to avoid possible contamination with ribonuclease (RNase). Before loading the samples, a pre-run was given at 5 mA/gel for ½ hr. Virus suspension was disrupted in 1.5 x buffer containing 10M urea, 4% SDS, 1% 2-Mercaptoethanol, 2mM EDTA, to which 0.001% bromophenol blue was added as a tracking dye.

The disrupted virus containing about 50 to 100 µg of nucleic acid was layered on the gels. Some gels were loaded with the disrupted virus that was treated with RNase at the rate of 1 µg/ml, and incubated at 25°C for 1 hour.

Electrophoresis was done at 5mA/gel at room temperature until the tracking dye reached near the bottom of the gel (about 3 hours). The gels were removed from the tubes as described in protein gels. They were suspended in 1M acetic acid for 15-30 minutes, stained in 0.2% toluidine blue, prepared in 0.4M acetate buffer, pH 4.7 for 15-30 min.

Destaining of the gels was done in several changes of distilled water in order to locate RNA bands.

IX. PRODUCTION OF ANTISERUM TO CMV

An Albino rabbit, weighing about 2 kg was used for immunization. One ml of purified virus (2-3 mg/ml) was emulsified with an equal volume of Fruend's incomplete adjuvant and injected into the rabbit intramuscularly in the thigh muscle of hind leg. A total of four injections were given at 10-day-intervals. Ten days after the last injection, the rabbit after 16 hr starvation was bled through a marginal ear vein by making an incision with a sterilized razor. The blood was collected in a test tube, allowed to clot for one hour at room temperature and kept at 4°C overnight. The serum was drawn with

a Pasteur pipette and centrifuged at 3000 rpm for 10 min in a clinical centrifuge to separate the serum from the blood plasma. Sodium azide (0.1%) was added as a preservative to the serum.

Ten-fold dilutions of the antiserum were made in normal saline and the titre was determined by employing the Ouchterlony agar double-diffusion test.

X. DETERMINATION OF THE SEROLOGICAL RELATIONSHIP BETWEEN THE PRESENT STRAIN OF CMV WITH OTHER KNOWN STRAINS

The serological affinity of the present strain of CMV with other strains was determined employing the Ouchterlony agar double-diffusion test. Antisera to eight strains of CMV, obtained from Dr. H.A. Scott (U.S.A.), Dr. H.E. Waterworth (U.S.A.), and Dr. R.I.B. Francki (Australia), along with the homologous antiserum were made to form precipitin bands with purified virus. Thickness of bands formed was taken as a method for determining serological affinity.

XI. SCREENING FOR DISEASE RESISTANCE

Chickpea germplasm lines that were reported to be resistant to other diseases; viz., wilt, root rots, Ascochyta blight and stunt (pea leaf roll virus) were screened for resistance to CMV and BYMV.

For each germplasm line, 10 seeds were sown in each 20 cm plastic pot. Ten-day-old seedlings were separately rub-inoculated with the standard extract of the two viruses. Observations on the number of plants infected were recorded for each line.

RESULTS

I. SYMPTOMATOLOGY IN CHICKPEA

A. Symptoms Produced by CMV on Chickpea

Two types of symptoms were produced by CMV in chickpea. In certain varieties, twisting of terminal bud occured 4-5 days after inoculation (Fig. 1a) which was followed by necrosis of terminal and axillary buds and wilting of the plant. Plants died 9-10 days after inoculation (Fig. 1b). In other varieties, inoculated plants after showing twisting and necrosis of terminal bud, did not wilt, but showed chlorosis followed by proliferation of branches. The new branches remained green which bore very small leaves and very few, small pods. Affected plants appeared bushy (Fig. 1c).

B. Symptoms Produced by BYMV on Chickpea

Twisting of the terminal bud occurred 6-7 days after inoculation which was very slight as compared with that by CMV infection. This was followed by initiation of very narrow leaves from new buds and was more evident 15 days after inoculation (Fig. 2a). As the time advanced, proliferation of both terminal and axillary branches occurred resulting into long and thin branches consisting of very narrow or filiform leaves. The leaves below the proliferated branches exhibited yellowing or interveinal chlorosis or mosaic depending on the genotype tested. The overall height of the plant was reduced. Such plants produced very few distorted flowers that were converted to very small pods (Fig. 2b). The seeds from infected plants were black, small and very much shrivelled (Fig. 2c).

II. HOST RANGE

The reactions of different plant species to CMV and BYMV are presented in Table 3 and Figures 3, 4, 5 and 6.

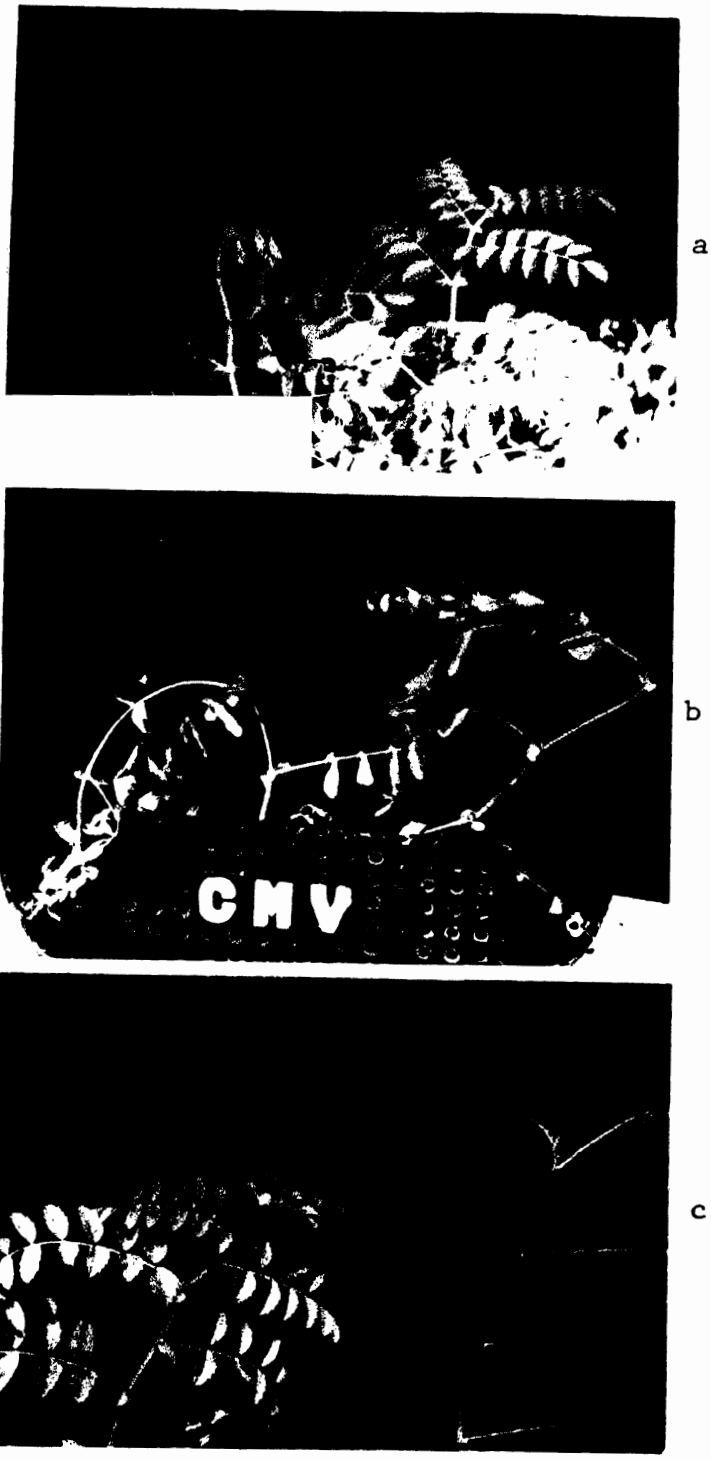


Fig. 1. Symptoms produced by cucumber mosaic virus on chickpea: (a) drooping of the terminal bud in infected plant (left), healthy plant on right; (b) wilting of the infected plant; (c) proliferation and bushiness of branches with small leaves in infected plant (left); healthy plant on right

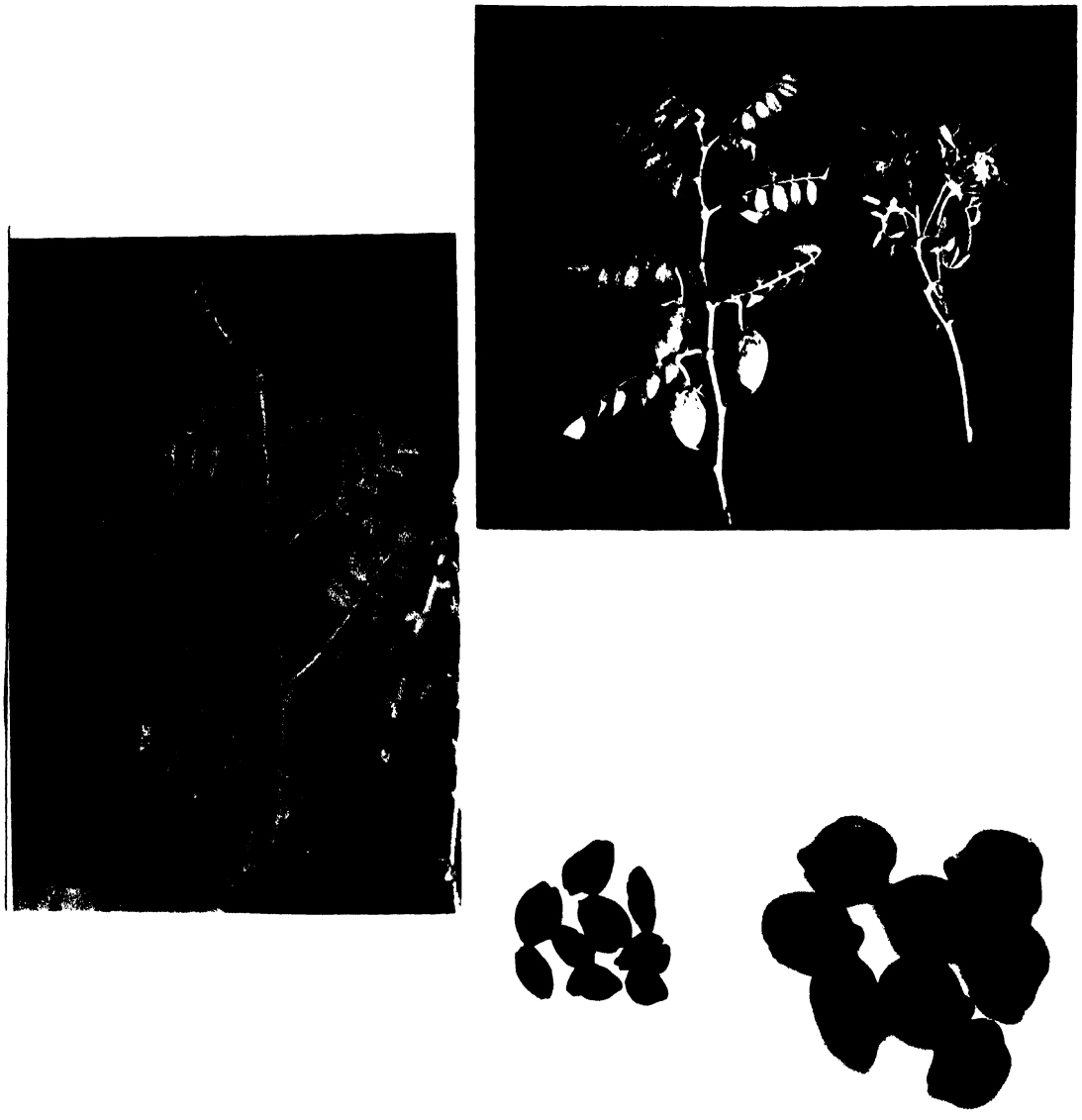


Fig. 2. Symptoms produced by bean yellow mosaic virus on chickpea: (a) an infected plant with narrow or filiform leaves; (b) showing proliferation of a branch with small pods (right), healthy branch on left; (c) discoloured, deformed and shrivelled chickpea seeds from infected plants (left), seeds from healthy plant on right.

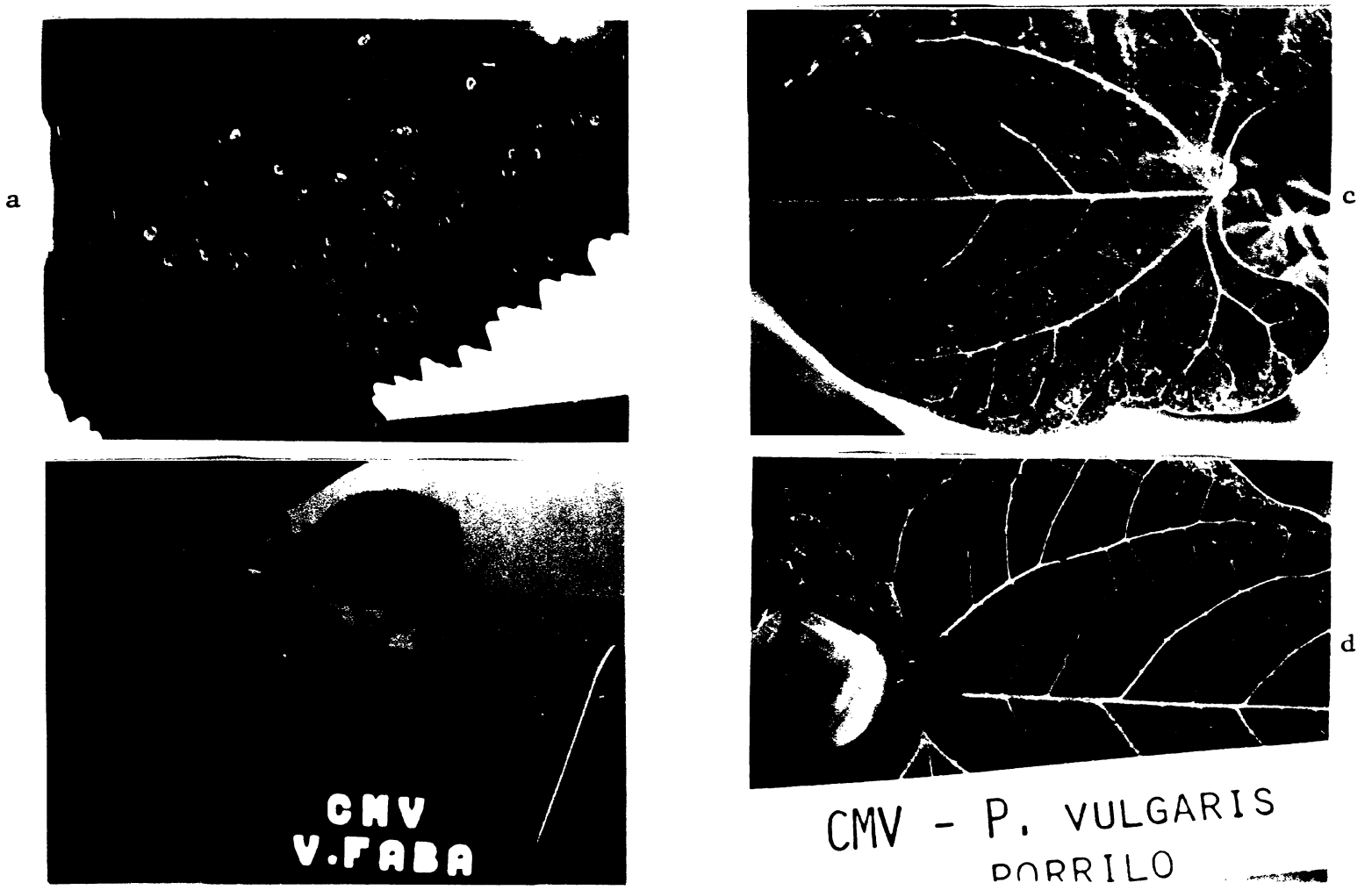


Fig. 3. Local lesions produced by cucumber mosaic virus: (a) *Chenopodium amaranticolor*, (b) Broad bean, (c) *Phaseolus vulgaris* var. Pinto, (d) *P. vulgaris* var. Porrilo

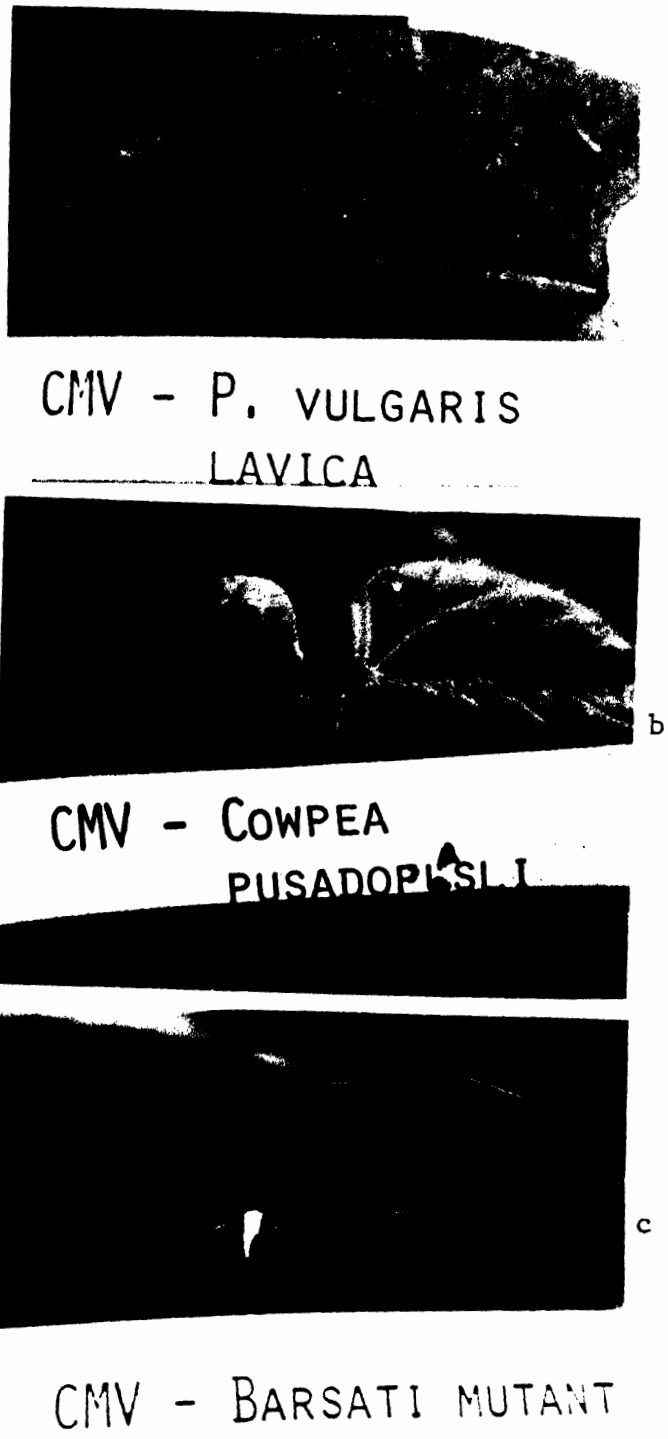


Fig. 4. Local lesions produced by cucumber mosaic virus: (a) *Phaseolus vulgaris* var. Lavica; (b) *Vigna unguiculata* var. Pusa Dophasli; (c) *V. unguiculata* var. Barsati mutant

5a

5c

5b

CMV - CUCUMBER LONG

6

Fig. 5. Systemic symptoms produced by cucumber mosaic virus on: (a) *Nicotiana rustica*; (b) cucumber and (c) peas

Fig. 6. Systemic symptoms produced by bean yellow mosaic virus on *P. vulgaris*

TABLE 3. Host range and symptomatology of cucumber mosaic and bean yellow mosaic viruses

Host	Symptoms	
	CMV	BYMV
AIZOACEAE		
Tetragonia expansa Murr.	CLL	-
AMARANTHACEAE		
Gomphrena globosa Murr.	-	-
CHENOPODIACEAE		
Chenopodium amaranticolor Coste et Ryne	NLL	-
C. murale L.	NLL	-
COMPOSITAE		
Chrysanthemum sp.	-	-
CUCURBITACEAE		
Cucumis sativus L.	Systemic, inducing large chlorotic patches	-
Cucurbita pepo L.	CLL	-
LEGUMINOSAE		
Arachis hypogaea L.	-	-
Cicer arietinum L.	Detailed symptoms are described in the text	
Cajanus cajan (L.) Millsp.	NLL	-
Canavalia ensiformis D.C.	-	Mos, Motl.
Macrotyloma uniflorum (Lam.) Verdc.	-	-
Phaseolus vulgaris L. 'Ababa'	NLL	Mos.
P. vulgaris 'Porrilo'	NLL	-
P. vulgaris 'Red kidney'	NLL	VN, Mos. Motl.
P. vulgaris 'Dwarf'	-	VN, TN, Mos, Motl, Ma
P. vulgaris 'Pinto'	NLL	VN, Mos, Motl, Ma.
P. vulgaris 'Munroe'	NLL	-
P. vulgaris 'Lavica'	NLL	-

contd.

TABLE 3. Contd.

Host	Symptoms CMV	BYMV
Pisum sativum L.	-	-
Vicia faba L. 'Early long pod'	NLL	-
Vigna unguiculata (L.) Walp. 'Early Ramshorn'	NLL	-
V. unguiculata 'Barsati mutant'	NLL	-
V. unguiculata 'Pusa Dophasli'	NLL	-
SOLANACEAE		
Capsicum annuum L.	-	-
Datura stramonium L.	CLL	-
Lycopersicon esculentum Mill.'Pusa Ruby'	-	-
Nicotiana tabacum L. 'Xanthi-nc'	Mos, Motl.	-
N. rustica L. 'White Pathar'	Mos, Motl.	-
Petunia hybrida Vilm.	-	-

CLL = Chlorotic local lesions; Ma = Malformation of leaves; Mos = Mosaic; Motl = Mottle; NLL = Necrotic local lesions; TN = Top necrosis; VN = Veinal necrosis; - = No symptoms.

III. *IN VITRO* PROPERTIES

A. Cucumber Mosaic Virus

1. Thermal inactivation point (TIP)

The assay plants inoculated with the standard extract treated at 45°, 50°, 55°, 60°, 65°, and 70°C produced local lesions whereas no local lesions developed with the extract heated at 75°C or more (Table 4) The TIP of the virus was thus between 70° and 75°C.

TABLE 4. Influence of heating the standard extract of cucumber mosaic virus at different temperatures for 10 minutes on infectivity

Exposure temperature (^{o}C)	Number of local lesions*
45	67 (4-243)
50	32 (3-112)
55	35 (4-134)
60	4 (1-13)
65	7 (0-36)
70	1 (0-2)
75	0
80	0
85	0
90	0
Control	77 (1-181)

*Average of six replications with range of lesions in parentheses.

2. Dilution end point (DEP)

Assay plants inoculated with 0, 10^{-1}, 10^{-2} and 10^{-3} dilutions of the standard extract produced local lesions. No local lesions were produced when the plants were inoculated with the extract diluted to 10^{-4} or above (Table 5). Thus the DEP of the virus was between 10^{-3} and 10^{-4}.

3. Longevity *in vitro*

The standard extract stored at room temperature (22^{o} - 24^{o}C) lost its infectivity on the fifth day (Table 6).

TABLE 5. Influence of diluting the standard extract of cucumber mosaic virus on infectivity

Dilution	Number of local lesions*
0	83 (47-103)
10^{-1}	74 (6-176)
10^{-2}	27 (4-50)
10^{-3}	7 (0-23)
10^{-4}	0
10^{-5}	0
10^{-6}	0
10^{-7}	0

*Average of six replications with range of lesions in parentheses.

TABLE 6. Influence of storage of the standard extract of cucumber mosaic virus at room temperature on infectivity

Day on which plants were inoculated	Number of local lesions*
Immediately after extraction of sap	85 (43-110)
First	67 (5-167)
Second	42 (4-82)
Third	14 (2-35)
Fourth	8 (0-13)
Fifth	0
Sixth	0
Seventh	0

*Average of six replications with range of lesions in parentheses.

B. Bean Yellow Mosaic Virus

1. Thermal inactivation point (TIP)

The chickpea plants inoculated with the standard extract heated at 45°, 50° and 55°C for 10 minutes produced disease symptoms (Table 7). However, no symptoms were noticed when the plants were inoculated with the extract heated at 60°C or more. The TIP of the virus was thus between 55° and 60°C.

2. Dilution end point (DEP)

Plants inoculated with the standard extract diluted to 10^{-1} and 10^{-2} produced disease symptoms but not with dilutions thereafter (Table 8). The DEP of the virus was thus between 10^{-2} and 10^{-3}.

3. Longevity *in vitro*

The virus sap when stored at room temperature (22-24°C) lost its infectivity on the 2nd day (Table 9).

TABLE 7. Influence of heating the standard extract of bean yellow mosaic virus at different temperatures for 10 minutes on infectivity

Exposure temperature (°C)	No. of plants inoculated	No. of plants infected
45	10	7
50	10	5
55	10	3
60	10	0
65	10	0
70	10	0
75	10	0
80	10	0
85	10	0
90	10	0
Control	10	10

TABLE 8. Influence of diluting the standard extract of bean yellow mosaic virus on infectivity

Dilution	No. of plants inoculated	No. of plants infected
0	10	9
10^{-1}	10	6
10^{-2}	10	2
10^{-3}	10	0
10^{-4}	10	0
10^{-5}	10	0
10^{-6}	10	0
10^{-7}	10	0

TABLE 9. Influence of storage of the standard extract of bean yellow mosaic virus at room temperature on infectivity

Day on which plants were inoculated	No. of plants inoculated	No. of plants infected
Immediately after extraction of plant sap	10	10
First	10	5
Second	10	0
Third	10	0
Fourth	10	0

IV. INSECT TRANSMISSION

The results on the transmission of the two viruses by the two aphid species are given in Table 10. Both the viruses could be transmitted by *Myzus persicae* and *Aphis craccivora* in a non-persistent manner though the latter was found to be more efficient than the former.

V. PURIFICATION

A. Cucumber Mosaic Virus

The purification procedure adopted in the present studies resulted in highly purified and infectious virus. When butanol was used for initial clarification, most of the chloroplast material could be eliminated and thus a clear amber-coloured supernatant was obtained after low speed centrifugation. The virus could easily be concentrated by PEG precipitation and differential centrifugation. The high speed centrifugation resulted in a glassy pellet and by which time almost all the green colour could be eliminated. Further purification by density gradient centrifugation resulted in a single, thick, light scattering zone in density gradient tube (Fig. 7a). When the gradient tube was subjected to fractionation in an ISCO fractionator, a single absorption peak could be obtained corresponding to the virus zone (Fig. 7b). The results of the infectivity assays of gradient fractions indicated that the infectivity was confined to the UV absorbing component (Fig. 7b). A clear glassy pellet was obtained in high speed centrifugation of the diluted virus zone and could be easily suspended in buffer.

The homogeneity of the purified virus could be further assessed spectrophotometrically. The absorption spectrum was found to be typical of nucleoprotein (Fig. 7c), with absorption maximum at

TABLE 10. Aphid transmission of cucumber mosaic and bean yellow mosaic viruses[a]

Aphid species	No. of aphids used per plant[b]	No. of plants inoculated[c]	No. of plants infected		Percent transmission	
			CMV	BYMV	CMV	BYMV
Myzus persicae	10-15	20	8	7	40	35
Aphis craccivora	10-15	20	9	9	45	45

a. Data from two trials.

b. The aphids were given one hour fasting period and one minute acquisition feed period.

c. Inoculation feeding period was 12 hours. Ten-day-old plants were used for inoculation.

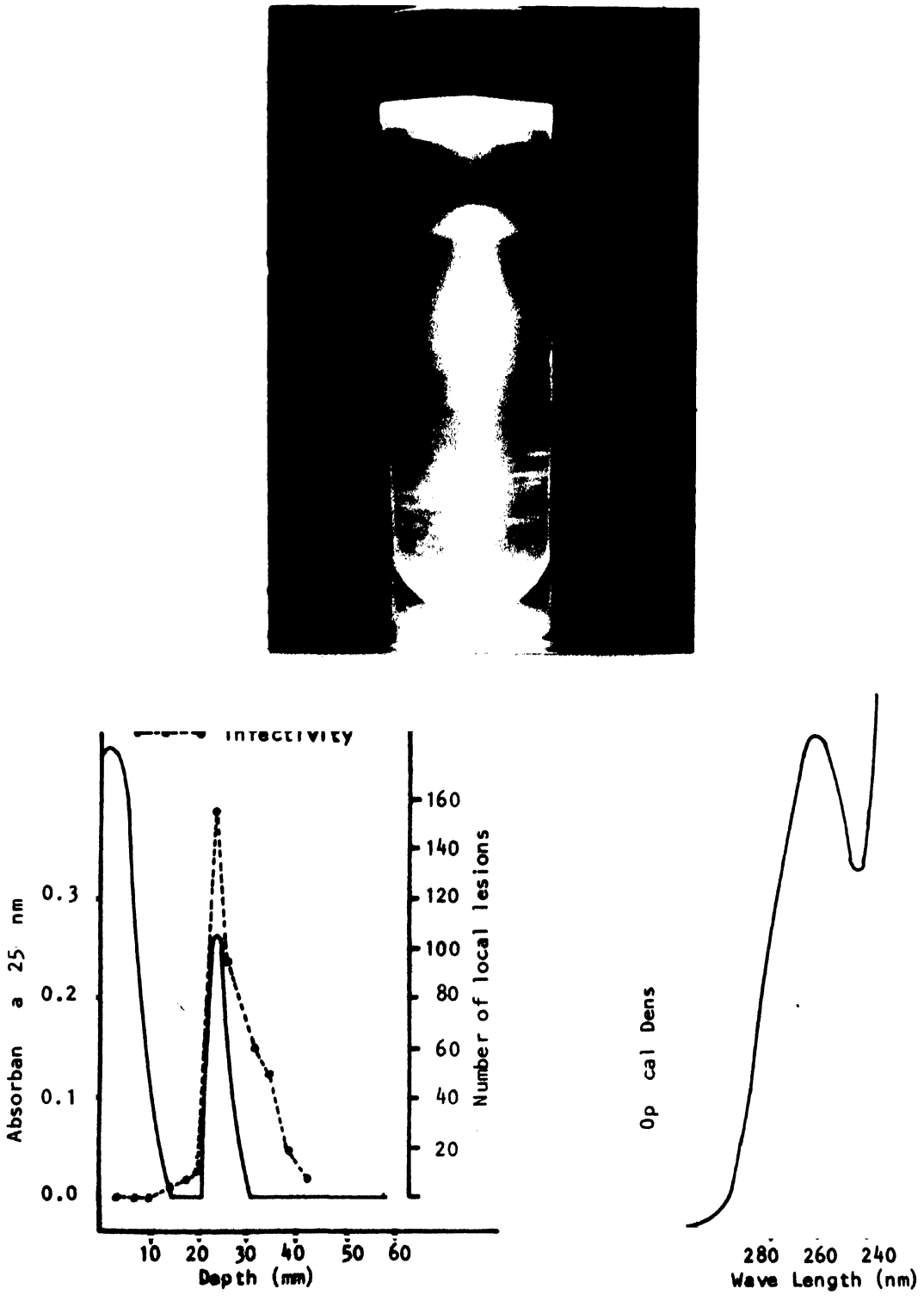


Fig. 7. a) Sucrose density gradient tube showing light scattering zone of CMV; b) the UV absorption (——) and infectivity(o–o–o–o) of fractions from density gradient column; c) the UV absorption spectrum of the purified CMV

260 nm and minimum at 240 nm. It had A_{260}/A_{280}, A_{280}/A_{260}, and A max/A min ratios of 1.55, 0.64, and 1.27, respectively.

The virus yield ranged from 8 to 10 mg/100 g of plant tissue when calculated spectrophotometrically.

B. Bean Yellow Mosaic Virus

Since a satisfactory local lesion host could not be found, the success of the purification procedure adopted was assessed only through the use of the electron microscope. With the experience gained from different purification procedures tried, the following conclusions could be drawn:

- Carbon tetrachloride was better than chloroform or butanol for preliminary clarification.
- Addition of carbon tetrachloride in the Waring blendor during homogenization was found to be better than adding it after homogenization.
- Use of PEG alone was found to be better than PEG + NaCl.
- Overnight incubation of the PEG dissolved preparation was found to be better than incubation for 2 hours.
- High speed centrifugal forces increased particle breakage.
- Use of a sucrose pad was found useful in eliminating most of the green material.

Out of several methods used, the one adopted in the present investigation (described under Materials and Methods) was found to be

satisfactory. But in density gradient centrifugation, the virus zone was not compact and clear. Rather, an inconspicuous, diffused zone was obtained. However, this zone, when pelleted consisted of high concentration of virus particles; though, there was some degree of breakage.

The optical characters and other physico-chemical properties could not be performed since the virus preparation, free of host components could not be obtained.

VI. ELECTRON MICROSCOPY

A. CMV Purified Preparation

The negatively stained preparation of the purified CMV contained a homogenous population of spherical particles which measured about 30 nm in diameter (Fig. 8).

B. Bean Yellow Mosaic Virus

1. Leaf dip preparation

The negatively stained leaf dip preparation revealed the presence of flexuous rods (Fig. 9). From the length distribution histogram (Fig. 10) it is clear that most of the particles measured between 740 and 760 nm with a modal length of 750 nm. The width was determined to be about 15 nm.

2. Purified preparation

A negatively stained preparation of the purified BYMV contained flexuous rods. Though there was a breakage of particles to some extent, aggregation was not observed (Fig. 11).

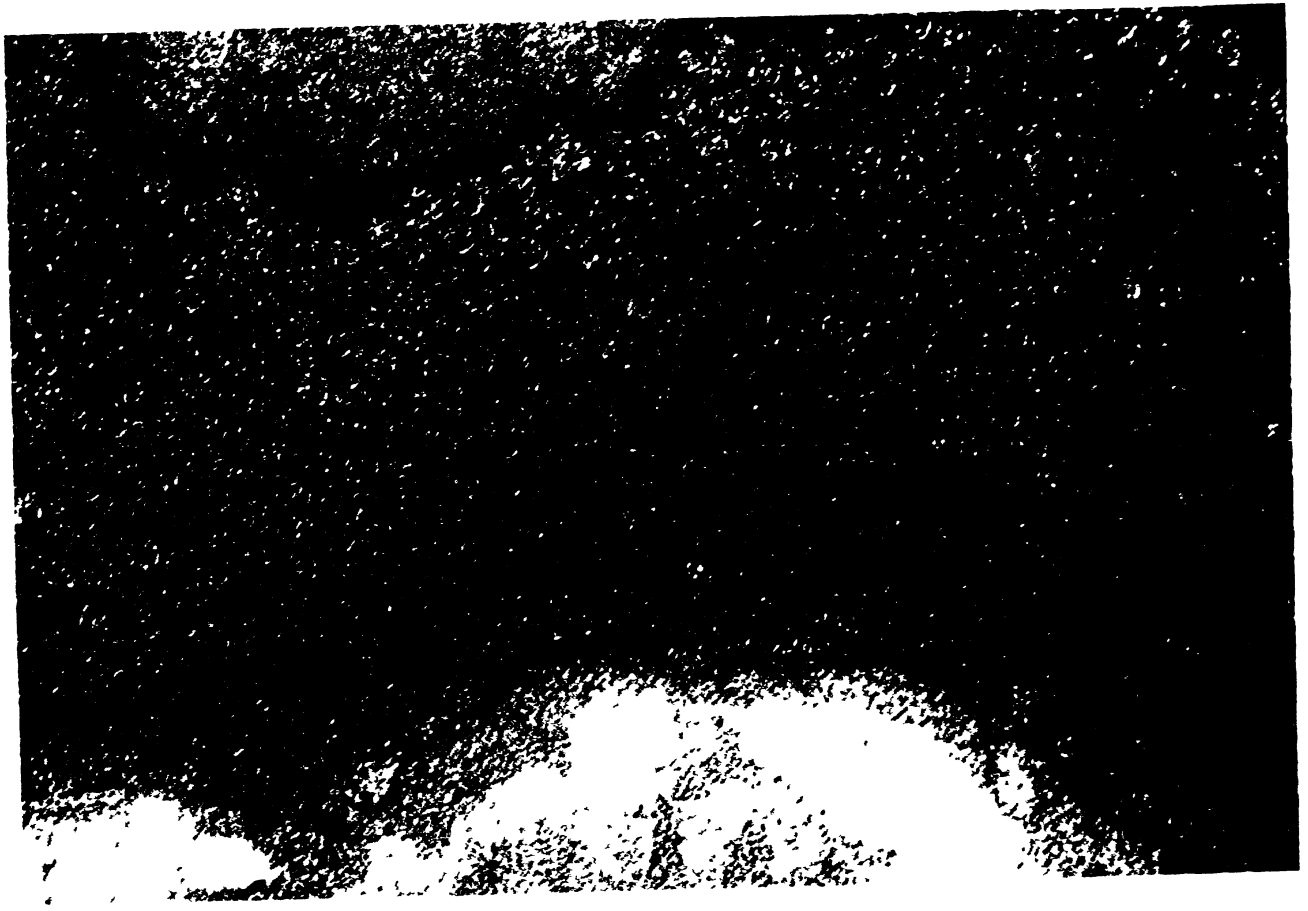


Fig. 8. An electron photomicrograph of purified CMV stained with 1% uranyl acetate (X 22,9000)

Fig. 9. An electron photomicrograph showing particles of bean yellow mosaic virus as observed in leaf dip preparation after staining with 1% phosphotungstic acid (X 45,700)

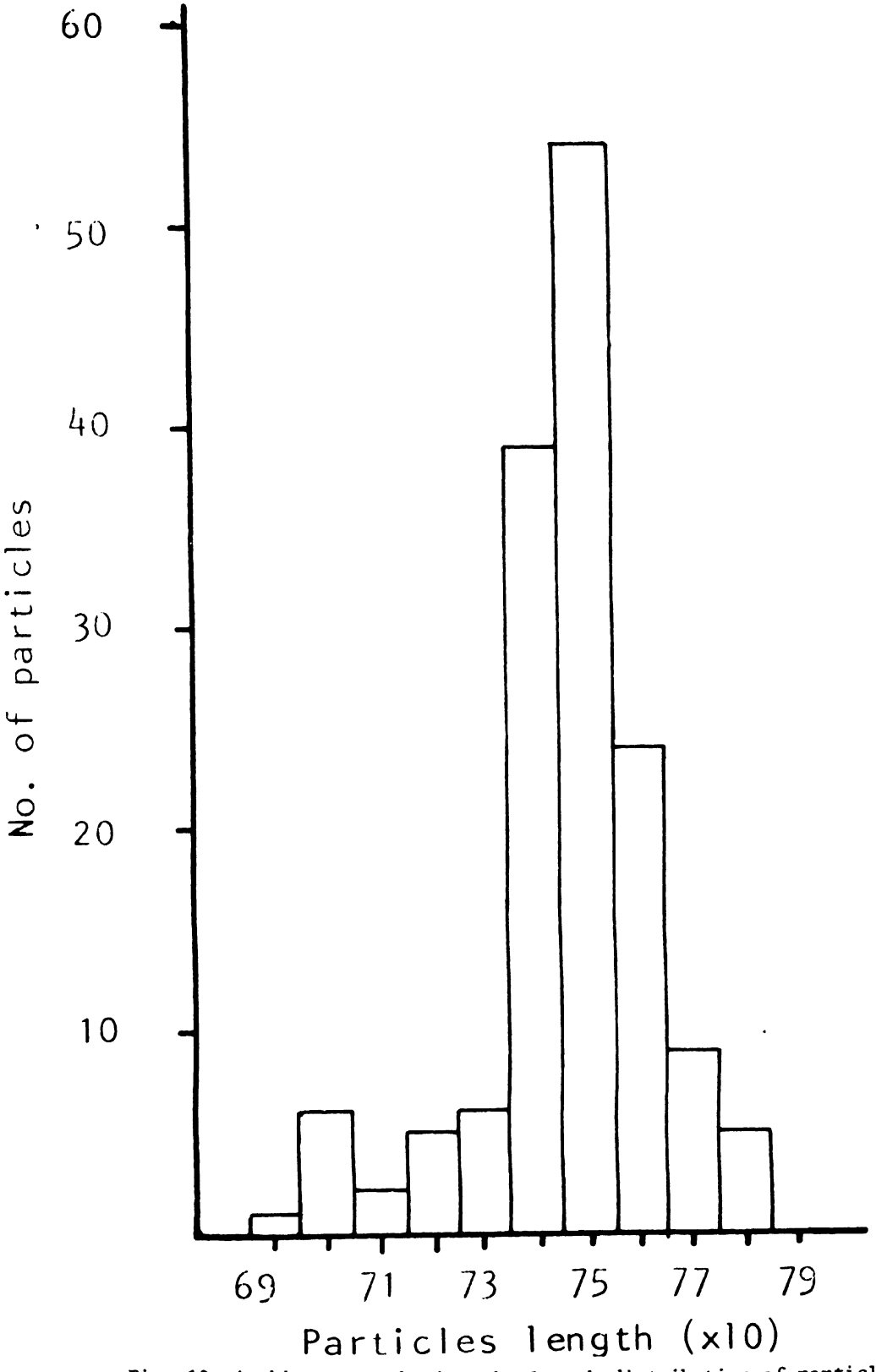


Fig. 10. An histogram showing the length distribution of particles of bean yellow mosaic virus from leaf dip

Fig. 11. An electron photomicrograph of purified BYMV stained with 1% phosphotungstic acid (X 80,000)

3. Ultrathin sections of BYMV-infected chickpea leaves

Electron microscopy of ultrathin sections of BYMV-infected chickpea leaves revealed the presence of pinwheel inclusions with curved plates, and laminate aggregates (Fig. 12).

/II. IDENTIFICATION OF VIRUSES BY SEROLOGY

A. Double-Diffusion in Agar

Clear precipitin bands could be observed when crude sap from virus-infected chickpea plants and purified preparations of CMV (Fig. 13a) and BYMV (Fig. 13b) were made to react with their respective antisera. The purified CMV sometimes produced two precipitin bands with its antiserum, a straight one near the antiserum well and a curved one near the antigen well (Fig. 13c). No reaction either with the healthy sap or buffer control was observed with antisera of the two viruses.

B. Enzyme-Linked Immunosorbent Assay (ELISA)

For identification of CMV by ELISA, the anti-CMV obtained from Dr. H.E. Waterworth, USDA, Maryland, U.S.A. was used (CMV-S). A positive reaction (development of yellow colour) could be observed both with infectious crude sap and purified virus at γ-globulin concentration of 1:100 and conjugate concentrations of 1:100 and 1:500 (Fig. 14). No reaction was observed either with the healthy sap or buffer.

The ELISA could not be performed with BYMV due to non-availability of good antiserum.

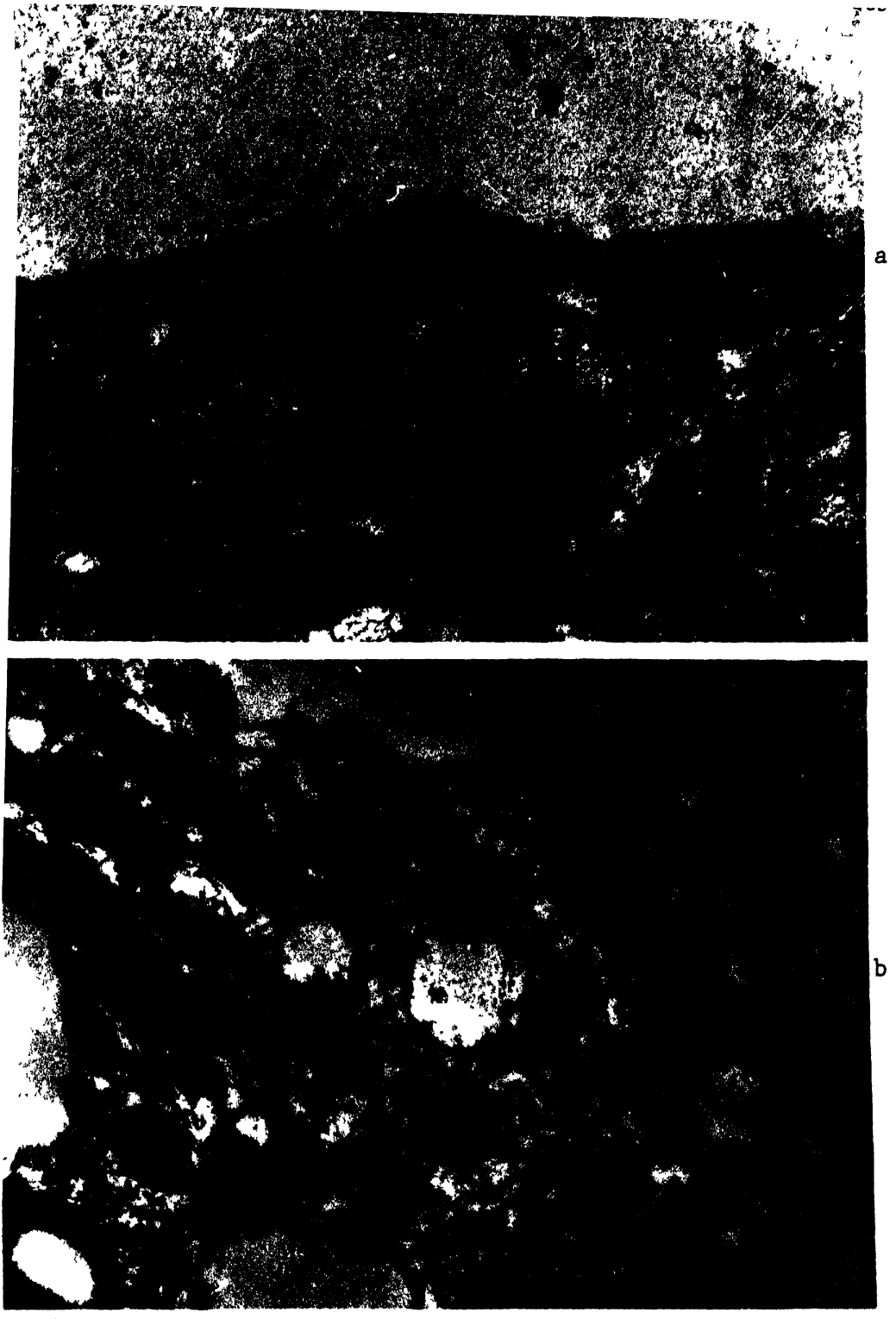
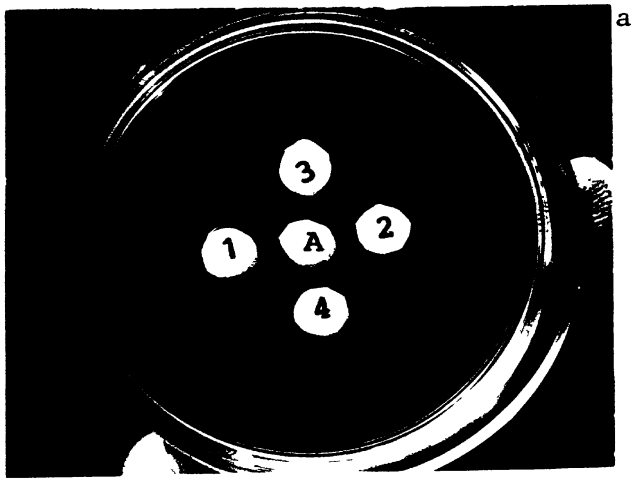


Fig. 12. Electron photomicrographs of ultrathin sections of BYMV-infected chickpea leaf showing: (a) pinwheels (PW) and (b) laminate aggregates (LA)



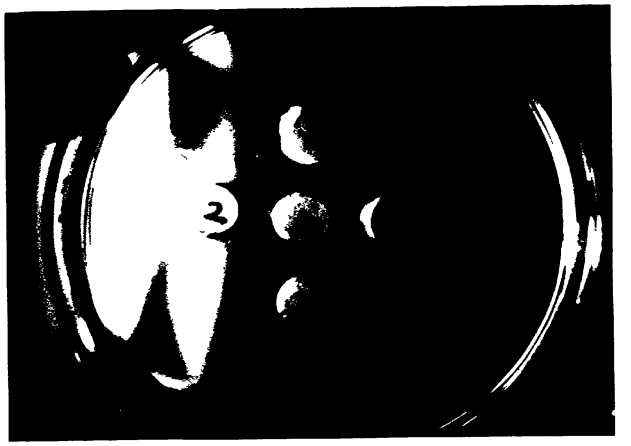


Fig.13. Agar gel double-diffusion test of purified CMV and infective crude sap. a) Each outer well contains: sap from CMV-infected chickpea (1), purified CMV (2), buffer (3), and sap from healthy chickpea (4). The central well contains antiserum to CMV(A). Note a positive reaction between purified CMV and infective sap with antiserum. b) Outer wells contain: buffer (1), purified virus (2), sap from healthy chickpea (3), sap from BYMV-infected chickpea (4). Note a positive reaction of purified virus with antiserum. Reaction of infective sap with antiserum has not come in the photograph. c) Both the outer wells contains purified CMV whereas the central well contains antiserum to CMV. Note the two types of precipitin bands

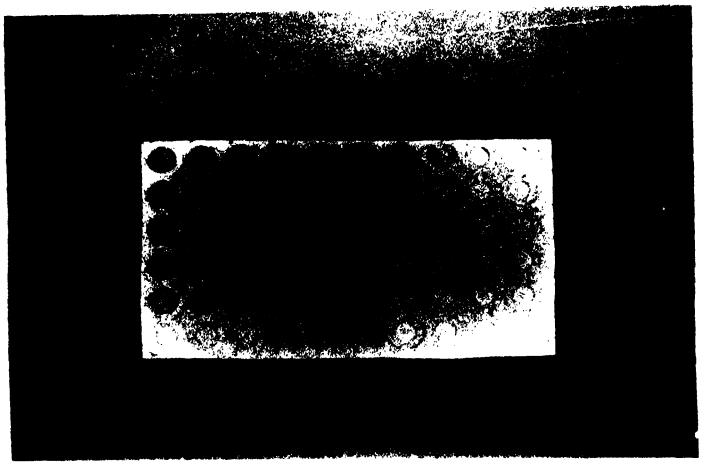


Fig. 14. Identification of cucumber mosaic virus by ELISA. INF - sap from infected chickpea; PV - purified virus; H - sap from healthy chickpea; CMV-H - homologous antiserum, CMV-S - antiserum to CMV strain S. Note a positive reaction with infective sap and purified virus. Also note a slight positive reaction of healthy sap with CMV-H at a conjugate concentration of 1:100 .

VIII. PHYSICO-CHEMICAL PROPERTIES OF THE PURIFIED CMV

A. Sedimentation Coefficient ($S_{20}W$)

The homogeneity of the purified CMV was indicated by the presence of a single Schleiren peak (Fig. 15) in the analytical ultracentrifuge. The $S_{20}W$ of the purified CMV was calculated to be 104S.

B. Buoyant Density

In the Cesium chloride equilibrium density gradient centrifugation, the purified CMV banded at a density level of 1.325 g/ml. The density levels and UV absorption of different fractions of the gradient are given in Fig. 16.

C. The PAGE of Coat Protein

The PAGE of SDS-disrupted coat protein of CMV revealed the presence of a single polypeptide. The banding pattern in the gels and the scanning pattern of the CMV-coat protein and marker proteins are given in Fig. 17a and 18. Based on the linear relationship between migration of marker proteins and the logarithm of their known molecular weights (Fig. 18d), the molecular weight of CMV-coat protein was estimated to be 25,000 daltons.

D. The PAGE of Nucleic Acids

The PAGE of SDS-disrupted CMV revealed the presence of four RNA species (Fig. 17b). The electrophoresis of the disrupted virus that was first treated with RNase did not produce any bands.

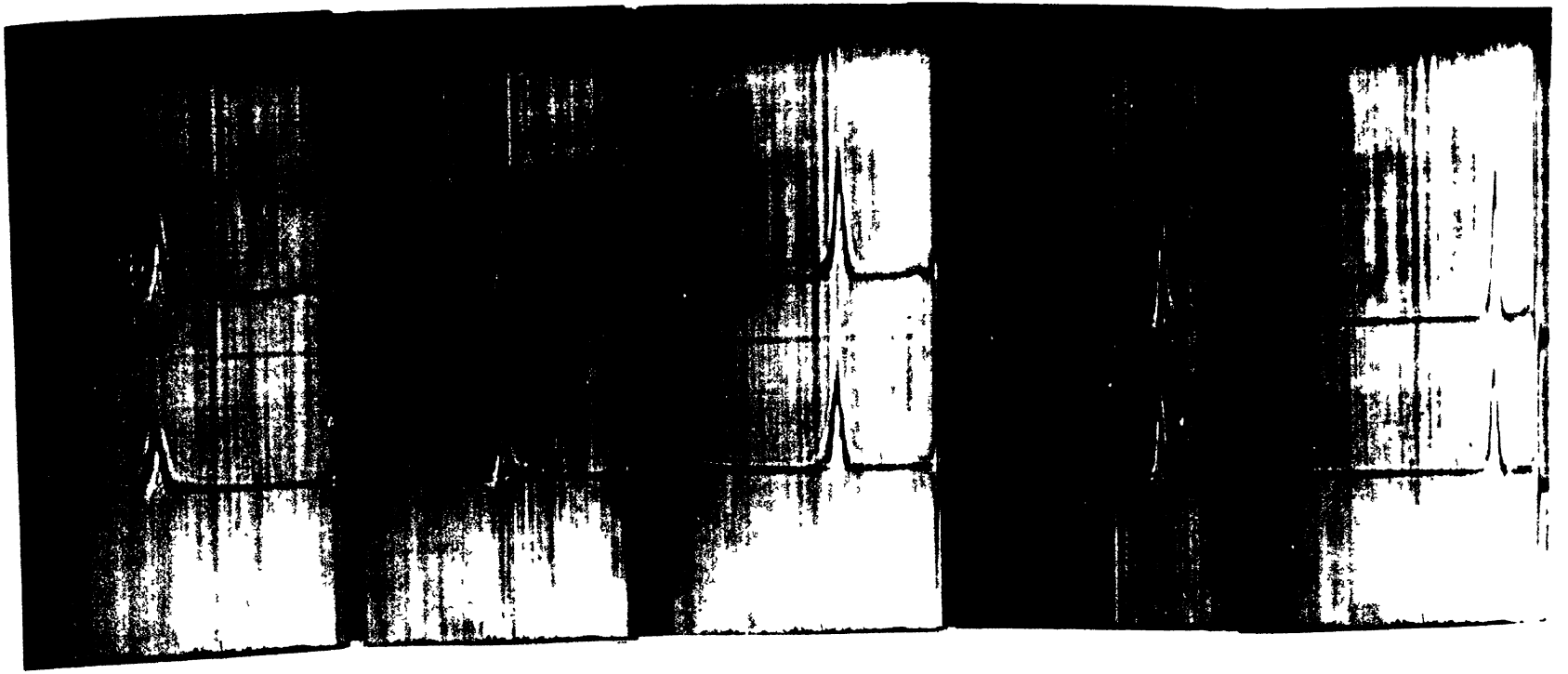


Fig. 15. Schleiren pattern of the purified CMV. Photographs were taken at 3 min intervals after reaching 30,000 rpm; sedimentation is from left to right. Upper peak is from the freshly purified virus and lower peak is from the purified virus stored for one week. Note a single peak in both the cases.

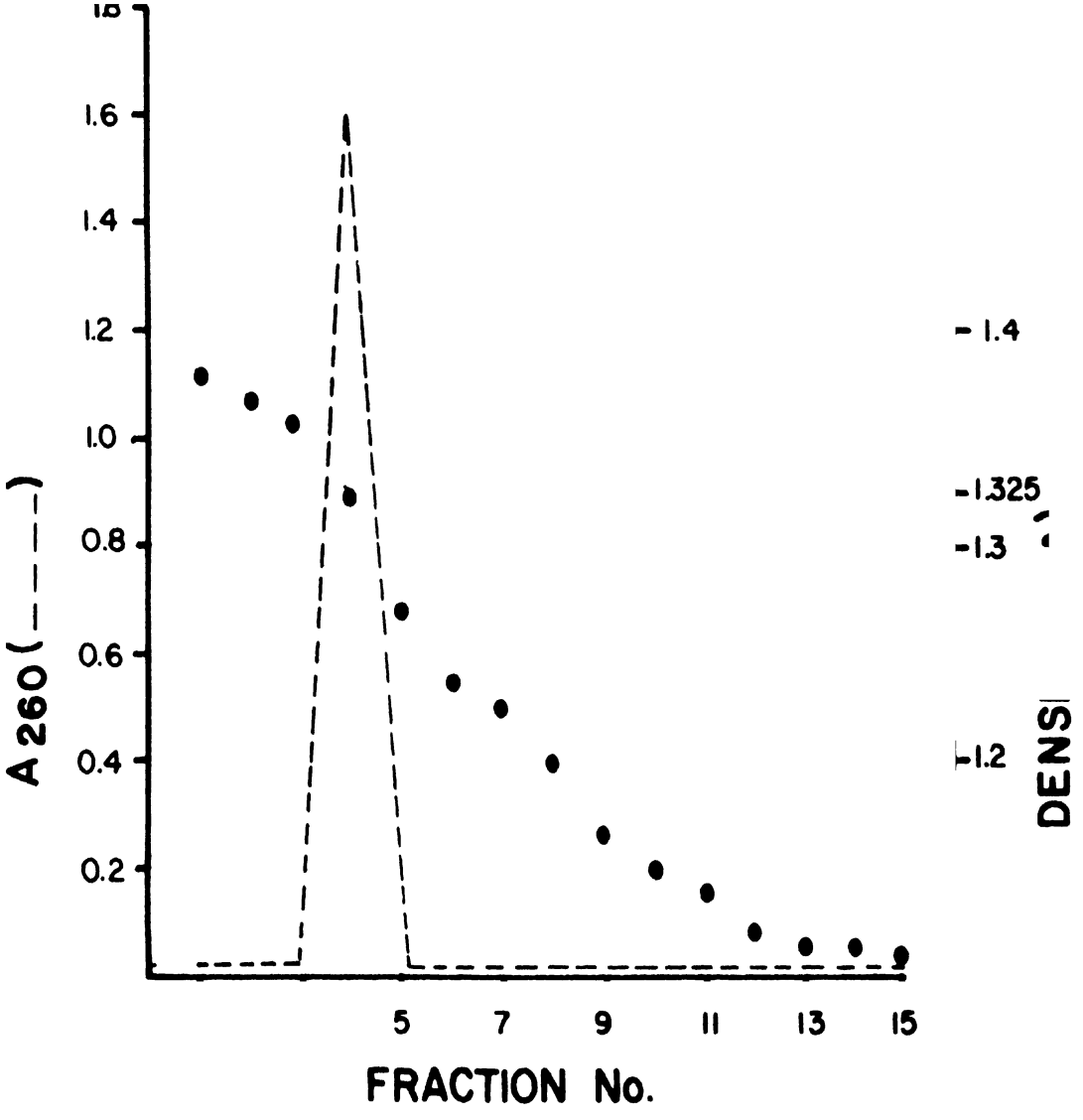
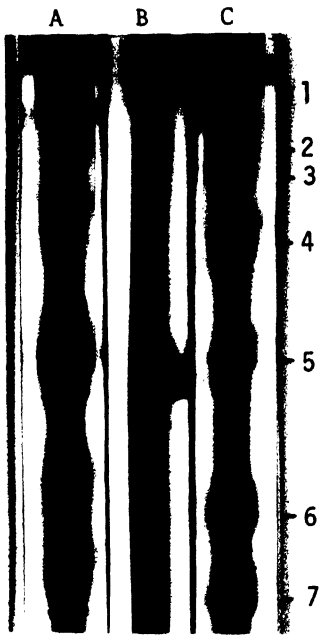


Fig. 16. The UV absorption (--------) and densities (●●●●●●) of fractions from CsCl gradient column (CMV). Note that the UV absorbing component had a buoyant density of 1.325 g/ml



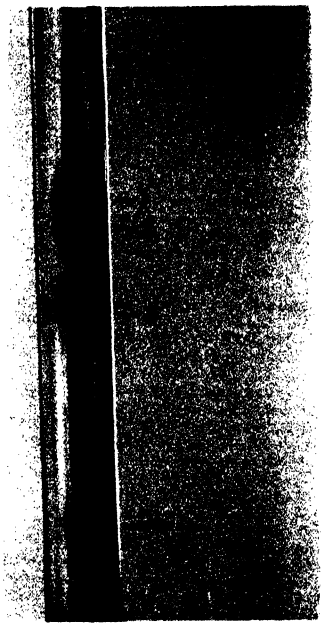


Fig. 17. a) The PAGE of (B) CMV-coat protein, (C) marker proteins and (A) coelectrophoresis of B and C. Marker proteins are: Phosphorylase B, mol.wt. 92,500(1); Bovine serum albumin, mol.wt. 66,200 (2 and 3 since this marker has split into two); Ovalbumin, mol.wt. 45,000(4); Carbonic anhydrase, mol.wt. 31,000(5); Soybean trypsin inhibitory, mol.wt. 21,500(6) and Lysozyme, mol.wt. 14,400(7). Note that virus protein has not resolved from carbonic anhydrase. Migration is from top to bottom.

b) The PAGE of CMV-RNAs, showing four RNA species. Migration is from top to bottom.

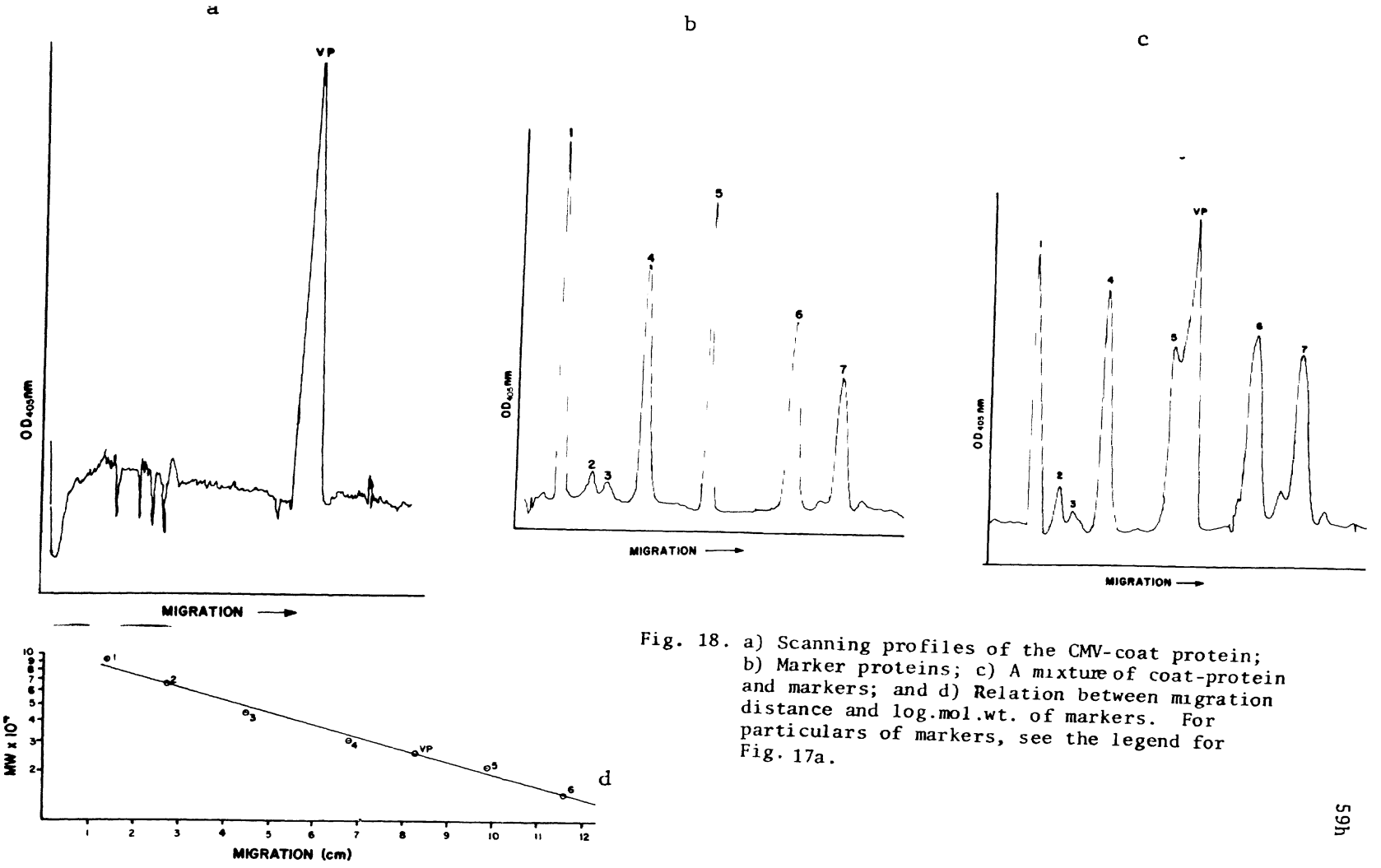


Fig. 18. a) Scanning profiles of the CMV-coat protein; b) Marker proteins; c) A mixture of coat-protein and markers; and d) Relation between migration distance and log.mol.wt. of markers. For particulars of markers, see the legend for Fig. 17a.

IX. PRODUCTION OF ANTISERUM TO CMV

The immunization schedule followed produced a reasonably high titred antiserum to CMV. In Ouchterlony double-diffusion test the titre was determined to be 1/1024 when tested with the purified virus. No reaction could be noticed with the healthy sap in this test. However, when tested by ELISA, a very negligible positive reaction was noticed at a conjugate concentration of 1:100; no reaction could be noticed at a conjugate concentration of 1:500 (Fig. 14).

X. SEROLOGICAL RELATIONSHIP BETWEEN THE PRESENT STRAIN OF CMV WITH OTHER KNOWN STRAINS

The intensities of the precipitin bands formed in Ouchterlony double-diffusion test when the purified CMV was made to react with antisera to different known strains of CMV are given in Table 11. The results indicate that the present CMV strain is relatively more closely related to CMV-C pool, followed by CMV-IX and M-CMV.

TABLE 11. Serological affinity of present CMV strain with other known strains of CMV

Strain	Source of antiserum	Reaction
CMV-C pool	H.A. Scott (U.S.A.)	+++
CMV-IX	H.E. Waterworth (U.S.A.)	++
CMV-S	H.E. Waterworth (U.S.A.)	+
U-CMV	R.I.B. Francki (Australia)	-
X-CMV	R.I.B. Francki (Australia)	-
Q-CMV	R.I.B. Francki (Australia)	-
M-CMV	R.I.B. Francki (Australia)	++
T-CMV	R.I.B. Francki (Australia)	+
Homologous	ICRISAT	+++

XI. SCREENING FOR DISEASE RESISTANCE

A. Cucumber Mosaic Virus

The data on the reactions of chickpea lines to CMV are presented in Appendix I. It is evident that only two lines, ICC-1781 and -8203, remained free from CMV infection, one line ICC-9006 showed 10% infection and the remaining 140 lines showed infection ranging from 33 to 100%.

B. Bean Yellow Mosaic Virus

The data on the reactions of the 106 chickpea lines to BYMV are presented in Appendix II. The results show that 9 lines; ICC-607, -6999, -1468, -2162, -2342, -3440, -3598, -4045, and -11550, remained free from BYMV infection, four lines; ICC-1416, -3916, -4093 and -4192, showed 10% infection, 12 lines; ICC-187, -1121, -1754, -3634, -3918, -3919, -3921, -3969, -4188, -6989, -7000 and -8207, showed infection between 10 and 20%. All the remaining 81 lines showed infection ranging from 22 to 100%.

DISCUSSION AND CONCLUSION

Two viruses, viz., cucumber mosaic virus (CMV) and bean yellow mosaic virus (BYMV) were found to infect chickpeas under natural conditions. Their identity was confirmed by studying their characteristics.

A detailed study of the symptomatology by CMV and BYMV in chickpea indicated that both the viruses caused a similar initial twisting of the terminal bud though the former caused a severe twisting which terminated in necrosis in contrast with a slight twisting without necrosis caused by the latter. The CMV, after terminal necrosis, caused two types of symptoms. In certain varieties, it caused wilting and death of the plants whereas, in others proliferation and bushiness of branches bearing small green leaves. The latter were commonly seen in mechanically inoculated chickpea and resembled the symptoms produced by CMV under field conditions. The infection by BYMV after initial twisting was followed by proliferation of both the terminal and axillary branches which are long and thin with narrow leaves. The leaves below the proliferated branches showed yellowing or interveinal chlorosis or mosaic depending upon the genotypes used.

The present strain of CMV appears to have a wide host range as it could infect 20 plant species belonging to five families; Aizoaceae, Chenopodiaceae, Cucurbitaceae, Leguminosae, and Solanaceae. Though it produced local lesions on several plants tested, *C. amaranticolor* was found to be very good assay host and was, therefore, used throughout the studies. Earlier, CMV was reported to produce systemic mosaic on cucumber and fern leaf symptoms on tomato (Francki *et al.*, 1979). On the contrary, the present strain of CMV could not infect tomato and produced large chlorotic patches on cucumber instead of a systemic mosaic. Waterworth and Povish (1975) also got similar results

with CMV-IX. However, the cultivars of cucumber and tomato used in the present studies were different from those of Waterworth and Povish. Though tobacco and pumpkin were reported to be good hosts for virus purification, it was found that chickpea supported better virus multiplication, and therefore was used for virus purification in the present studies.

Though BYMV is known to infect several non-legumes (Bos, 1970a), the present strain appears to have a very restricted host range as it could not infect any plant species outside the Leguminosae. It also produced veinal necrosis of inoculated leaves and top necrosis of some french bean cultivars. The severe yellow mosaic strain of BYMV (Rex and Zaumeyer, 1953) which also produced veinal necrosis and top necrosis of french beans could also not infect plants outside Leguminosae.

Based on the results of *in vitro* properties, the present strain of CMV appears quite stable. On the contrary, BYMV appears to be very unstable. However, it is evident from the literature that *in vitro* properties greatly depend on the virus source and test plant (Bos, 1970a).

Transmission of both the viruses by aphids did not exceed 45%. It is well documented that CMV transmission efficiency varies with plant species (Simons, 1955; Simons, 1957). Similarly, with CMV and BYMV, Kaiser and Danesh (1971b) found that transmission by different aphid species was usually less than 20% when chickpea was used both as source and test plant, whereas it was invariably higher when sweet clover and cucumber were used for CMV and BYMV, respectively. However, in the present studies the effect of different host species on the transmission efficiency of aphids was not studied.

The purification procedure adopted for CMV appeared efficient as judged by the UV absorption in ISCO fractionator and spectrophoto-

meter, infectivity, and electron microscopy. A single, sharp peak obtained in the ISCO scanning pattern was an indication of intact virus, without aggregation or degradation. In infectivity tests, the fractions present only in the UV absorbing component were found infective. Thus, there was a close correlation between UV absorbing component and infectivity which reveals that the zone in the sucrose gradient consisted of infective virus. The UV absorbing pattern of final purified virus in spectrophotometer was also typical of nucleoprotein. Considerably higher yields (80-100 mg/kg of tissue) were obtained by the purification procedure adopted. Yields of 100-500 mg/kg tissue have been reported in the literature (Waterworth and Povish, 1975; Francki *et al.*, 1979), though the yield depended on virus strain and host plant (Francki *et al.*, 1979).

The particle diameter (30 nm) obtained for the present CMV strain was in agreement with other reports (Francki *et al.*, 1966 and Debrot *et al.*, 1974).

An efficient purification procedure could not be evolved for BYMV which belongs to the potato virus Y (potyvirus) group. Viruses of the potyvirus group are difficult to purify because of their tendency to aggregate, both end to end and side by side (Shepherd and Pound, 1960). Nevertheless, there have been several reports on the purification of various viruses of this group. The general conclusions from these reports are that only small amounts of homogenous virus preparations were obtained with rather complicated procedures and that the methods successfully used with a particular virus could not generally be applied for purification of other elongate viruses. In the present studies, various methods used for BYMV purification did not yield a high purity virus. However, virus preparation of satisfactory purity could be obtained by the procedure adopted. Urea to the concentration ranging from 0.2 to 1M has been used in purification of different potyviruses to avoid aggregation of the particles

(Damirdagh and Shepherd, 1970; Uyeda *et al.*, 1975). In the present studies, the aggregation of BYMV particles could be avoided by using 0.2M urea in a suspending medium. However, the breakage of the virus particles could not be avoided completely despite the use of considerably low centrifugal forces. The diffused light scattering zone obtained after a density gradient centrifugation might be due to the breakage of virus particles. Withdrawal of the entire diffused zone might have resulted in the contamination of the virus with plant constituents. With the result, the virus free of host material could not be obtained. For this reason, the physico-chemical properties carried out for CMV could not be performed for this virus. The modal length obtained for this virus was in agreement with that reported for other strains of BYMV (Uyeda *et al.*, 1975; Kaiser and Danesh, 1971a). Pinwheel inclusions observed in ultrathin sections of BYMV-infected chickpea leaves were typical of potyviruses (Martelli and Russo, 1977) to which BYMV belongs.

In Ouchterlony double-diffusion test, CMV sometimes produced two precipitin bands. Scott (1968) and Devergne and Cardin (1970) also observed formation of two precipitin lines, a curved one near the antigen well, caused by more or less intact virus, and a straight one near the antiserum well caused by degraded virus. Sodium dodecyl sulfate-treated BYMV, however, produced only a single precipitin band.

The reason for a slightly higher sedimentation value (104S) obtained for the present CMV strain than the reported values of 98.6 (Francki *et al.*, 1966) and 101 (Tomlinson *et al.*, 1959) is not clear.

The buoyant density of 1.325 g/ml obtained for the present CMV strain was in close agreement with the values of 1.361 and 1.359 for CMV-S and CMV-D strains, respectively (Lot and Kaper, 1976). Essentially no UV absorbing material was present except in the virus band, indicating the high degree of buoyant homogeneity of the purified product.

The molecular weight of the CMV coat protein obtained in the present studies (25,000) was in close agreement with the values of 24,500 (Habili and Francki, 1974) and 25,200 (van Regenmortel *et al.*, 1972).

The PAGE of SDS-disrupted CMV revealed the presence of four RNA species. The RNAs of CMV have been variably described as comprising 4-6 different sized molecules. The presence of fith and sixth RNA components was claimed (Kaper and West, 1972; Peden and Symons, 1973) but was disputed when these components could not be consistently extracted from different strains of CMV (Lot *et al.*, 1974). However, more recent work has demonstrated that at least one of these components (RNA 5) is consistently present in large proportions in CMV-W (Wood and Coutts, 1975), and also in strains D and R (Lot and Kaper, 1976). Kaper and Waterworth (1977) discovered that RNA 5 is the causal agent for a lethal necrosis disease of tomato plants. Though the presence of fifth and sixth RNAs was doubtful, the presence of the four largest components has never been in doubt (Kaper and Tousignant, 1977). The present strain of CMV also contained 4 RNAs. With CMV-Commelina strain, Kaper and Tousignant (1977) demonstrated that more than one passage in tobacco was required to build up electrophoretically detectable quantities of RNA 5. Thus, they attributed that certain strains of CMV may contain undetectable quantities of RNA 5 whose synthesis can be generated upon propagation in certain hosts. However, the effect of propagation of CMV in different host plants on generation of RNA 5 was not studied in the present studies.

A good titred (1/1024) antiserum to CMV produced in the present studies indicated that the immunization schedule followed was good. The immunogenicity of CMV was reported to be poor (Francki *et al.*, 1979), but could be enhanced by fixation with formaldehyde (Francki and Habili, 1972) or glutaraldehyde (Rao, 1982). Using the fixed virus as an antigen, Rao (1982) produced antiserum to six

strains of CMV with a titre ranging from 1/128 to 1/512. Tomlinson *et al.* (1958) produced antiserum to unfixed CMV which had a titre of 1/1024. However, they used partially purified virus and thus the resultant antiserum might have contained antibodies to plant proteins also which might have contributed to the high titre of antiserum. In the present studies, no reaction with the healthy sap was detected in agar double-diffusion test, and hence, the reaction was attributed to the virus only. In ELISA, a slight reaction could be noticed with the healthy sap also. However, when the reactions to the infective and healthy sap were compared, the colour development in case of healthy sap was very negligible. Even this reaction however was not observed when conjugate concentration was increased from 1:100 to 1:500.

The natural occurrence of these two viruses was found to be less than 1%. Considering the cropping pattern in India which includes several cucurbitaceous and leguminous plants, which not only harbour these viruses but also aid in the vector population buildup, there exists a potential possibility for these viruses to become widespread in the future. The natural occurrence of CMV has already been reported by Dhingra *et al.* (1979). Though the natural occurrence of BYMV was not reported, the survey results indicated that it was present in several places in India such as Faizabad and Pantnagar (Uttar Pradesh), Junagarh (Gujarat), Parbhani (Maharashtra), and Tapparwaripora (Jammu and Kashmir) (Dr. S.P.S. Beniwal, personal communication). Therefore, keeping the threat posed by these viruses in view, the elite chickpea germplasm accessions were screened for resistance to these two viruses, and a number of accessions were found free from infection. These accessions after confirming their resistance, could be used in chickpea breeding program to incorporate resistance into commercially acceptable chickpea cultivars to thwart any potential danger caused by these viruses to chickpea cultivation in future.

SUMMARY

Two mechanically transmissible viruses, viz., cucumber mosaic virus (CMV) and bean yellow mosaic virus (BYMV) were found to naturally infect chickpeas. Both the viruses were identified by studying their host range, physical properties, insect transmission, electron microscopy and serology. Elite chickpea germplasm accessions that were earlier found to be resistant to wilt, root rot, Ascochyta blight and promising to stunt (pea leaf roll virus) were screened for resistance to these viruses. The results are summarized below:

1. Initially, both the viruses produced twisting of terminal bud in chickpea. Subsequently, CMV either produced wilting and death of plants or proliferation and bushiness of branches bearing very small green leaves and very few small pods, whereas BYMV produced proliferation of branches with narrow leaves, followed by production of distorted flowers that were converted into very small pods having shrivelled and black seeds.

2. CMV appeared to have a wide host range infecting the plants of Aizoceae, Chenopodiaceae, Cucurbitaceae, Leguminosae and Solanaceae, whereas BYMV could not infect hosts outside Leguminosae.

3. The CMV had thermal inactivation point between 70^{o} and $75^{o}C$, the dilution end point between 10^{-3} and 10^{-4} and longevity *in vitro* between 4 and 5 days. The corresponding figures for BYMV are between 55^{o} and $60^{o}C$, 10^{-2} and 10^{-3}, and 1 and 2 days.

4. Both viruses could be transmitted by two aphid species, viz., *Aphis craccivora* and *Myzus persicae* in a non-persistent manner.

5. The purification procedure developed for CMV yielded 8-10 mg virus/100 g of infected tissue. A satisfactory purification procedure was also developed for BYMV.

6. The CMV measured about 30 nm in diameter, whereas the length and width of BYMV were found to be 750 and 15 nm, respectively. Ultrathin sections of BYMV-infected chickpea leaves revealed the presence of pinwheel inclusions and laminate aggregates.

7. The sedimentation coefficient of purified CMV was determined as 104S, and the buoyant density as 1.325 g/cm^3.

8. The CMV coat protein is made up of single polypeptide with a molecular weight of 25,000 daltons.

9. The CMV-RNA contains four RNA species.

10. An antiserum to CMV produced in a rabbit had a titre of 1/1024 as determined in agar double-diffusion test.

11. The present chickpea strain of CMV was found to be closely related to CMV-C pool, followed by CMV-1x and M-CMV.

12. Out of 143 chickpea germplasm accessions tested for resistance to CMV, two (ICC-1781 and -8203) remained free from infection, one (ICC-9006) showed 10% infection, whereas all others showed infection ranging from 33 to 100%. For BYMV, 106 accessions were tested and nine remained free from infection, four showed 10% infection, 12 showed infection between 10 to 20%, whereas the remaining 81 showed infection ranging from 22 to 100%.

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APPENDIX

I. Reaction of chickpea lines to cucumber mosaic virus

Sl. No.	ICC No.	Number inoculated	Number infected	Percent infection
1	2	3	4	5
1.	76	10	9	90
2.	102	10	9	90
3.	267	9	6	67
4.	434	10	5	50
5.	438	11	6	55
6.	519	9	8	89
7.	858	10	5	50
8.	999	11	10	91
9.	1069	10	10	100
10.	1121	9	8	89
11.	1305	6	4	67
12.	1416	10	9	90
13.	1467	10	7	70
14.	1468	9	2	22
15.	1754	7	3	43
16.	1762	5	4	80
17.	1781	5	0	0
18.	1910	10	9	90
19.	1913	10	3	30
20.	1918	10	7	70
21.	2083	10	7	70
22.	2160	10	9	90
23.	2162	10	5	50
24.	2165	8	7	88
25.	2270	10	9	90

contd

1	2	3	4	5
26.	2342	8	7	87
27.	2354	10	10	100
28.	2441	6	5	83
29.	2450	9	7	78
30.	2461	10	3	30
31.	2506	7	7	100
32.	2566	10	5	50
33.	2660	10	9	90
34.	2858	11	7	64
35.	2862	10	8	80
36.	3103	9	8	90
37.	3181	10	8	80
38.	3439	9	5	56
39.	3440	6	5	83
40.	3597	10	8	80
41.	3598	8	8	100
42.	3634	10	10	100
43.	3843	10	10	100
44.	3916	10	10	100
45.	3918	10	8	80
46.	3919	10	10	100
47.	3921	10	8	80
48.	3932	8	8	100
49.	3969	10	7	70
50.	4030	10	7	70
51.	4045	10	8	80
52.	4083	6	6	100
53.	4093	8	8	100
54.	4181	6	4	67
55.	8188	9	7	78

contd.

1	2	3	4	5
56.	4192	8	8	100
57.	4472	66	6	100
58.	4616	9	9	100
59.	4847	10	6	60
60.	4850	10	4	40
61.	5035	9	9	100
62.	5313	6	6	100
63.	5566	6	5	83
64.	6103	9	8	89
65.	6304	8	7	88
66.	6306	10	10	100
67.	6336	8	8	100
68.	6366	9	9	100
69.	6381	10	9	90
70.	6411	9	7	78
71.	6455	10	8	80
72.	6718	9	9	100
73.	6813	10	10	100
74.	6988	10	7	70
75.	6989	8	8	100
76.	6999	8	8	100
77.	7000	10	9	90
78.	7904	10	4	40
79.	7909	10	8	80
80.	7926	10	8	80
81.	7928	10	4	40
82.	8001	11	7	64
83.	8003	10	5	50
84.	8004	9	7	78
85.	8068	8	7	88

contd.

1	2	3	4	5
86.	8087	10	8	80
87.	8088	10	10	100
88.	8095	8	4	50
89.	8096	8	4	50
90.	8097	8	4	50
91.	8098	10	6	60
92.	8099	10	10	100
93.	8181	8	6	75
94.	8132	10	10	100
95.	8134	10	10	100
96.	8137	10	5	50
97.	8148	10	10	100
98.	8175	8	6	75
99.	8203	4	0	0
100.	8205	10	6	60
101.	8206	10	10	100
102.	8210	6	2	33
103.	8207	8	6	75
104.	8585	9	6	67
105.	8622	10	7	77
106.	8933	10	6	60
107.	8957	10	8	80
108.	8960	10	10	100
109.	8971	10	4	40
110.	8979	11	9	82
111.	8980	9	8	89
112.	8982	9	6	67
113.	8985	10	7	70
114.	8988	10	10	100
115.	9006	10	1	10

contd.

1	2	3	4	5
116.	9023	9	8	89
117.	9032	10	9	90
118.	9033	10	6	60
119.	9055	8	7	88
120.	9142	10	6	60
121.	9143	10	8	80
122.	9146	10	10	100
123.	9147	10	8	80
124.	9149	10	10	100
125.	9150	10	10	100
126.	9198	10	6	60
127.	9202	10	10	100
128.	9203	8	6	75
129.	9245	10	6	60
130.	9249	10	4	40
131.	9251	10	10	100
132.	9252	10	4	40
133.	9319	10	10	100
134.	9326	7	5	71
135.	9368	10	7	70
136.	9369	10	10	100
137.	9374	10	8	80
138.	9394	5	3	60
139.	10803	10	10	100
140.	10823	10	6	60
141.	11531	10	5	50
142.	11550	11	10	91
143.	11551	11	10	91

II. Reaction of chickpea germplasm lines to bean yellow mosaic virus

Sl. No.	ICC No.	Number inoculated	Number infected	Percent infection
1	2	3	4	5
1.	76	9	2	22
2.	162	10	10	100
3.	187	9	1	11
4.	607	10	0	0
5.	843	11	3	27
6.	1069	10	6	60
7.	1121	10	2	20
8.	1136	8	2	25
9.	1305	9	3	33
10.	1416	10	1	10
11.	1467	10	6	60
12.	1468	11	0	0
13.	1754	9	1	11
14.	1762	9	3	33
15.	1781	10	3	30
16.	2160	7	3	43
17.	2162	10	0	0
18.	2165	9	2	22
19.	2270	8	4	50
20.	2342	10	0	0
21.	2441	8	7	88
22.	2506	7	2	29
23.	3440	10	0	0
24.	3597	8	2	25
25.	3598	10	0	0
26.	3634	9	1	11

contd.

1	2	3	4	5
27.	3916	10	1	10
28.	3918	10	2	20
29.	3919	7	1	14
30.	3921	10	2	20
31.	3932	10	4	40
32.	3969	10	2	20
33.	4018	9	2	22
34.	4020	8	7	88
35.	4030	9	5	56
36.	4045	10	0	0
37.	4083	8	2	25
38.	4093	10	1	10
39.	4174	9	3	33
40.	4181	9	2	22
41.	4187	10	5	50
42.	4188	10	2	20
43.	4192	10	1	10
44.	4472	10	4	40
45.	4616	8	3	38
46.	5035	10	6	60
47.	5313	10	5	50
48.	5566	9	4	44
49.	6103	8	8	100
50.	6304	9	8	89
51.	6306	10	3	30
52.	6336	8	6	75
53.	6781	10	5	50
54.	6813	9	4	44
55.	6988	10	4	40
56.	6989	8	1	13

contd.

1	2	3	4	5
57.	6999	8	0	0
58.	7000	10	2	20
59.	7904	10	6	60
60.	7907	10	10	100
61.	7909	10	8	80
62.	6926	8	4	50
63.	7928	10	10	100
64.	8079	10	6	60
65.	8087	8	6	75
66.	8088	10	8	80
67.	8095	8	6	75
68.	8096	10	4	40
69.	8098	10	10	100
70.	8099	10	8	80
71.	8127	10	10	100
72.	8131	10	4	40
73.	8132	10	10	100
74.	8134	10	10	100
75.	8137	8	8	100
76.	8148	10	10	100
77.	8175	10	6	60
78.	8203	8	4	50
79.	8205	10	8	80
80.	8206	10	8	80
81.	8207	10	2	20
82.	8210	8	6	75
83.	8959	10	10	100
84.	8960	10	10	100
85.	9142	10	10	100
86.	9143	10	8	80

contd.

1	2	3	4	5
87.	9146	10	10	100
88.	9147	10	10	100
89.	9150	10	8	80
90.	9198	10	10	100
91.	9202	10	10	100
92.	9203	10	4	40
93.	9245	10	10	100
94.	9249	10	8	80
95.	9252	10	10	100
96.	9319	10	4	40
97.	9326	10	6	60
98.	6363	10	6	60
99.	9368	10	6	60
100.	9369	8	6	75
101.	9774	8	8	100
102.	9379	8	4	50
103.	9394	10	10	100
104.	9402	10	6	60
105.	9403	10	10	100
106.	11550	10	0	0

VITA

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