AN ABSTRACT OF THE THESIS OF

Robert F. Davis for the degree of Doctor of Philosophy in Botany and Plant Pathology (Plant Virology) presented on March 17, 1981

Title: Occurrence, Transmission, and Properties of Strains of Cucumber Mosaic Virus that are Seed-Transmitted in Phaseolus vulgaris

Abstract approved: Redacted for Privacy

Dr. Richard O. Hampton

A survey of major bean (Phaseolus vulgaris) breeding programs and seed producing areas in Idaho, Washington, and Oregon was conducted to determine the occurrence of cucumber mosaic virus (CMV), recently reported to be seedborne in beans. Some 259 plants, sampled from breeding lines of diverse germplasm, were found by enzyme-linked immunosorbent assay (ELISA), bioassay, or gel double-diffusion serology to be free of seedborne CMV. CMV was, however, detected in 1 of 32 Plant Introduction (PI) bean accessions tested by the same methods.

Seed-transmission rates of selected isolates (isolates will hereafter refer to those reportedly seedborne and one not reported to be seedborne in beans) were determined under field and greenhouse conditions. An isolate from PI No. 271998 (originating in Spain) was found to be seed-transmitted in up to 49% of the progeny of infected 'Topcrop' bean plants. Seed-transmission depended on early inoculation of plants, i.e., less than four weeks after planting. Rates of transmission under our conditions differed from values reported by others. Most notably, seed-transmission of CMV-F, reported at a rate of 54% in seeds of 'Topcrop' (61), was found in our studies to be 3.3% in this cultivar. CMV was distributed uniformly throughout seedlings arising

from infected seed but relative virus concentration was most consistently higher near the growing point. Symptoms were mild or masked in plants arising from infected seed.

Cucumber (Cucumis sativus 'Lemon') and tobacco (Nicotiana tabacum 'Xanthi') plants infected with CMV were detected by gel serology using standard procedures. To obtain gel serological reactions with infected bean or cowpea tissue, a 0.5 M citrate buffer, pH 6.5 with 0.1% sodium thioglycollate and 0.1% Triton X-100, was employed. CMV was readily detected in infected plant tissues by ELISA. Purified CMV was detected by gel serology at concentrations of 0.05-0.1 mg/ml and by ELISA at concentrations of 6 ng/ml.

Isolates were compared for differences in host range, infectivity, serological relatedness, electrophoretic mobility of protein and nucleic acid species, and presence of minor or satellite RNA components. No differences among isolates were found in any of the above properties, except that CMV-Pg seemed to produce lower yields in beans and tobacco and that its major RNA components appeared to fragment into unique electrophoretic species. Otherwise, the greater seed-transmission rate of CMV-Pg was the principal distinction among these isolates.

The nucleic acid components of CMV-Pg (seedborne) and CMV-Le (non-seedborne) were separated by sucrose density gradient centrifugation. Pseudorecombinants were prepared among RNA components of these isolates for continuing studies of the genomic determinants of CMV seed-transmissibility in beans.

Occurrence, Transmission, and Properties of Strains of Cucumber Mosaic Virus that are Seed-Transmitted in *Phaseolus vulgaris*

by

Robert Francis Davis

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Commencement June 1981

APPROVED:

Redacted for Privacy

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Date thesis presented on March 17, 1981

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SALLY KIDDER DAVIS

ACKNOWLEDGEMENTS

I wish to express sincere gratitude to Dr. Richard O. Hampton, my major professor and close friend. Dr. Hampton's encouragement and careful guidance in all aspects of my graduate training were invaluable.

My appreciation is extended to Dr. Thomas C. Allen, Dr. Dallice I. Mills, Dr. Ralph S. Quatrano, Dr. Henry W. Schaup, and Dr. Denis P. Lavender for serving as members of my graduate committee and for providing additional assistance.

I would also like to thank my loving parents, Mr. Robert E. Davis and Mrs. Grace E. Davis, and my very dear friends, Mr. John Turbitt, Mrs. Thelma Turbitt, and the late Dr. Ralph J. Shay, for their inspiration and assistance in the attainment of my goals.

I reserve a very special thanks for my wife, Sally, whose love and support I have appreciated most of all.

TABLE OF CONTENTS

Table of Contents, cont.

Table of Contents, cont.

LIST OF FIGURES

LIST OF TABLES

List of tables, cont.

LIST OF APPENDICES TABLES

Occurrence, Transmission, and Properties of Strains of Cucumber Mosaic Virus that are Seed-Transmitted in *Phaseolus vulgaris*

Chapter One

Thesis Introduction and Cucumber Mosaic Virus Literature Review

INTRODUCTION

Transmission through seed has been reported for over 90 viruses in one or more of their host species, representing more than 20% of the adequately described viruses and 300 virus-host combinations. Seed-transmission of about 35 of these viruses occurs in plants of the Fabaceae (Leguminosae) family, the largest group of crops involved (76). Seed-transmission represents the only known means of natural spread for some viruses, such as barley stripe mosaic virus and bean southern mosaic virus (75). In others, infection by seedborne virus, even at low rates, can result in severe disease situations due to vector activity (33). Seed-transmission is also recognized as primarily responsible for the geographic spread of viruses, i.e., bean common mosaic virus (5), and as a major factor in virus survival and epidemic development of serious plant diseases (87).

The following premises have been asserted (76) about seed-transmission: 1) successful seed-transmission generally depends on invasion of the embryo by virus (through gametophytic tissue) and retention of viable virus during seed maturation; 2) seed-transmission occurs with viruses of nearly every morphological group including rhabdoviruses and viroids; 3) seedborne viruses generally infect parenchyma tissue

and cause mosaic symptoms; 4) most known vectors, except leafhoppers and fungi, can also transmit one or more of these viruses; and 5) viruses remain viable as long as does the seed. Information about specific physical and molecular characteristics, however, is lacking for most of the viruses, hindering understanding of mechanisms involved in this phenomenon.

Cucumber mosaic virus (CMV) is a classic and significant virus in many respects. It is one of the earliest and best characterized plant virus. Some isolates contain a satellite RNA molecule capable of altering pathogenicity (see literature review). In addition, CMV infects a wide range of plants, occurs world-wide, and is transmitted mechanically, by aphids, and through the seed of some hosts (28,75). CMV has been reported to infect bean plants (Phaseolus vulgaris) in a few cases since 1941 (6,44,95). Until 1974, however, CMV was not known to be seed-transmitted in beans. Since that time, several isolates worldwide have been reported to be seedborne in beans. Seed transmission, wide host range, high infectivity, and partial masking of symptoms make this virus potentially dangerous to the bean seed industry and to crop production.

Beans are grown worldwide and represent a principal crop in many countries. Arid-irrigated areas of the Pacific Northwest, are vital to the production of high quality bean seeds for U.S. crop and export needs.

One purpose of this thesis, was to determine the occurrence of seedborne CMV in the Pacific Northwest bean seed industry. A survey for seedborne CMV was therefore conducted in key bean seed producing areas and breeding programs of Idaho, Washington, and Oregon. This

survey, conducted over a two year period, involved beans of diverse germplasm types. Also surveyed were 32 selected Plant Introduction bean accessions of international origin.

Another major intent of this thesis was to investigate seed-transmission of CMV in beans and identify virus/host factors associated with this phenomenon. For this latter purpose, selected seedborne isolates were compared with an isolate not seedborne in beans.

Seed-transmission is known to be affected by many variables, notably virus, virus strain, host species and cultivar, environment during seed production, and time of plant inoculation (5,76,87). Effects of these variables on seed-transmission of CMV in beans was studied to assess the requisites of maximum seed-transmission. Symptom development and virus distribution in seedlings infected from seed were monitored to establish behavioral modes for seedborne CMV.

Differentiation of CMV isolates by host reactions on selected plant species, legumes and non-legumes, was attempted. To identify cultivars useful in breeding for resistance and develop experience in monitoring occurence of seedborne CMV isolates, several bean cultivars were inoculated and observed for resistance or sensitivity in field plots. Virus concentration with respect to time and symptom expression was determined. Bean cultivars selected for CMV sensitivity were subsequently planted as indicators of naturally occuring CMV isolates.

CMV isolates were purified, because purification of virus is a prerequisite for many sensitive and reliable techniques in plant virology. After purification of isolates, antiserum was produced and utilized for development of enzyme-linked immunosorbent assay for CMV. Isolates were also compared by gel double-diffusion serology to deter-

mine degree of serological homology. Electrophoretic mobility and numbers of protein and nucleic acid species were also compared. Finally, pseudorecombinants were prepared between the separated viral genomes of a seedborne isolate and an isolate not seedborne in beans. These pseudorecombinants will be tested in a continuing study to evaluate genomic determinants of CMV seed-transmissibility in beans.

CUCUMBER MOSAIC VIRUS LITERATURE REVIEW

History. Cucumber mosaic virus (CMV), reported in 1916 by Doolittle (20) and Jagger (43), was one of the earliest plant viruses to be described. As a result of definitive research on this virus, beginning with the classic works of Price (79,80), CMV is one of the most completely characterized plant viruses to date. A recently discovered feature of CMV which appears unique among plant viruses thus far is the association with some isolates of a small satellite RNA molecule which is capable of causing increased virulence (51). This phenomenon is treated in more detail near the end of this review.

General properties. According to the current method of classification proposed in 1971 by Harrison et al. (40), CMV is the type member of the Cucumovirus group. Other members include peanut stunt virus and tomato aspermy virus (92). The cryptogram of CMV (R/1:1.3/18 + 1.1/18 + 0.8-0.3/18:S/S:S/C,Ve/Ap) indicates, in abbreviated form, that this virus contains about 18% single-stranded RNA divided into four segments (ranging in size from 1.3-0.3 million daltons) which are encapsidated into three separate particles; the nucleoproteins are basically spherical in shape, and aphids are vectors of the virus in seed plants. The three nucleo-

protein particle types are about 28-30 nm in diameter and sediment in rate zonal sucrose density gradients at the same rate (25). CMV is distributed worldwide, especially in temperate regions, and has an extremely wide host range. Douine et al. (21) reported that 775 plant species in 365 genera and 85 families were susceptible to CMV. The CMV host range includes many crops of economic value and weed species. Diseases caused by CMV include mosaics of cucurbits and celery, blight of spinach, fern leaf of tomato, and numerous others (89). An edition of the journal Annales de Phytopathologie (Volume 11, No. 3) has been entirely devoted to reporting extensive research on the epidemiology and ecology of CMV in vegetable growing areas of southeast France.

Natural infection of beans by CMV was first reported in 1941 (95). An isolate infectious to beans (CMV-Le), reported in 1952 in Japan, was included in these studies (41). Hampton et al. (37) developed a diagnostic host range key for distinguishing among viruses infectious to legumes, including CMV.

Transmission. CMV is readily transmitted mechanically and by over 60 species of aphids in a non-persistent manner (53). Whole virus but not RNA in membrane systems was aphid transmissible (78). CMV is also transmitted through seeds of 19 plant species (67), a remarkably low number in comparison to the number of species infected by this virus.

Bos and Maat, in 1974, were the first authors to report seed-transmission of CMV in beans. The reported isolate (CMV-B_{32}), originating in eastern Spain, was found to be seedborne in about 7% of the seeds from bean cultivar 'Metis' and not seedborne in 11 other cultivars tested. Seed remained infective after 27 months of storage.

All bean cultivars were susceptible to infection. These authors concluded that this virus was potentially dangerous to bean crops because of mild and partially masked symptoms induced, high infectivity, wide host range, and seed-transmission (10). Four other isolates of CMV seedborne in beans have since been reported. These isolates, the country and year in which they were reported, and seed-transmission rates are as follows: CMV-B, New York, 1976, 0.3% (81); CMV-Pr, Puerto Rico, 1977, 1.5% (63); CMV-F, France, 1977, 9-54% (61); and CMV-Za8, Yugoslavia, 1979, 20% (1). No resistance to CMV was found by Provvidenti in 90 bean cultivars and 105 bean PI accessions tested. Provvidenti noted severe pod distortion in bean plants infected with CMV-B (81).

Purification. The first efficient method of purifying CMV was developed by Scott in 1963 (86). A modification of this method, for more rapid purification, was developed by Lot et al. in 1972 (59), and has been widely used. Mossop et al. (68) developed another method for CMV isolates that cannot be purified by the method of Lot et al. The light absorbance ratio of A_{260}/A_{280} for purified CMV preparations is 1.7, corrected for light scattering (25), or 1.65 uncorrected (28). Absorbance of CMV at 260 nm per 1 mg/ml, 1 cm light path, is 5.0 (26).

Serology. CMV is a weak immunogen but can be improved in this respect by fixation with formaldehyde (24). The complex serological relationships among selected CMV isolates and other Cucumoviruses have been elucidated by Devergne and Cardin (15,16). These authors have reported the existence of twelve categories of antibodies of fairly high specificity, representing four serological types, which can be classified

into two main serogroups, designated DTL and ToRS. These groups are serologically distinguishable by the formation of spurs between heterologous antigens in gel double-diffusion serology. Their serological classifiction is in agreement with certain biological properties of CMV isolates, i.e., all members of the ToRS group (but not DTL group members) produce necrotic ringspot lesions on Nicotiana tabacum 'Xanthi' (15). CMV-F, the seedborne isolate from France, has been reported to belong to the DTL serogroup (61).

Several serological methods have been used successfully to detect CMV in infected plants. Devergne, Cardin, and Quiot compared gel double-diffusion serology, radial immunodiffusion, and enzyme-linked immunosorbent assay (ELISA) and found ELISA to be the only method for reliably detecting CMV in natural infections, in which the titer is often low (17).

Virions. The virions of CMV consist of 18% (by particle weight) single-stranded RNA (47) surrounded by a protein shell of 180 identical subunits about 24,500 daltons in size (34) arranged into pentamer-hexamer clusters to form an icosahedral particle about 28-30 nm in diameter (22) with a central core of 12 nm diameter free of RNA (42). Molecular weight estimates vary from 5.0 to 6.7 million daltons and the isoelectric point is pH 4.7 (25). Virions are easily dissociated into RNA and protein by low concentrations of sodium dodecyl sulfate, indicating that structural stability depends on RNA-protein interactions (7,45). Strains of CMV have been found to differ in particle electrophoretic mobility, degradation by lithium chloride, and reaction with trinitrobenzenesulfonic acid (46,57).

RNA. CMV has been shown to contain RNA which is highly infectious when extracted (19) and is functionally divided or multipartite, i.e., the three largest species are required for infectivity (58,74). Plant viruses with multipartite genomes recently have been reviewed (27,82, 93,94). The molecular weights of RNA 1-4 have been reported to be 1.01-1.30, 0.89-1.13, 0.68-0.78, and 0.33-0.34 million daltons, respectively, depending on the isolate and method of estimation (50, 52,74).

The degree of sequence homology between the major RNA species has been investigated by Gould and Symons (32) using hybridization with complementary DNA (cDNA) probes. They have shown that RNA 1 and 2 are unique sequences, each having about 300 nucleotides in common with RNA 3. The complete nucleotide sequence of RNA 4 is contained within RNA 3 (32). Hybridization with cDNA (30) and competitive hybridization (77) have also been used to determine RNA sequence homology between isolates of CMV. RNA pseudorecombination experiments have also shown RNA 3 to contain the genes for coat protein (35) and aphid transmissibility (65).

Schwinghamer and Symons studied 'in vitro' translation of the major RNA's and developed a scheme for translation, showing that protein coat is coded for by RNA 3 and 4 (85). The four major RNA species can be aminoacylated at the 3'-end with tyrosine by plant-derived aminoacyl tRNA synthetases (54) and are 'capped' with 7-methyl guanosine at their 5'-ends (90).

In addition to the four major RNA species, minor RNA species of lower molecular weight (0.05-0.10 million daltons) have been reported (31,49,65). Some of these have been shown to be 'satellite' RNA

molecules since they are encapsidated with CMV-RNA, are completely dependent on CMV for replication, and contain totally unique base sequences (18,31,66). One of these satellites, CARNA 5, is capable of increasing the virulence of CMV in tomato, inducing a lethal necrotic disease in that host (51).

Chapter Two

Survey for Seedborne Cucumber Mosaic Virus in Selected *Phaseolus vulgaris* Germplasm and Breeding Lines in Idaho, Washington, and Oregon

PREFACE

Chapter Two is presented in its entirety as accepted for publication in *Plant Disease*. The research reported therein was initiated and conducted jointly by all authors under a cooperative agreement between the USDA SEA-AR and Oregon State University and Washington State University. The survey of *Phaseolus vulgaris* germplasm and breeding lines in Idaho and Oregon and selected *P. vulgaris* Plant Introduction accessions was conducted by R. Davis and R. Hampton. The survey of breeding lines in Washington was performed by Z. Weber, H. Pospieszny, and M. Silbernagel.

Results of this research are summarized in Table 2-1 of the manuscript, but are supplemented in Appendices I and II.

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Contribution of the U. S. Department of Agriculture, Science and Education Administration, Agricultural Research, in cooperation with the Agricultural Experiment Station, Oregon State University, Corvallis. Technical Paper No.5429 of the latter.

Accepted for publication: October 24, 1980.

ABSTRACT

Two hundred fifty-nine bean plants (Phaseolus vulgaris) from principal bean breeding lines grown in Idaho, Washington, and Oregon were tested in 1978 and 1979 for seedborne cucumber mosaic virus (CMV). All plants were free of seedborne CMV as determined by ELISA, bioassay, or serology. By the same methods seedborne CMV was detected in 1 of 32 Plant Introduction (PI) bean accessions of international origin. Future monitoring for seedborne CMV may be desirable in view of current international exchanges of bean germplasm which necessarily involve potential dissemination of this virus.

INTRODUCTION

Approximately 69,000 hectares in the Pacific Northwest were devoted to production of edible beans (Phaseolus vulgaris) in 1979, representing a wholesale value, including production for seed, of about 100 million dollars. Approximately 80% of the snap bean seed produced in the U.S.A. is grown in this area, adding significantly to the regional

value of this crop.

Historically, the principal seedborne virus causing economic damage to the bean industry has been bean common mosaic virus (BCMV,9). This virus is distributed worldwide wherever beans are grown, continues to cause significant crop losses, and receives emphasis in development of resistant cultivars. Hopefully, preventive measures can be developed to preclude new seed-transmitted cucumber mosaic virus (CMV) isolates from posing a parallel disease problem for beans.

It has been reported (25) that CMV infects plants of over 40 dicotyledonous and monocotyledonous families, is readily transmitted mechanically, and is vectored by more than 60 aphid species in a nonpersistent manner (29). Strains of CMV that cause economic loss and are particularly infectious to legumes have existed in Japan for some time (41). In the past decade, two previously unknown properties of this virus, of particular interest to us, have been described.

First, in 1974 a CMV isolate from eastern Spain was reported to be seed-transmitted in beans at a rate of 7% (10). Despite the extensive host range of this virus, prior to 1974 CMV had been reported to be seed-transmitted in only 12 hosts, three of which were legumes (75). Since 1974, three isolates of CMV from New York (81), Puerto Rico (63), and France (61) have been reported to be seed-transmitted in certain bean cultivars at frequencies of 0.3, 1.5, and 30%, respectively.

Second, a low-molecular-weight, CMV-associated satellite RNA (referred to as CARNA 5 by Kaper and Waterworth) has been implicated in altering pathogenicity of isolates naturally infectious to tomato (51). Diaz-Ruiz and Kaper (18) concluded that this CMV satellite was a helper-dependent RNA capable of inducing severe necrosis in tomato

in the presence of CMV. Satellite RNA has been found in several CMV strains and isolates (31,48,56,66,96), and some of these molecules appear to be physically uniform by present techniques (48,66,83). In addition, it has been reported that the satellites from different CMV strains can be freely exchanged between their helpers (48,51). Hence, CMV-satellite RNA contributes unique and unknown capabilities to an otherwise well characterized plant virus. Seed transmission of CMV in P. vulgaris could result in local and international dissemination of this insidious satellite RNA. Quantifying CMV in the Northwest bean seed industry has allowed us to simultaneously monitor CMV-satellite RNA.

The work reported here was prompted by the strategic role and economic value of this industry, recent reports of CMV transmission in bean seed, extensive international movement of bean germplasm, and the potential of CMV-satellite RNA to increase CMV virulence (previous ref. to tomato necrosis).

MATERIALS AND METHODS

In 1978, 28 leaf samples, representing diverse germplasm types, were taken from among thousands of bean plants in eight breeding plots of seven seed companies located in important bean-seed-producing areas in eastern Washington and southern Idaho. Sixty-two samples were also taken from USDA (Prosser, Washington) and Oregon State University (Corvallis) breeding programs. Plants sampled exhibited mild mosaic, stunting, or yellowing symptoms characteristic of CMV infection, but unlike those induced by bean common or bean yellow mosaic viruses, curly top virus, alfalfa mosaic virus, or tobacco streak virus, which

are not uncommon to any of these areas.

Samples from Idaho were ground in buffer and rub-inoculated onto three bean cultivars, Bountiful, Black Turtle, and Limelight. Two weeks later, tissue triturates from these hosts were applied to cucumber plants (Cucumis sativus 'Improved Long Green') in order to increase chances of detecting CMV isolates. Samples taken in Oregon were assayed directly onto cucumbers.

Samples from Washington were similarly processed and used to rub-inoculate bean cv. Bountiful and Monroe, cucumber cv. Boston Pickling, pea (Pisum sativum) cvs. Alaska and Dark Skin Perfection, and cowpea (Vigna unguiculata) cv. California No. 5 (Ramshorn). Selected plants were also tested for CMV by leaf-dip or serologically specific electron microscopy, and/or gel double-diffusion serology, in each instance using our antiserum to isolate CMV-B.

In 1979, 73 plant samples were taken from seed company breeding plots in eastern Washington, southern Idaho, and western Oregon, and 96 samples were collected from USDA and Oregon State University breeding programs. Samples from southern Idaho and western Oregon were assayed for CMV on cowpea, Chenopodium amaranticolor (Corvallis strain), and 'Improved Long Green' cucumber. These samples were also assayed for CMV by enzyme-linked immunosorbent assay (ELISA) as previously described by Clark and Adams (13), with minor modifications. Antiserum to isolate CMV-B (81), was produced from virus purified by the method of Lot et al.(59). Coating immunogammaglobulin (I_gG) and alkaline phosphatase-I_gG conjugate were each used for ELISA at concentrations of 1 ug/ml.

Samples taken in eastern Washington were processed and assayed as

in 1978, samples from Oregon State University breeding plots were assayed solely by ELISA.

Seeds of 32 P. vulgaris Plant Introduction (PI) lines, selected to represent areas from which seed-transmitted isolates of CMV have been reported or are considered likely to occur, were obtained from the W-6 Regional Plant Introduction Station in Pullman, Washington, and tested either by bioassay on Chenopodium and cucumber plants or by ELISA.

CMV-infected (positive) and CMV-free (negative) controls were routinely included in CMV assays. Sensitivity of Chenopodium assay and ELISA methods was determined by testing dilution series of CMV-infected tissues and purified virus.

RESULTS AND DISCUSSION

CMV was not detected in any of the 259 bean plants sampled in Idaho, Washington, and Oregon, but one of 32 Plant Introduction (PI) accessions of beans of U.S. and international origin (Table 2-1) contained seedborne virus serologically identified as CMV. Two of 56 seeds of PI 271998, from Spain, contained this virus designated CMV-Pg. CMV-infected control plants were detected with 100% accuracy. Although the extent of sampling among Pacific Northwest bean breeding programs was somewhat limited, and the appearance of CMV-induced symptoms in diverse bean germplasm types was unknown to us, samples were taken judiciously from among thousands of plants on the visual criteria available to us. Likewise, it was our objective to determine whether seedborne CMV had become a significant problem in breeding programs aimed at developing new, improved bean cultivars for the U.S.A. The absence of CMV in the 259 samples taken from three States

Table 2-1. Results of sampling *Phaseolus vulgaris* germplasm in Idaho, Washington, and Oregon and Plant Introduction (PI) lines for seedborne cucumber mosaic virus (CMV)

YEAR	LOCATION	SOURCE	No. SEED CO.'s OR PI LINES	No. PLANTS SAMPLED	ASSAY METHOD[a]	RESULTS[b]
1978	So. Idaho	Seed Co.'s	6	26	B	0/26
	E. Washington	Seed Co.'s	2	2	B,M,S	0/2
	Prosser	Breeding Programs	-	59	B,M,S	0/59
	Corvallis	Breeding Programs	-	3	B	0/3
1979	So. Idaho	Seed Co.'s	4	43	B,E	0/43
	W. Oregon	Seed Co.'s	1	4	B,M,S	0/4
	E. Washington	Seed Co.'s	4	26	B,M,S	0/26
	Prosser	Breeding Programs	-	56	B,M,S	0/56
	Corvallis	Breeding Program	-	40	B,E	0/40
1979-1980	U.S. PI collection, Pullman, Washington	PI lines, U.S.	6[c]	188	B,E	0/188
		PI lines, Int.	26[c]	1070	E	2/1070[d]

[a] B = Bioassay, see text for hosts. M = Electron microscopy of selected hosts. S = Gel double-diffusion serology of selected hosts. E = Enzyme-linked immunosorbent assay (ELISA).

[b] No. samples in which CMV was detected/no. samples tested.

[c] 6 *Phaseolus* lines of U.S. origin, 26 lines of international origin.

[d] 2/56 plants infected of PI 271998 from Spain.

suggests that CMV has not yet become a problem to the Pacific Northwest bean seed industry. Surveillance for CMV is continuing, using highly susceptible bean cultivars as trap crops.

By ELISA methodology, CMV was readily detected in infected fresh tissue controls diluted 1000-fold (100 fold by healthy plant tissue, 10-fold by buffer), in desiccated tissue at 100,000-fold dilution with buffer, and in the purified state at 6 ng/ml. The corresponding values for bioassay on Chenopodium plants were 10-fold, 100-fold, and 700 ng/ml for fresh and desiccated infected tissue, and purified virus, respectively. Thus, ELISA was roughly 1000 times more sensitive than bioassay for detection of CMV.

Certain characteristics of the CMV isolates seedborne in beans may have limited our ability to detect them during this survey, e.g., symptoms induced in beans by these isolates typically consist of mild mosaic or veinbanding, which can be caused by other factors or may be masked, and these isolates may be seed-transmitted in beans at frequencies that would easily escape attention. However, in our opinion, expected low frequencies of CMV seed transmission would be partially off-set relative to detection potential in our survey by the readiness with which the virus once established is aphid transmitted to healthy plants. In addition, the use of highly sensitive ELISA methodology should have facilitated detection of this virus even at low concentrations.

Despite the ostensible absence of seedborne isolates in the Pacific Northwest bean-seed-producing areas, CMV is a likely candidate for inadvertent introduction into beans through the international movement of bean germplasm for development of new improved cultivars,

unless preventive measures are instituted. We believe disease prevention is increasingly a strategic measure for successful bean production. In retrospect, substantial losses to the pea seed industry might have been avoided if pea seedborne mosaic virus (38) had been detected and prevented at an early stage, as the opportunity with CMV presently provides. Concern for the threat of seedborne CMV to the bean seed industry and a continuing surveillance for this virus are warranted by pathogenic implications of CMV satellite RNA in tomatoes, recent reports of seed transmission of CMV through beans, lack of CMV-resistant Phaseolus germplasm, and the occurrence of CMV in at least one international PI bean accession.

Chapter Three

Host Range/Reactions and Virus Titer Development in *Phaseolus vulgaris*

ABSTRACT

Host ranges of selected legume-infecting cucumber mosaic virus (CMV) isolates on 20 plant species in 17 genera and 7 plant families were compared and found to be similar. Thirty-two bean (*Phaseolus vulgaris*) cultivars representing diverse germplasm were infected when inoculated in the field with CMV-B. Timing and severity of CMV-induced symptom development, virus concentration, and susceptibility to field infection (aphid transmission) varied among cultivars. Virus concentration was estimated to be maximal in infected field-grown beans approximately two weeks after inoculation. Virus concentration in greenhouse-grown plants increased to a peak about one week after inoculation, remained at that level the second week, and declined during the third and fourth weeks to an equilibrium level, to the limit of detection in this system. Implications of these results to sampling for detection of CMV are discussed.

INTRODUCTION

In 1974 a cucumber mosaic virus (CMV) isolate from eastern Spain ($CMV\text{-}B_{32}$) was reported to be seed-transmitted in beans (*Phaseolus vulgaris*) at a rate of 7% (10). Since 1974, the following isolates have been reported to be seed-transmitted in beans at the given frequencies: CMV-B, New York, 0.3% (81); CMV-Pr, Puerto Rico, 1.5% (63); CMV-F, France, 9-54% (61); CMV-Za8, Yugoslavia, 20% (1); and CMV-Pg,

U.S. Plant Introduction accession 271998-Spain (Chapter Two, p.15). Like other CMV isolates, these isolates infect a wide range of hosts, and are easily transmitted both mechanically and by aphids in a non-persistent manner. Resistance to this virus among Phaseolus vulgaris sources may be non-existent. Provvidenti (81) reported only two Phaseolus species resistant to CMV-B in 21 species tested.

Described here are results of host range studies conducted in our area with the seedborne isolates as well as an isolate considered non-seed-transmissible in beans, CMV-Le from Japan (41). The relationship of time and virus concentration in plants of selected bean cultivars following mechanical inoculation was also investigated.

MATERIALS AND METHODS

Virus isolates. CMV-Pg was isolated from a PI accession (No. 271998) of P. vulgaris obtained from the Western Regional Plant Introduction Station in Pullman, Washington (Chapter Two, p.15). Other CMV isolates used were obtained from the following individuals: CMV-B from Dr. R. Provvidenti (81); CMV-B_{32} from Dr. L. Bos (10); CMV-Pr from Dr. J. P. Meiners (63); CMV-F from Dr. J. C. Devergne (61); and CMV-Le from Dr. T. Inouye (41). Isolates were propagated in P. vulgaris 'Bountiful', and desiccated for use as standard inoculum sources.

General host range. All of the 23 hosts (except Phlox drummondii) used to differentiate legume viruses (37) were tested for reactions to some of the CMV isolates used. Also tested were Cucumis sativus cvs. Improved Long Green and Lemon, Glycine max cv. Bonsai, Nicotiana hybrida, Nicotiana tabacum cv. Xanthi, and 25 additional cultivars of

P. vulgaris (Table 3-1). The total host range tested represents 20 species, 17 genera, and 7 plant families, including legumes and non-legumes.

Plants were dusted with 400-mesh carborundum and rub-inoculated with fresh or desiccated virus-infected tissue ground in 0.02 M phosphate buffer, pH 7. Inoculum was assayed onto indicator plants to monitor infectivity. Two or more plants of each host were inoculated with each isolate tested. Plants mock-inoculated with buffer served as comparisons. Symptoms were recorded at least weekly for several weeks following inoculation. In some cases symptomless infections were tested by back inoculation onto indicator hosts or by enzyme-linked immunosorbent assay, ELISA (Chapter Two, p.14).

Bean cultivars. Susceptibility of selected bean cultivars to CMV-B and the range of field symptoms induced by CMV-B were determined in field plots near Corvallis in 1979. Replicated plots of 32 bean cultivars were inoculated for direct comparison with non-inoculated plants (Table 3-2). Symptom development was monitored weekly and symptomless infection was tested by back inoculation onto indicator hosts. Seeds were harvested from nine cultivars for seed-transmission determinations (Chapter Four).

Titer versus time - field. Infected 'Bountiful' bean plants from the above plots were sampled weekly from the uppermost or youngest fully expanded trifoliate leaves. Non-inoculated plants were similarly sampled. Leaf samples were immediately desiccated and stored over Anhydrone at -10 C. Samples were assayed by ELISA serology at a dilution of 1:80,000 (wt/vol) with buffer, a dilution determined to be

within the range of linearity of this system.

Titer versus time - greenhouse. Virus titer versus time was also measured in CMV-B-infected 'Bountiful' plants in an aphid-free greenhouse. Groups of ten 'Bountiful' plants were each rub-inoculated with CMV isolates B, Le, or F when the unifoliate leaves were just fully expanded. One group was left non-inoculated. One week after inoculation a number seven (13 mm) cork borer was used to remove leaf discs from unifoliate leaves of the 10 replicate plants. These were combined and desiccated. At weekly intervals thereafter for six weeks leaf discs were removed from the youngest fully expanded trifoliate leaf. Thus, each leaf set was sampled uniformly among plants per time of sampling (see relation of plant parts and virus concentration, Chapter Four). When sampling was completed, a dilution series of healthy and infected desiccated tissue ground in buffer was tested by ELISA serology to determine relative virus concentrations during the sampling period. All samples were then tested at the determined dilution of 1:100,000 (wt/vol).

RESULTS AND DISCUSSION

General host range. All 12 legume and 8 non-legume plant species included in this study, except Glycine max, were infected with the isolates tested (Table 3-1). These results are in general agreement with data previously published for the individual CMV isolates and demonstrated the wide host range of this virus. This information represents an attempt to differentiate CMV isolates by symptomatology on selected hosts. Analyses of these data indicated that CMV isolates were not consistently differentiated by symptoms produced on these hosts.

Table 3-1. Host reactions of greenhouse-grown plants to mechanically inoculated cucumber mosaic virus (CMV) isolates

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
Chenopodium amaranticolor (Corvallis strain)	LLc[a] / -*	LLc / -*	LLc / -*	(LLc) / -*	LLc / -*	LLc / -*
Cucumis sativus Chicago Pickling	LLc / Mo,(VChl)	0 / 0	LLc / Mo,(VChl)	(LLc) / Mo,Vc	LLc / Mo	0 / 0
Lemon	LLc / Mo	LLc / Mo	LLc / Mo	0 / 0	LLc / Mo	LLc / Mo
Improved Long Green	LLc / Mo	LLc / Mo	LLc / Mo	0 / 0	LLc / Mo	LLc / Mo
Datura stramonium (R. Fulton strain)	Mo,Chl,N / (Chl)	0 / 0	Mo,Chl,N / (Chl)	0 / 0	0 / 0	0 / 0
Glycine max Bonsai	-* / -*	0 / 0	-* / -*	-* / -*	-* / -*	0 / 0
Bragg	- / -	- / -	- / -	-* / -*	-* / -*	0 / 0
Davis	- / -	- / -	- / -	-* / -*	-* / -*	0 / 0
Gomphrena globosa A. F. Ross strain	LLn / Chl,VChl	LLn / Chl	LLn / Chl	-* / -*	-* / -*	0 / 0

Table 3-1, cont.

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
Lycopersicon esculentum Marglobe	LL (Mo)	0 0	LL Mo,LLn	LL,N Mo,Stu	LLn,N Stu	0 0
Medicago sativa DuPuits	-* S	-* S	-* S	0 0	0 0	0 0
Nicotiana glutinosa Corvallis strain	Mo Mo,Ma	0 0	Chl,Mo,N LLc,Mo	-* -*	0 0	0 0
Nicotiana hybrida	-* (Mo,Vc),Stu	0 0	-* Mo	-* Mo	-* (Mo,Vc),Stu	0 0
Nicotiana tabacum Samsun NN	ℓ Mo	0 0	(Mo) Mo	-* (Mo)	0 0	0 0
Xanthi	ℓ Mo	ℓ Mo	ℓ Mo	0 0	0 0	ℓ Mo
Petunia hybrida King Henry	-* VChl,Mo	0 0	-* Mo,(VChl)	-* (Mo)	-* Mo	0 0
Phaseolus vulgaris - DRY BEANS Big Bend RM	Chl,Ep Chl,Mo,Vb	o 0	Ep Chl,Mo,N,Vb	-* Stu*	0 0	0 0
Black Turtle	Chl,Ep,LLn Mo,LR,Stu	Ep Chl,Mo,(Vb)	Chl,Ep,(LLn) LR, Stu	(LLn) Mo,(LLn)	-* -*	0 0

Table 3-1, cont.

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
Chief	Chl,Ep / Chl,Stu,Vb	0 / 0	Chl,Ep / Mo,Stu	(VN) / -*	0 / 0	0 / 0
Columbia Pinto	0 / 0	0 / 0	Ep / LR	0 / 0	0 / 0	0 / 0
Earliwax	Ep / Vb,VChl	0 / 0	Ep / Vb,VChl	ChlS,Ep / Mo,Vb,Stu	0 / 0	0 / 0
Pinto 111	Ep,(Mo,VC) / Mo,Stu,(Vb)	(Ep) / Mo	Ep,Mo,VC / Mo,Vb	0 / 0	Ep,LLn / Chl,LLn,Mo	0 / 0
Pinto UI 114	Ep / Mo,N	0 / 0	Ep / Stu,Vb,VN	-* / Stu*	0 / 0	0 / 0
Red Kidney	Ep,(VChl) / Mo,LR,Stu,Vb	0 / 0	Ep,(VChl) / Stu,Vb	(Ep)* / Mo,Vb	-* / (VC)*	0 / 0
Sanilac	Chl,N / Mo,VChl	0 / 0	-* / -*	-* / (VChl)	0 / 0	0 / 0
Viva Pink	Chl,Ep / ChlS,Stu	0 / 0	Chl,Ep / Chl,Stu,Vb	Chl / Stu,Vb	0 / 0	0 / 0
P. vulgaris - SNAP BEANS Asgrow 290	(ChlS) / Chl,Stu,Vb	0 / 0	Ep,(ChlS) / Stu,Vb,VN	Chl / Stu,Vb	0 / 0	0 / 0

Table 3-1, cont.

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
Asgrow BBL 274	(Ep) / Stu	(Ep) / Ma,Stu	Ep / Ma,Mo,Stu	0 / 0	0 / 0	0 / 0
Asgrow Checkmate	-* / Ma,Mo,Vb	0 / 0	Ep / Ma,Mo,Vb	0 / 0	0 / 0	0 / 0
Asgrow Eagle	-* / Mo,Stu,Vb	-* / Mo,Stu	Ep / Stu,Vb	0 / 0	0 / 0	0 / 0
Bountiful	Ep / (Mo,Vb),Stu	Ep / (Mo,Vb),Stu	Ep / (Mo,Vb),Stu	Ep / (Mo,Vb),Stu	Ep / (Mo,Vb),Stu	Ep / (MO,Vb),Stu
Early Gallatin	Ep,VChl,N / Stu,Vb	0 / 0	Ep,VChl,N / Vb	Chl / Chl,Mo,Vb	0 / 0	0 / 0
IBRN 4	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	0 / 0	0 / 0	0 / 0
IBRN 9	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	0 / 0	0 / 0	0 / 0
IBRN 19	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	(Ep,LLn) / (Mo)	0 / 0	0 / 0	0 / 0
Limelight	Chl,Ep,LLn / ChlS,Stu,Vb	0 / 0	Chl,Ep,LLn / Stu,Vb	-* / Stu	0 / 0	0 / 0

Table 3-1, cont.

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
OSU 58	-* Mo,Vb	(Ep) Mo,Vb	Ep Mo,Vb	0 0	0 0	0 0
OSU 190	Mo Vb	0 0	Chl,Ep Vb	N Ma,Vb	0 0	0 0
OSU 1604	ChlS,Ep LR,Ma,Stu,Vb	0 0	-* -*	-* -*	0 0	0 0
OSU 3525	ChlS,Ep LR,Mo,Stu,Vb	0 0	Ep Ma,Mo	Chl Chl,Mo	0 0	0 0
RB Majestic	(Ep) Vb,(Mo)	(Ep) Vb,(Mo)	Ep Vb,(Mo)	0 0	0 0	0 0
Slenderette	-* Stu	-* Stu	Ep Mo	0 0	0 0	0 0
Tendercrop	ChlS Stu,Vb	0 0	ChlS Mo,Stu	ChlS,Ep Mo,Stu,Vb	0 0	0 0
Twilley Greenpak	(Ep) Ma,Mo	(Ep) Mo	Ep Ma,Mo	0 0	0 0	0 0
Pisum sativum Perfected Wales	N Mo,VChl	-* -*	N Chl,Mo,VC	-* -*	-* Chl	0 0

Table 3-1, cont.

HOST	CMV ISOLATE					
	Le	F	B	Pr	B_{32}	Pg
Dark Skin Perfection	N / Chl,N,VChl	ℓ / Mo	N / Chl,Mo,N	-* / -*	-* / Chl	0 / 0
Spinacea oleracea Bloomsdale Long Standing	Mo,N / LC,Ma,Mo,Stu	0 / 0	LLc,Mo,N / Mo,Stu	(ChlS) / LR,Ma,Mo,Stu	-* / -*	0 / 0
Trifolium sp. white	-* / -*	0 / 0	-* / -*	-* / -*	-* / -*	0 / 0
red	-* / Mo	0 / 0	-* / Mo,Ma	-* / LLc	-* / -*	0 / 0
Vicea faba Bell	(LLn) / Chl,Mo,VChl	(LLn) / S	(LLn) / N,Stu,VC	(LLn) / N	-* / N	0 / 0
Vigna unguiculata Calif. No. 5 (Ramshorn)	LL / Mo,Stu	LL / Mo	LL / Mo,Stu	(LL) / -*	LL / Stu	0 / 0

[a] Key to symbols: 0 = Not tested; - = No symptoms; ( ) = Variable, symptom not always expressed; * = No back inoculation to test for latent infection; Chl = Chlorosis; ChlS = Chlorotic spots; Ep = Epinasty; ℓ = Latent, localized; LC = Leaf curl (upward); LL = Local lesions, unspecified, LLc (chlorotic), LLn (necrotic); LR = Leaf roll (downward); Ma = Malformation (distortion, crinkle, savoying, strap leaf, fern leaf); Mo = Mottle or Mosaic; N = Necrosis, general; NS = Necrotic spots (restricted systemic necrosis, zones, spots, flecks); s = Latent, systemic; Stu = Stunt; Vb = Vein banding (chlorotic band on each side of vein); VC = Vein clearing (loss of normal color, chlorosis of vein); VChl = Vein chlorosis; VN = Vein necrosis. Systemic symptoms/local symptoms.

However, it was noted that several cultivars of beans reacted with a more pronounced epinasty of the primary leaves when inoculated with CMV-B in the greenhouse, compared with CMV-Le, F or buffer. This mild variation in symtomatology, however, was viewed as insufficient distinction among isolates and was not pursued. Thus, host range results did not provide an appropriate symptomalogical marker for differentiating isolates to be used in pseudorecombination experiments (Chapter Seven). Figure 3-1 shows typical CMV-induced symptoms on three indicator species, cucumber (Cucumis sativus 'Lemon'), cowpea (Vigna unguiculata 'Ramshorn'), and Chenopodium amaranticolor Corvallis Strain, and on 'Bountiful' beans.

Bean cultivar reations. The 32 cultivars of field-grown beans inoculated with CMV-B displayed a full range of reactions from symptomless to severe leaf malformations, vein banding, blistering, and stunting (Table 3-2). The plants are grouped in Table 3-2 according to the type and severity of symptoms. Symptom Group I contained four cultivars that remained symptomless; the 15 cultivars in Group II produced mild and infrequently expressed leaf symptoms; Group III plants included three cultivars that were markedly stunted but with few or no leaf symptoms; and the ten plants in Group IV developed severe vein-banding, mottle, mosaic, stunt, and leaf malformation resembling 2, 4-D injury. Figure 3-2 shows symptoms expressed by eight of the cultivars in Group IV collected one month after inoculation.

Virus concentration versus symptom development. Although some cultivars remained symptomless, all cultivars were susceptible to CMV as indicated by bioassay or ELISA serology. However, results of the bio-

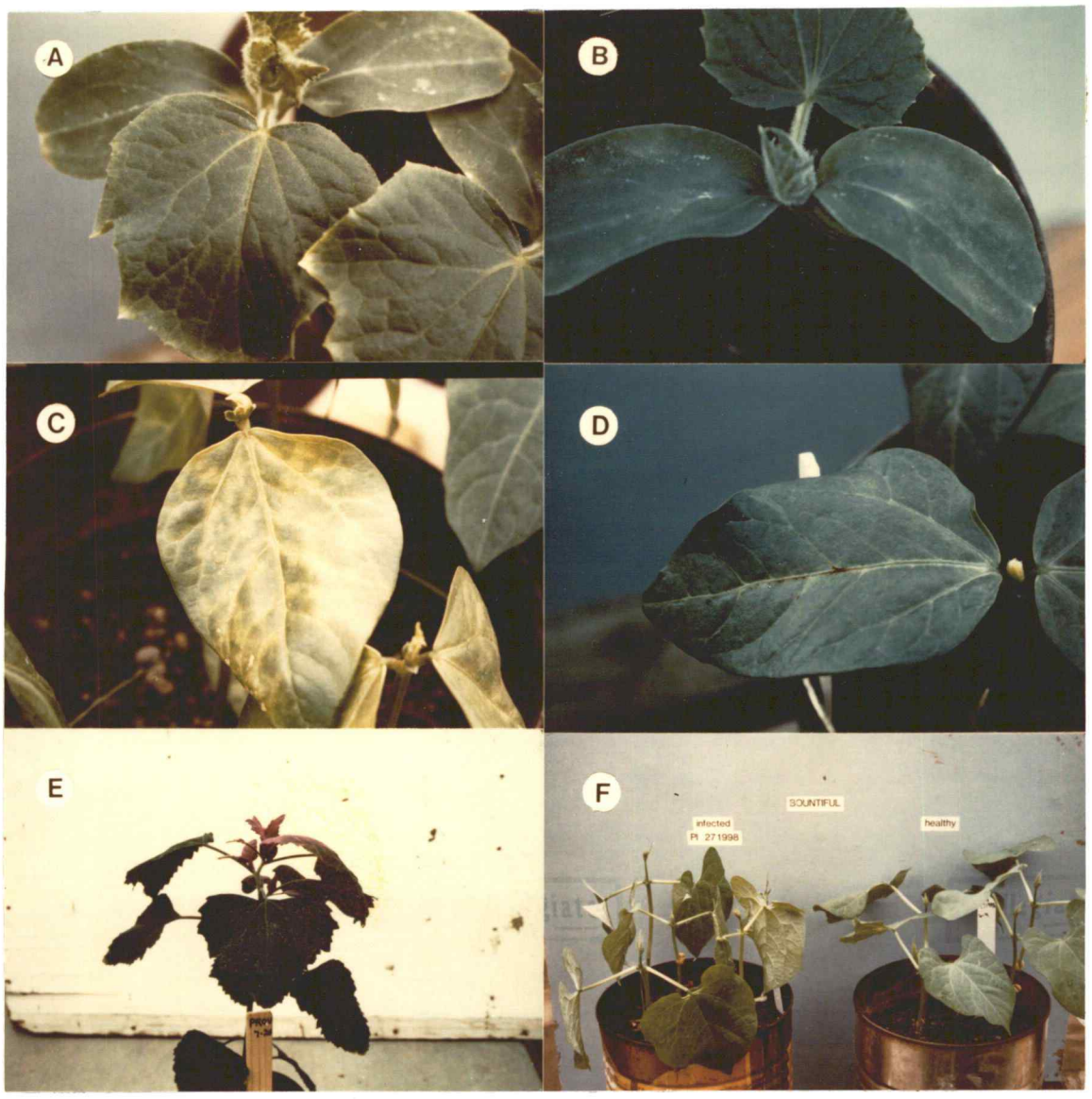


Figure 3-1. Typical host reactions of selected species to CMV-infection. A, Non-inoculated cucumber (Cucumis sativus 'Lemon'); B, CMV-B infected cucumber with typical chlorotic spots on primary leaves; C,D, symptom variation in infected cowpeas (Vigna unguiculata 'Ramshorn'), C, CMV-B infected cowpea showing chlorotic spots, D, necrotic spots and veinal necrosis on CMV-B_{32} infected cowpea; E, Chenopodium amaranticolor (Corvallis strain) displaying chlorotic local lesions; F, CMV-Pg (PI 271998) infected bean (Phaseolus vulgaris 'Bountiful') plants with epinasty of the primary leaves are shown on the left.

Table 3-2. Host reactions and infectivity index of field-grown bean cultivars mechanically inoculated with CMV-B.

SYMPTOM[a] GROUP	CULTIVAR[b]	SYMPTOMS[c]	INFECTIVITY INDEX[d]			COMMENTS[e]
			COWPEA	CUCUMBER	CHENOPODIUM	
I	BBL Advance	-/-	2/5	3/4	-	
	Vitagreen	-/-	-	-	+(1)	
	Michelite	-/-	-	4/4	-	
	Kentucky Wonder	-/-	4/4	3/4	++	
II	Bountiful	-/(Chl,Mo,Stu,Vb)	2/4	2/5	+	
	Gallamour	-/(Vb)	-	-	+(2)	
	GV-50	-/(Mo,Stu,Vb)	-	1/4	+	
	Royal Burgundy	-/(Stu,Vb)	-	-	-	
	Charlevoix	-/(Vb)	-	1/4	+(1)	
	Sutter Pink	-/(Mo,Stu)	-	-	+	
	Dwarf Hort 1976	-/Stu,(Vb)	-	-	-	
	Santa Ana	-/(Stu,Vb)	3/4	2/3	++	
	Early Blue	-/(Vb)	2/4	4/4	++	
	NK EXP 186	-/(Mo,Vb)	2/4	2/4	+	
	Oregon 58	-/(Mo,Vb)	5/5	4/4	++	
	Bush Romano	-/(Mo,Vb)	-	-	-	
	Medford	-/(Ma,Mo,Vb)	4/4	4/4	++	
	Astro	-/(Mo,Stu,Vb)	1/5	1/3	-	
	Spartan Arrow	-/(Mo,Vb)	0	0	0	
III	Stretch	-/Stu	3/5	2/4	++	
	Gabriella	-/Stu	2/4	2/3	++	
	Sunrise	-/(Mo),Stu	3/4	2/3	-	
IV	Harvester	-/Ma,Mo,Stu,Vb	0	0	0	E
	Majestic	-/Ma,Vb	2/4	3/4	++	E,S

Table 3-2, cont.

SYMPTOM[a] GROUP	CULTIVAR[b]	SYMPTOMS[c]	INFECTIVITY INDEX[d] COWPEA	CUCUMBER	CHENOPODIUM	COMMENTS[e]
IV	BBL 274	-/Ma,Vb	-	-	-	M,S
	Eagle	-/Stu,Vb	-	-	-	M,R
	Slenderette	-/Ma,Stu,Vb	-	2/4	+(1)	E,R
	Checkmate	-/Stu,Vb	-	-	-	M
	Greenpak	-/Ma,Vb	-	-	-	E,S
	Goldrush	-/Ma,Mo,Stu,Vb	0	0	0	E,S
	Green Isle	-/Ma,Mo,Vb	-	-	-	E,R
	NK EXP 519-6	-/Ma,Mo,Stu,Vb	0	0	0	E,R

[a] I = Symptomless. II = Mild or infrequently expressed symptoms. III = Strong stunt. IV = Severe foliar symptoms expressed frequently.

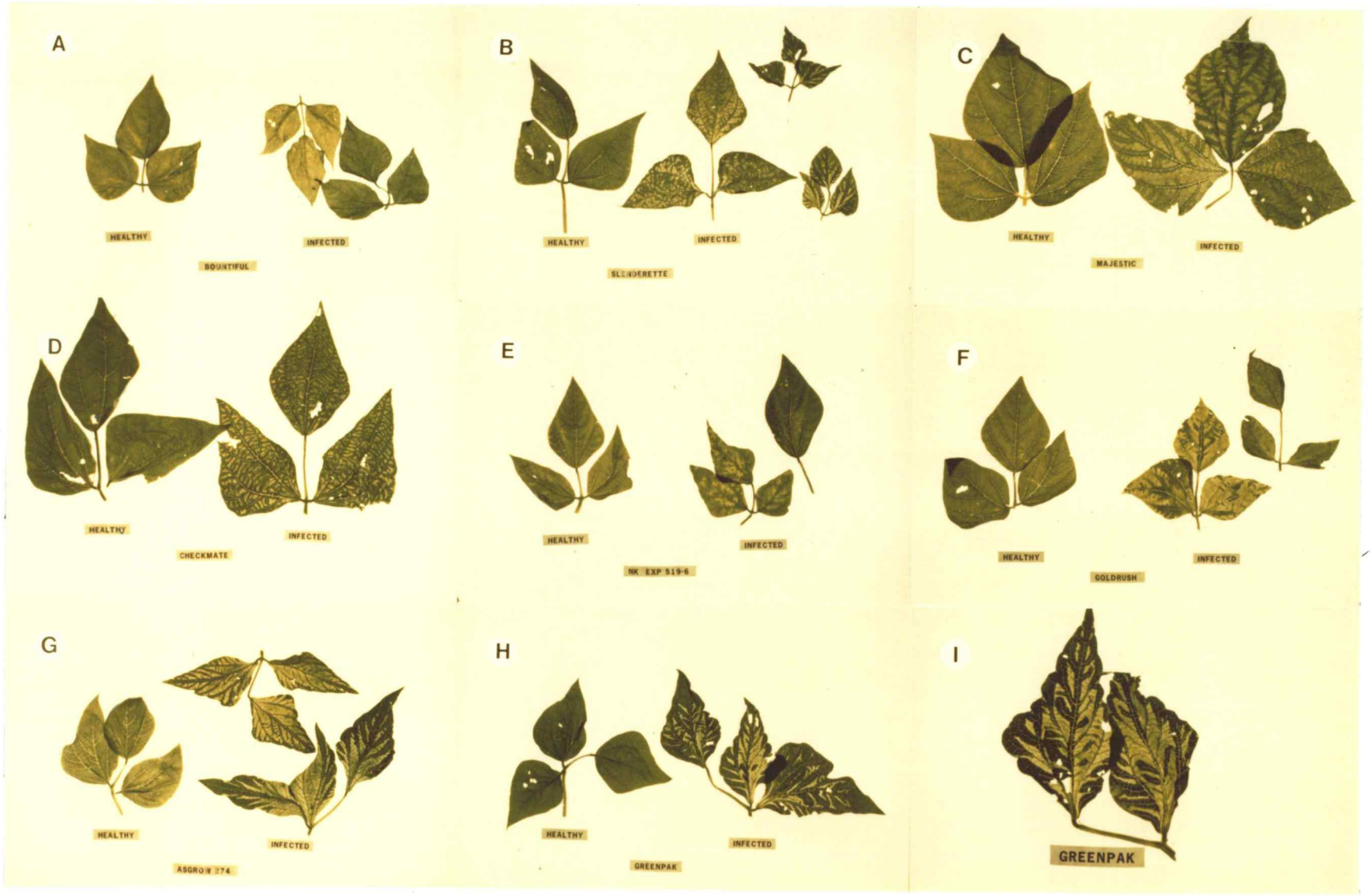
[b] Approximately 10 plants in a 10-foot row were inoculated, others were left non-inoculated.

[c] Local/Systemic symptoms. See key to symbols in Footnote [a] of Table 3-1.

[d] Results of back inoculation onto cowpea (Vigna unguiculata 'Ramshorn'), cucumber (Cucumis sativus 'Lemon'), and Chenopodium amaranticolor (Corvallis strain) five weeks after inoculation of beans. Number of half-leaves infected/number inoculated. - = 0 lesions or no infection; + = 1-10 lesions; ++ = 11-50 lesions; number of lesions in parentheses; 0 = not tested.

[e] Symptom development timing and spread of disease. E = Early season symptom development. M = Midseason symptom development. R = Recovery from symptoms. S = Spread of symptoms to adjacent non-inoculated plants.

Figure 3-2. Symptom expression in selected field-grown Phaseolus vulgaris cultivars one month after mechanical inoculation with CMV-B. A-I, Healthy and infected leaves are labelled. A, Bountiful with latent infection; B, Slenderette showing mosaic; C, Majestic with moderate veinbanding; E,F, NK EXP 519-6 and Goldrush, respectively, with mottle; G,H, Asgrow (BBL) 274 and Greenpak, respectively, showing leaf malformation and veinbanding; I, Greenpak, close-up of symptoms.


A
HEALTHY
INFECTED
BOUNTIFUL
B
HEALTHY
INFECTED
SLENDERETTE
C
HEALTHY
INFECTED
MAJESTIC
D
HEALTHY
INFECTED
CHECKMATE
E
HEALTHY
INFECTED
NK EXP 519-6
F
HEALTHY
INFECTED
GOLDRUSH
G
HEALTHY
INFECTED
ASGROW 274
H
HEALTHY
INFECTED
GREENPAK
I
GREENPAK

assay conducted five weeks after inoculation revealed that plants without symptoms (Group I) contained high concentrations of virus, but more surprisingly five out of seven cultivars with strong symtoms (Group IV) did not appear to contain virus in amounts detectable at that time by bioassay on three indicator plant species (Table 3-2). Shock and Goodman (88) have reported a similar situation with bean golden mosaic virus, in which virus titer was low in bean leaves with well developed symptoms when compared with leaves at a similar developmental state that had shown less severe symptoms. Thus, symptomatology may not be the most efficient means of selecting bean plants samples for CMV testing.

Symptom timing and virus spread. The timing of symptom development and spread of virus infection to adjacent plants in Group IV cultivars were observed. Symptoms appeared in cultivars at different times and the longevity of symptoms varied, with some cultivars recovering. In addition, a variable amount of infection in non-inoculated plants was noted (more than 50% in some cases) among cultivars. Based on this high degree of virus spread bean cultivars,Majestic, BBL 274, Greenpak, and Goldrush were concluded to be uniquely favorable sources of CMV for aphid vectors (Table 3-2).

Trap-crop indicator cultivars. An effort was made to discern cultivars that could serve as trap-crop indicators of CMV infections. Four cultivars (Greenpak, Checkmate, Goldrush, and Harvester) were selected on the basis of strong reactions to CMV infection in the field (Table 3-2) and the greenhouse (results not shown). These cultivars did not develop CMV symptoms during the summer of 1980 when planted in bean

producing areas of Oregon, Washington, and Idaho, suggesting that CMV was not prevalent in aphid populations during that time.

Titer versus time - field. ELISA assay of the desiccated tissue collected from field grown CMV-B infected Bountiful plants suggested that the maximum virus concentration occured about two weeks after inoculation (Table 3-3). However, the validity of assay results were limited by apparent secondary spread and resultant symptomless virus infection in non-inoculated plants used as controls.

Titer versus time - greenhouse. A more controlled study conducted in the greenhouse with CMV-B, Le and F revealed a surprising degree of uniformity in virus titer among Bountiful plants infected with these isolates (Table 3-4). When tissue from infected and non-infected plants sampled weekly was tested by ELISA assay at dilutions determined to be within the range of a linear response with this system, it was found that virus levels for all three infected tissues were highest the first two weeks after inoculation, dropping to one-third that level on the third week and decreasing to the limit of detection on the fourth and subsequent weeks. These results suggest that virus titer in greenhouse-grown beans reaches a maximum within two weeks, then declines to an equilibrium level. The development of the bean plants grown in the greenhouse under our conditions is much more rapid than in the field, i.e., plants in the greenhouse remain in a vegetative state for a shorter period before flowering. Considering this difference in rate of development, it is quite plausible that the production of maximum virus levels would be shifted in field-grown plants by as much as two to three weeks.

Table 3-3. Relative CMV titer in bulk samples of field-grown CMV-B infected plants of 'Bountiful' beans, as measured sequentially by ELISA serology (A_{405} nm)

	TIME AFTER INOCULATION (DAYS)				
INOCULUM	14	31	34	41	48
CMV-B	.84 ± .16	.53 ± .20	.41 ± .07	.48 ± .13	.49 ± .02
NON-INOC	.05	.79 ± .30[a]			

[a] Plants infected by natural field spread, titer determinations discontinued on controls.

Table 3-4. Relative CMV titer in juvenile foliage of 'Bountiful' bean plants as measured sequentially by enzyme-linked immunosorbent assay serology (A_{405} nm)

	TIME AFTER INOCULATION (WKS)[a]						
INOCULUM	1	2	3	4	5	6	7
CMV-B	1.32 ± .71[b]	1.27 ± .38	0.53 ± .12	0.09 ± .04	0.02 ± .02	0.06 ± .03	0 ± .02
CMV-F	1.60 ± .49	1.58 ± .99	0.56 ± .08	0.11 ± .05	0.07 ± .03	0.11 ± .03	0.02 ± .01
CMV-Le	1.46 ± .64	1.58 ± .86	0.45 ± .12	0.06 ± .05	0.04 ± .03	0.03 ± .03	0.01 ± .01
AVERAGE[c]	1.46 ± .29	1.48 ± .41	0.51 ± .01	0.09 ± .05	0.04 ± .03	0.06 ± .04	0.01 ± .01
NON-INOC	0 ± .05	0 ± .04	0 ± .02	0 ± .02	0.03 ± .02	0.03 ± .05	0 ± .01

[a] Unifoliate leaves of ten plants were sampled uniformly one week after inoculation. At weekly intervals thereafter samples were collected from newly expanding trifoliate leaves. Samples were desiccated and assayed simultaneously at 100,000-fold dilutions.

[b] ELISA A_{405} values are means of three determinations, standard deviations given.

[c] Average ELISA A_{405} values for CMV-B, F, and Le at each week

Summary. In summary, no host range differences were found among the CMV isolates tested. No bean cultivars that were resistant to infection by CMV have been discovered in these studies or related studies by Dr. M. J. Silbernagel (personal communication) or Dr. R. Provvidenti (81). Although all beans cultivars tested were CMV susceptible, time and severity of symptom development varied widely, as did the propensity to provide inoculum for secondary virus spread by aphids. Although visual detection is greatly facilitated by using plants that react with strong symptoms to the virus, assays based on virus titer might be more successful with plants containing symptomless infection. In future use of "trap cultivars" for monitoring CMV incidence, both hypersusceptible and tolerant, CMV-supportive bean cultivars should be chosen.

Some of the variables that could be expected to affect virus synthesis and titer include susceptibility of plants, age of plants when infected, age of plant when sampled, specific virus isolate, and environmental factors. While preliminary experiments indicate a maximum buildup of virus in one to two weeks after mechanical inoculation, additional experiments in the field involving these variables could determine more precisely the dynamic relationship between time and virus concentration under natural infection conditions.

Chapter Four

Seed Transmission and Distribution of Cucumber Mosaic Virus in *Phaseolus vulgaris*

ABSTRACT

Transmission of cucumber mosaic virus (CMV) in seeds from mechanically inoculated bean (*Phaseolus vulgaris*) cultivars was investigated. CMV-B was transmitted through 2.4% and 1.8% of the seed of field-grown 'Tendercrop' and 'Red Kidney', respectively. CMV-F was transmitted through 3.3% and CMV-Pg through 49% of the seeds of greenhouse-grown 'Topcrop' beans. Plants infected through seed-transmission did not display symptoms consistently or for more than two weeks after systemic tissue developed. Seed-transmission did not occur in seeds from plants inoculated as long as four or six weeks after planting. Virus distribution was found to be uniform within plants infected through seed-transmission but concentration was most consistent in tissue near the growing point. Detection, control, and significance of seedborne CMV are discussed.

INTRODUCTION

The value of the bean seed industry in the Pacific Northwest and reports of CMV isolates seedborne in beans were discussed earlier (Chapter Two, pp.11-12). Although reports of CMV seed-transmission in beans have come from different parts of the world (1,10,61,63,81), seed-transmissibility of these isolates in beans grown in the Pacific Northwestern U.S.A. had not been accessed. Investigation of this phenomenon, it was concluded, would 1) provide supportive information on

the potential of CMV to be seedborne in beans, 2) aid in estimation of the significance of this phenomenon to this area and elsewhere, 3) seek to identify bean cultivars with genetic resistance to the seed-transmission process, for use in breeding programs, and 4) seek to elucidate viral-host genetics that contribute to high rates of seed-transmission. The latter objective was undertaken to identify viral genomic determinants of seed-transmissibility, by comparing seed-transmission rates of RNA pseudorecombinants produced between seedborne and non-seedborne isolates (Chapter Seven).

Clear symptoms in bean plants contracting CMV by means of infected seed have been reported for CMV-B_{32} (10), CMV-Pr (63), and CMV-Za8 (1). Detection of seedborne CMV infections is facilitated by clear symptom development in bean plants grown from CMV-infected seed. However, in the event that symptoms in such plants are mild or masked, knowledge of the distribution of virus within the plant was sought to allow more efficient sampling for CMV detection.

Reported in this chapter are results of seed-transmission evaluations of CMV isolates B, F, Pg and Le (non-seedborne control) in seeds from mechanically inoculated bean cultivars. Symptom development and virus distribution in seedlings infected from seed are also presented.

MATERIALS AND METHODS

Virus isolates. Information regarding the origin and maintenance of isolates is outlined in Chapter Three, p.20.

Seed-transmission.

Experiment I. In July, 1978, 100 seeds each of bean cultivars

Tendercrop, OSU-1604, and Red Kidney were planted about two inches apart in 16-foot row-plots at the Lewis-Brown Horticulture Farm, Corvallis. Two weeks later, plants were dusted with 400-mesh carborundum and rub-inoculated, using cotton applicators, with tissue homogenates of CMV-B-infected cowpea (Vigna unguiculata 'Ramshorn') or Limelight beans. One foot of each row was mock-inoculated with 0.02 M phosphate buffer, pH 7, to serve as control. Infectivity of inoculum was confirmed on indicator plants - cowpea, cucumber (Cucumis sativus 'Improved Long Green'), and Chenopodium amaranticolor (Corvallis strain). Inoculated plants were assayed periodically to confirm infection and symptoms were recorded. Pods were harvested when mature (after the first frost), then dried and stored at room temperature. Seeds were planted three to six months later in one-gallon cans, five seeds per can, 80 cans at a time. Seedlings were examined visually, some photographed, and bulk tested in groups of two cans each on indicator plants. Plants in groups assaying positively were retested individually on indicator plants. Those plants producing positive results were confirmed again by another indicator assay and tested serologically in double-diffusion gels of 0.4% ionagar (Chapter Five, p.59).

Experiment II. Nine of the thirty-two bean cultivars tested for reactions to CMV-B (Chapter Three) — Early Blue, Harvester, Santa Ana, Bush Blue Lake (BBL) Advance, BBL 274, Greenpak, Majestic, Oregon 58, and Sutter Pink — were selected from among the four symptom groups (Table 3-2) for determination of seed-transmission. Pods were shelled when dry and seeds were stored at room temperature for up to six months before being tested. Seeds were planted for testing as described in

Experiment I. Seedlings were bulk assayed in groups of ten[1] or less by enzyme-linked immunosorbent assay (ELISA) two weeks after planting as previously described (Chapter Two, p.14). The ELISA test was repeated on individual plants in groups suspected of containing virus. Photographs were taken when appropriate.

Experiments III-VI. For these experiments beans were planted in one-gallon cans, four plants per can, and grown in aphid-free greenhouses under supplemental artificial lighting. Plants were carborundum-dusted and rub-inoculated with virus-infected tissue ground in phosphate buffer. In Experiments III and IV, approximately 200 'Tendercrop' and 'Topcrop' bean plants, respectively, were inoculated with CMV-F and the same number with CMV-Le, two weeks after planting. The effect of inoculation time on seed-transmission was studied in Experiment V by inoculating about 40 'Topcrop' bean plants in the primary leaf stage, about two weeks after planting, with CMV-F; 60 plants at pre-bloom stage, four and one-half weeks after planting; and 60 plants at pod-forming stage, six weeks after planting. In Experiment VI, 40 'Topcrop' bean plants were inoculated with CMV-Pg two weeks after planting and 60 plants were inoculated four weeks after planting.

Pods were harvested when mature, shelled when dry, and seeds were stored at room temperature for up to six months. Seeds were planted and assayed by ELISA serology as described for previous experiments. Photographs of infected seedlings were taken, symptoms were observed.

[1] Results of standardization experiments showed that the ELISA system is capable of detecting 1 infected plant in 100 healthy plants.

Virus distribution. One of the two seedlings from PI accession 271998 that were found to be infected with seedborne CMV, designated CMV-Pg (Chapter Two, p.15), was tested by ELISA three weeks after planting, for an estimate of the distribution of virus. Starting at the roots, tissue was removed from the following locations (as illustrated in Figure 4-2): root system, hypocotyl above root system (two sections), cotyledon (two sections), unifoliate leaf (six sections), petiole of unifoliate leaf (two sections), stem section of first internode, petiole of trifoliate leaf, two leaflets of trifoliate leaf (four sections each), stem section in second internode, and tissue at the growing point. Tissue from each of the sampling locations (including sections) was cut out with a sterile razor blade, weighed, and 0.03 g placed in a single well of a Gilford microtiter ELISA plate. To each well, 0.3 ml of the ELISA virus buffer was added and tissue triturated with a glass rod. A non-infected plant from the same seedlot was assayed similarly to serve as a control.

Several plants infected with CMV-Pg through seed-transmission were obtained from Experiment VI. Three of these plants showing leaf malformation symptoms (nos. 17,51,65), three symptomless infected plants (nos. 38,43,69), and two non-infected plants (nos. 1,9) were assayed for virus distribution. Plant samples were taken from the following locations one month after planting: growing point 1), second trifoliate 2), first trifoliate 3), unifoliate 4), and roots 5). Tissue was ground in buffer (1:10, w/v) and tested by ELISA.

RESULTS

Seed-transmission. Results of seed-transmission are summarized in

Table 4-1.

CMV-B. CMV-B was transmitted through 6 of 253 seeds of inoculated, field-grown 'Tendercrop' bean plants, a rate of 2.4%, as determined by indicator indexing and gel double-diffusion serology. One of the six infected seedlings displayed mild mosaic symptoms, others were symptomless. Virus was also detected in 6 of 335 seeds of 'Red Kidney', a rate of 1.8%. None of these six infected plants showed discernible symptoms. No seed-transmission of CMV was detected in 126 seeds of 'Limelight'. In Experiment II, no seeds of the following CMV-B inoculated field-grown beans were found to be CMV-infected (numbers tested in parentheses): OSU 1604 (126), Early Blue (79), Santa Ana (83), BBL Advance (96), Sunrise (49), Michelite (48), BBL 274 (40), Greenpak (37), Majestic (37), Oregon 58 (41), and Sutter Pink (36).

CMV-Le. Virus infection was not evident by ELISA assay or symptom expression in seedlings of any of the 144 seeds taken from inoculated'Tendercrop' bean plants grown in the greenhouse (Experiment III), or in 136 seeds of 'Topcrop' plants (Experiment IV).

CMV-F. No virus was detected by symptomatology or ELISA serology in 148 seedlings of inoculated 'Tendercrop' bean plants (Experiment III), or 149 seedlings of inoculated 'Topcrop' plants (Experiment IV). In Experiment V, virus was detected by ELISA in 2 of 61 seedlings from 'Topcrop' plants inoculated two weeks after planting, but not in 99 and 98 seedlings from plants inoculated four and one-half and six weeks, respectively, after planting. One of the infected seedlings showed stunt, mosaic, and leaf malformation symptoms for about two weeks after emergence, but recovered shortly thereafter. The other infected

Table 4-1. Seed-transmission of cucumber mosaic virus (CMV) isolates mechanically inoculated onto selected Phaseolus vulgaris cultivars

EXP	ISOLATE	CULTIVAR	LOCATION	PLANTING DATE	INOC[a] (WK)	ASSAY[b] METHOD	RESULTS[c]	%ST
I	CMV-B	Tendercrop	Field	7-11-78	2	B,S	6/253	2.4
		OSU 1604				B,S	0/126	0
		Red Kidney				B,S	6/335	1.8
II		Early Blue		6-15-78	2	E	0/65	0
		Harvester				E	0/79	0
		Santa Ana				E	0/83	0
		BBL Advance				E	0/96	0
		Sunrise				E	0/49	0
		Michelite				E	0/48	0
		BBL 274				E	0/40	0
		Greenpak				E	0/37	0
		Majestic				E	0/37	0
		Oregon 58				E	0/41	0
		Sutter Pink				E	0/36	0
III	CMV-Le	Tendercrop	Green-	8-31-79	2	E	0/144	0
IV		Topcrop	house	3-4-80	2	E	0/136	0
III	CMV-F	Tendercrop	Green-	8-31-79	2	E	0/148	0
IV		Topcrop	house	3-4-80	2	E	0/149	0
V		Topcrop		5-27-80	2	E	2/61	3.3
					4½	E	0/99	0
					6	E	0/98	0
VI	CMV-PG	Topcrop	Green-	6-26-80	2	E	44/89	49
			house		4	E	0/190	0

[a] Age of plants at inoculation (weeks).

[b] B = Bioassay on cowpea, cucumber, or Chenopodium (see text for species). S = Gel double-diffusion serology of selected individuals. E = Enzyme-linked immunosorbent assay (ELISA). Groups of 10 seedlings were bulk assayed 2-3 weeks after planting, plants of suspect groups were then assayed again individually.

[c] No. samples in which CMV was detected/no. samples tested.

plant remained symptomless.

CMV-Pg. In this experiment seed-transmission occured at a 49% incidence, i.e., 44 of 89 seedlings from 'Topcrop' bean plants inoculated two weeks after planting were determined by ELISA to be CMV-infected. Downward leaf curling, twisting, and mosaic symptoms were observed on trifoliate leaves of 16 of the infected plants, while 28 remained symptomless. Photographs of a non-infected and three infected plants (Figure 4-1, A, B-D, respectively) show these symptoms in increasing degrees of severity. Symptom intensity was maximal about one week after unfolding of the first set of trifoliate leaves, then declined as the plants developed. By maturity, very few of the infected plants showed distinguishing symptoms. In contrast, no seed-transmission of CMV occurred in 190 seedlings of plants inoculated four weeks after planting.

Virus distribution. An estimate of virus distribution in a three-week-old bean plant of Plant Introduction accession No. 271998 infected from seed with CMV-Pg is shown diagrammatically in Figure 4-2. ELISA (A_{405}) values for tested tissues were compared for infected (seed-borne) and healthy seedlings (in parentheses). There was general uniformity of A_{405} values among samples taken from the same leaf or stem area. The highest relative CMV concentration appeared to be in the primary leaf and adjacent upper stem with tissues above being intermediate and tissues below the primary leaf being lower. Symptoms of seedborne CMV infected bean plants (although not frequently expressed) are illustrated in Figure 4-1, E, F, showing, respectively, a trifoliate leaf of a non-infected bean (6-2) and infected bean plant (6-7) before

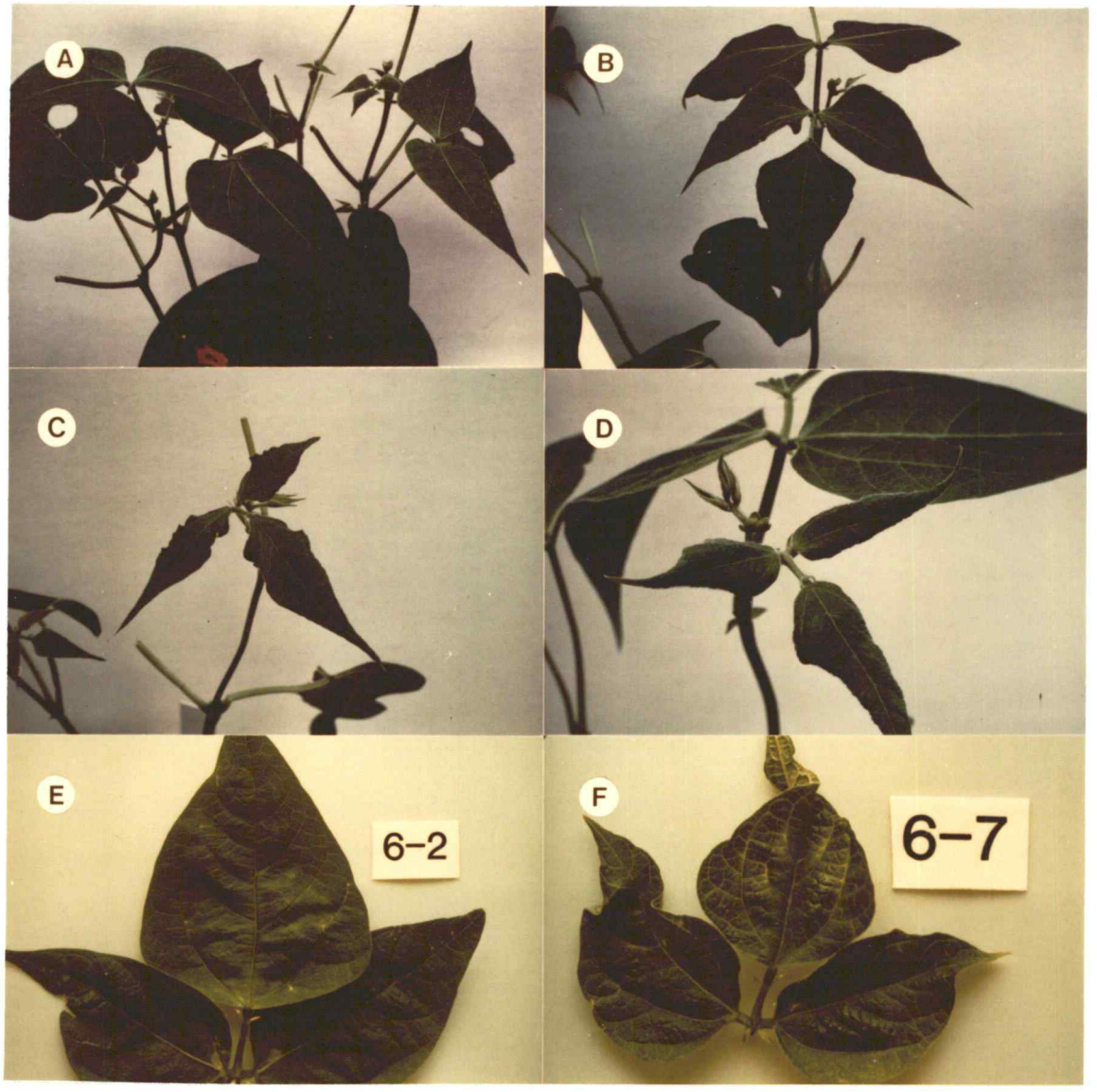


Figure 4-1. Symptoms induced in beans infected through seed-transmission with CMV-Pg. A-D, 'Topcrop' bean plants 23 days after planting. A, non-infected control. B-D, infected plants showing leaf curling and mosaic in three degrees of severity. EF, PI 271998 healthy (6-2) and infected (6-7), respectively, three weeks after planting.

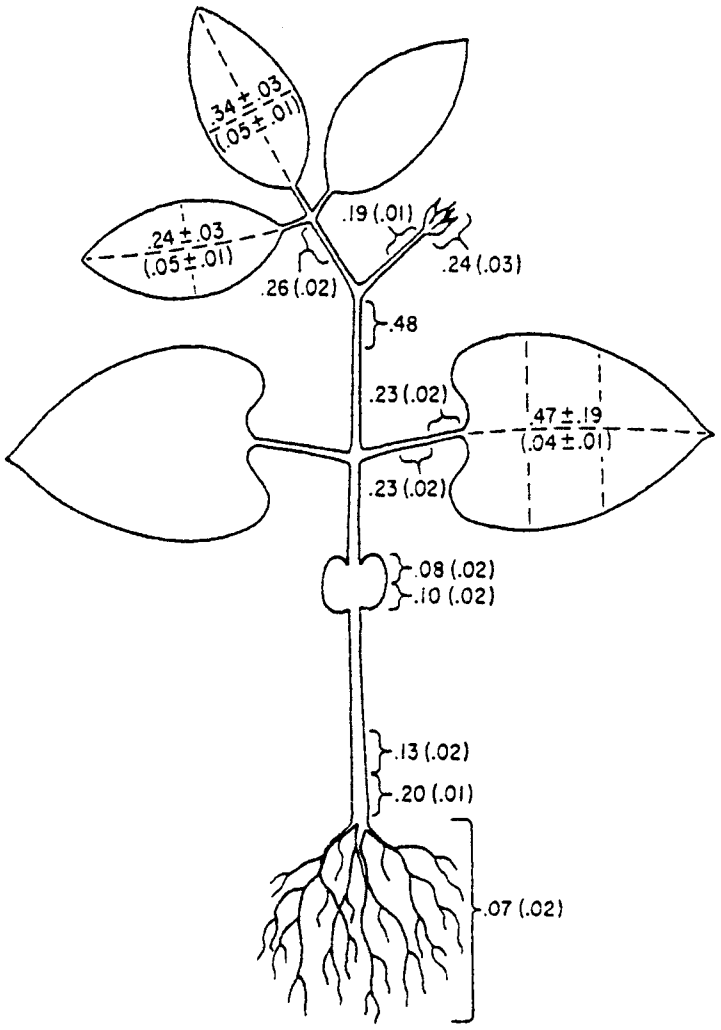


Figure 4-2. ELISA (A405) values showing an estimate of virus distribution in a three-week-old bean plant (PI 271998) infected with CMV-Pg through seed. Values for non-infected plant given in parentheses. Dotted lines show individual sections of leaves tested, standard deviations given. See text for complete details.

sampling. Symptoms consisted of slight leaf twisting at the apex of the leaflets, blistering, and mild mosaic.

Virus distribution was estimated by ELISA in one-month-old 'Top-crop' bean plants infected with CMV-Pg through seed-transmission (Table 4-2). One of the infected plants (no. 38) gave weakly positive results in this test. The other five plants (nos. 17,43,51,65,69) produced strong positive results in all tissue locations with some variation among locations and among plants at the same location. The widest range (A_{405}) within a single plant occured between the unifoliate and root tissue of plant no. 65 and spanned 0.64 (A_{405}) units, more than 60% of the absorbance range in this experiment. The least variation in virus content among plants existed in tissue at the growing point or meristem. There were no apparent differences between plants with (nos. 17,51,65) and without (nos. 38,43,69) symptoms, except no. 38 with a much lower virus content. Absorption (405 nm) values for non-infected controls were consistently 0.04 or less.

DISCUSSION

Significance to bean industry. Seed-transmissibility of CMV in beans under field growing conditions in the U.S. Pacific Northwest has been adequately demonstrated in these studies. Planting CMV-infected seed provides an inoculum source for secondary spread of infection by aphids. Aphids are efficient vectors and often present in substantial numbers in this region during the bean growing season. Effects of this disease on bean yields have not been demonstrated. Furthermore, Provvidenti (81) reported pod distortion of bean plants infected with CMV-B, thus reducing crop quality.

Table 4-2. Relative absorbance (405 nm) of ELISA colorimetric reactions obtained by testing one-month-old Phaseolus vulgaris 'Topcrop' plants infected with CMV-Pg through seed-transmission and healthy plants at five tissue locations. Results show estimates of virus distribution in infected plants

	PLANT No.								
	NON-INFECTED PLANTS		PLANTS ARISING FROM INFECTED SEED						
			WITH SYMPTOMS			SYMPTOMLESS			
LOCATION[a]	1	9	17	51	65	43	69	38	AVERAGE[b]
MERISTEM	.03	.04	.56	.55	.65	.58	.67	.06	.60± .05
TRIFOL 2	.01± .01	.02± .01	.26± .04	.57± .06	.43± .08	.52± .04	.57± .04	.09± .02	.45± .13
TRIFOL 1	0± .01	0± 0	.33± .05	.42± .08	.44± .06	.54± .06	.59± .07	.11± .01	.45± .10
UNIFOL	0± 0	.01± .01	.34± .11	.42± .02	.36± .03	.61± .04	.43± .07	.06± .01	.43± .12
ROOTS	0± .01	0± .01	.25± .02	.31± .08	1.01± .16	.31± .01	.87	.03	.46± .32
AVERAGE[c]	0± .01	.01± .02	.31± .09	.42± .10	.54± .25	.50± .12	.59± .13	.08± .03	

[a] Meristem = tissue within one centimeter of the growing point. Trifol 1 and 2 = first and second trifoliate leaves expanding, respectively. Unifol = unifoliate leaf. Roots = primary and secondary roots including hypocotyl.

[b] Average A_{405} value at each location of plant no.s 17, 51, 65, 43, and 69; note highest value with least variation is for meristem tissue.

[c] Average A_{405} value for each plant at all tissue locations; note generally low variation among location.

CMV-Pg, isolated from PI 271998, seems well adapted to beans grown in this area and is capable of extensive transmission through seeds (49%). Introduction of this isolate into bean breeding lines could threaten bean production and export markets. Because CMV produces mild or masked symptoms in some cultivars and the particular seed-transmission potential of CMV-Pg, elimination of CMV from infected seedlots could prove difficult.

Detection. Temporary symptom development in some of the plants arising from CMV-Pg infected seed indicated the efficacy of early visual assessment in detecting infected plants from CMV-infected seedlots. However, for accurate determinations of seed-transmission, a sensitive assay method, such as ELISA serology, is required. Assay of meristem tissue would provide the most reliable CMV-detection results.

Control. The most efficient means of preventing the development of this disease in beans would probably be elimimation and exclusion of virus from bean germplasm. Monitoring germplasm for the presence of CMV before introduction into breeding programs (particularly PI accessions known to contain CMV or originating in countries from which seed-borne CMV has been reported) would provide an initial strategy for elimination. Monitoring breeding lines for CMV escaping previous detection or having been introduced by aphids would further facilitate preemptive control. Use of cultivars resistant to the seed-transmission process (exemplified by OSU 1604 and many other bean cultivars), bean plantings that avoid CMV transmission by aphids early in plant development, and control of aphids could comprise general control measures.

Chapter Five

Purification, Serology, and Electron Microscopy of Cucumber Mosaic Virus Isolates Seedborne in *Phaseolus vulgaris*

ABSTRACT

Yields of purified cucumber mosaic virus (CMV) up to 320 mg per kg infected bean (*Phaseolus vulgaris* 'Bountiful') tissue were obtained by two purification methods compared. Possible factors affecting yield, which varied drastically, are discussed. Although both methods provided similar virus yields, the preferred method was more time efficient and produced more stabile virions. CMV isolates B, Le, B_{32}, F, and Pg were purified by this method. The ratio of A_{260}/A_{280} nm values agreed closely with the published average value of 1.65 for CMV (28).

Antisera to CMV-B, Le, and B_{32} were produced with reciprocal titers of 1024, 256, and 256, respectively. The highest titer was obtained in a rabbit injected several times over a longer period, both intramuscularly and intravenously.

Gel double-diffusion serological tests reliably detected CMV in tobacco (*Nicotiana tabacum* 'Xanthi') and cucumber (*Cucumis sativus* 'Lemon' and 'Improved Long Green') plant tissues ground in phosphate buffered saline solution. Detection of CMV in bean tissue was facilitated by tissue maceration in 0.5 M citrate buffer, pH 6.5, 0.1% sodium thioglycollate, and 0.1% Triton X-100. In general, antisera reacted more strongly to virus fixed with 1% formaldehyde. Purified CMV was detected by gel serology at concentrations of 0.05-0.1 mg/ml. CMV isolates B, Le, B_{32}, F, and Pg were found to be serologically indistinguishable, suggesting that they all probably belong to the DTL serogroup proposed

by Devergne and Cardin (15).

Enzyme-linked immunosorbent assay (ELISA), utilizing globulin from antiserum produced in these studies, was found to be capable of detecting 6 ng/ml of purified CMV. ELISA also detected CMV at a 1000-fold dilution of sap, 100,000-fold dilution of desiccated tissue, and in preparations containing 1 part CMV-infected to 100 parts healthy plant tissues. Procedural modifications and detection limitations of ELISA are discussed.

CMV isolates B, Le, B_{32}, F, and Pg, examined by electron microscopy after negative staining with phosphotungstic acid (PTA), were affirmed to consist of isometric particles about 33-37 nm in diameter. CMV particles were more intact and uniform when fixed with 1% formaldehyde and stained with PTA at pH 3 than when unfixed or stained with PTA at pH 5 or 7. Storage of virus in 20 mM tetradosium ethylenediaminetetraacetate (EDTA), pH 7, also improved virus stability and longevity.

Serologically specific electron microscopy (SSEM) using grids coated with a 100-fold dilution of antiserum increased the number of particles and uniformity of their distribution on grids. Decoration of particles by a second antiserum coating did not enhance particle detail or visibility.

INTRODUCTION

CMV purification and antiserum production, which are prerequisite to development of serological detection methods and serological comparisons of isolates, are described in this chapter. Electron microscopy and serological tests including gel double-diffusion and enzyme-

linked immunosorbent assay (ELISA), and serologically specific electron microscopy (SSEM) are investigated and discussed. CMV isolates B, Le, B_{32}, F, and Pg were compared for serological homology.

MATERIALS AND METHODS

Virus isolates. Details concerning virus isolates were presented in Chapter Three, p. 20.

Virus propagation. CMV isolates were propagated in bean, cucumber, and tobacco plants. Primary leaves of bean cultivars Bountiful, Black Turtle, Limelight, Columbia Pinto, and Red Kidney were inoculated about two weeks after planting; cotyledons of cucumber cultivars Lemon and Improved Long Green were inoculated about ten days after planting; and tobacco cultivars Samsun NN and Xanthi were inoculated when plants were five to ten centimeters high. Tobacco plants were maintained in a growth chamber at 25 C under 16 hr of artificial light; all others were maintained in aphid-free greenhouses with approximate diurnal temperatures of 20/16 C. Plants were harvested for purification about seven to fourteen days after inoculation.

Purification. Two methods of purification were used. Method No. One was originally described by Scott (86) in 1963, with the following modifications added by Mink and Ehara (personal communication): 1) 0.5 M citrate buffer contained 0.01 M EDTA and 0.1% mercaptoethanol; 2) tissue extracted with two volumes citrate buffer and two volumes chloroform; 3) first high-speed pellet ground in mortar, clarified twice for 15 min at 5400 g; 4) second high-speed pellet resuspended and spun through a 10-40% sucrose gradient, in 5 mM borate pH 9.0 buf-

fer for two hours at 24,000 rpm in an SW 27 rotor (Beckman); and 5) virus band removed and centrifuged for two hours at 105,000 g, then resuspended.

Method No. Two as described by Lot et al. in 1972 (59) was modified (H. T. Hsu, personal communication) as follows: 1) tissue is homogenized only once in citrate-chloroform; 2) elimination of low-speed spin after resuspension of PEG pellet; 3) first high-speed pellet ground in mortar with 20 mM EDTA, pH 7.0, followed by two low-speed centrifugations; 4) second high-speed pellet layered onto 10-40% sucrose gradients in EDTA and centrifuged for two hours at 24,000 rpm in an SW 27 rotor; and 5) from first high-speed centrifugation through storage, virus is kept in EDTA buffer. All reagents and glassware were kept at 0-4 C. This method is outlined in detail in Table 5-1.

Antiserum production. Male New Zealand White rabbits were injected with CMV-B, Le, or B_{32} according to the schedule outlined in Table 5-2. This table shows the route of injection, amount of virus injected (in mg), and the day (starting with the first injection as Day Zero). CMV-B was injected seven times into the marginal ear vein (IV) and three times in the hind leg muscles (IM) for a total of 10.3 mg over a four-month period. Rabbits injected with CMV-B_{32} and CMV-Le received a total of 8.2 mg each in three IM injections over the course of six weeks. All virus preparations were diluted in 0.01 M phosphate, pH 7, 0.85 M NaCl (PBS) and stabilized with 1% formaldehyde prior to injection to optimize immunogenicity (24) and mixed with Freund's incomplete adjuvant (1:1, v/v) prior to IM injections.

The rabbit injected with CMV-B was bled nine times in a four-month

Table 5-1. Method No. Two for purification of cucumber mosaic virus.[a]

DISCARD		RETAIN
	1)	Homogenize tissue in 2 volumes each of 0.5 M citrate pH 6.5 with 0.1% sodium thioglycollate and chloroform
	2)	Low speed 10 min at 3000-4000 g
Chloroform, tissue	3)	Remove supernatant, add PEG 6000 to final concentration of 10% (w/v)
	4)	Stir 30-45 min, low speed 10 min at 12,000-16,000 g
Supernatant	5)	Resuspend pellet in 0.05 M citrate pH 7.0 with 0.2% Triton X-100, stir 30-45 min
	6)	High-speed 2 hr at 78,000 g or 1.5 hr at 105,000 g
Supernatant	7)	Grind pellet in mortar and pestle with 0.02 M EDTA pH 7.0
	8)	Low-speed 15 min at 12,000 g
	9)	Remove supernatant (S1), low-speed pellet again with additional EDTA buffer
Pellet	10)	Remove supernatant (S2), combine S1 and S2, high-speed 1.5 hr at 105,000 g
Supernatant	11)	Resuspend pellet overnight in EDTA buffer
	12)	Layer 0.5-1.0 ml virus solution onto fresh or thawed 10-40% linear sucrose gradients prepared in EDTA buffer, high-speed 2 hr at 24,000 rpm in Beckman SW 27 rotor
	13)	Collect band manually with syringe and bent needle, dilute solution in EDTA, high-speed 1.5 hr at 105,000 g
Supernatant	14)	Resuspend pellet in EDTA buffer

[a] All reagents and glassware are kept at 0-4 C

Table 5-2. Rabbit injection schedule and reciprocal titer of three antisera produced to CMV isolates B, Le, and B_{32}

CMV-B			CMV-Le and CMV-B_{32}[a]		
DAY[b]	INJ. ROUTE OR BLEEDING	RECIPROCAL[c] DILUTION	DAY[b]	INJ. ROUTE OR BLEEDING	RECIPROCAL[c] DILUTION
0	IV - 0.1 mg		0	IM - 6.2 mg	
2	IV - 0.1 mg		9	Bleeding	16
4	IV - 0.1 mg		14	Bleeding	64
27	IV - 0.2 mg		20	IM - 1.0 mg	
29	IV - 0.2 mg		22	Bleeding	16
31	IV - 0.2 mg		30	Bleeding	256
38	Bleeding	64	35	Bleeding	64
40	IM - 2.0 mg		44	IM - 1.0 mg	
48	Bleeding	32	55	Bleeding	128
54	Bleeding	8			
56	IM - 2.3 mg				
62	Bleeding	128			
66	IM - 1.0 mg				
73	Bleeding	256			
91	IM - 4.1				
101	Bleeding	128			
103	Bleeding	512			
109	Bleeding	1024			
124	Bleeding	512			

[a] Information given applies to an antiserum produced to CMV-Le and a separate antiserum produced to CMV-B_{32}, both of which were produced identically and resulted in identically titered antiserum.

[b] Starting with the first injection as Day 0.

[c] Reciprocal dilution as determined by microprecipitin test.

period. Rabbits injected with CMV-B_{32} and CMV-Le were each bled six times in two months. All bleedings were done aseptically by cardiac puncture. Blood was allowed to clot at room temperature for one to two hours before being loosened from the flask by agitation. The blood was kept at 4 C for one to two days to allow maximum clot shrinkage and serum yield. Serum was removed with a sterile pipet, spun for ten minutes at top speed in a clinical centrifuge, and supernatant was made 0.01% with sodium azide as a bactericide. Serum was stored at 4 C or -10 C.

Microprecipitin test. Each antiserum preparation was tested for reaction end point by microprecipitin titration in 10-cm-square plastic petri dishes (2). Incubation of test dishes in a moist chamber obviated mineral oil over-lays. Reactions were recorded after 12-48 hours.

Ouchterlony test. Gel double-diffusion serology was performed using the Ouchterlony test. A 12 ml solution of 0.4% ionagar, 0.01 M phosphate pH 7.0, 0.85% sodium chloride, 0.2% sodium azide was poured into a 10-cm-square plastic petri dish. Well patterns as shown in Figure 5-1 were cut with a number two cork borer (6 mm), agar was removed by aspiration, and approximately 25 ul of the reactants diluted in PBS or other buffers were added to each well. Plates were incubated in a moist chamber for four to ten days and reaction lines were recorded.

ELISA test. ELISA was performed basically as described by Clark and Adams (13) with some modifications (14). The exact protocols used in this work for the purification of immunogammaglobulin (IgG) from anti-

serum, enzyme-conjugation, and the ELISA test are outlined in Tables 5-3 and 5-4. Two methods were used in purifying IgG from crude antiserum (Table 5-3), one involving ammonium sulfate precipitation of protein followed by fractionation of globulin molecules on a DEAE cellulose column, the other involving fractionation of the crude serum on a DEAE AFFI-GEL BLUE (BIO-RAD) column. Only one method of enzyme-conjugation was used. Two ELISA systems were used: an automatic system using a Gilford PR-50 EIA Automatic Analyzer, which was used for all steps except sample grinding (Table 5-4); and a manual system using 96-well Dynatech microtiter plates which were read at 405 nm on a Beckman Model 25 Spectrophotometer equipped with a sipper cell. The Gilford plates consist of a base with five removable strips and ten wells per strip. Gilford plates were often fastened together when several were being processed.

Electron microscopy. Purified virus was applied to carbon-stabilized formvar-coated copper grids (PELCO No. 1 GC 200), negatively stained by PTA, examined by a Phillips EM 300 transmission electron microscope, and photographed on three-and-a-half by four inch film plates. Grids were coated with formvar (about 0.25% polyvinyl formal in ethylene dichloride) by standard techniques (62). To increase stability and shelf life of grids, a thin layer of carbon was deposited on the grids in a vacuum evaporator (62). Purified virus solutions fixed with 1% formaldehyde were applied in drops to the grids for two minutes, then removed with blotter paper. PTA (2%) pH 3 was applied for two minutes and removed by blotting.

SSEM. Techniques for SSEM were identical to EM except for sample

Table 5-3. Purification and enzyme-conjugation of immunogammaglobulin from antiserum for use in enzyme-linked immunosorbent assay

PURIFICATION METHOD No. ONE	PURIFICATION METHOD No. TWO
1. Add 9 ml DD H_2O to 1.0 ml serum	1. Dialyze 1 ml serum 3 times against 500 ml TRIS (0.02 M Tris-HCl, 0.028 M NaCl, 0.02% NaN_3 pH 8.0)
2. Slowly, dropwise, add 10 ml saturated $(NH_4)_2SO_4$ while stirring, leave 30-60 min	2. Prepare a 7 ml AFFI-GEL BLUE (BIO-RAD No. 153-7307) column according to mfg directions, pretreat with 8 M urea, equilibrate with TRIS
3. Low-speed 5 min at 6,000 rpm	
4. Resuspend pellet in 2 ml ½-PBS[a]	
5. Dialyze 3 times against 500 ml ½-PBS (2 hr-2 hr-overnight)	
6. Filter through 3-5 cm DEAE cellulose column propared to mfg directions, equilibrated with ½-PBS	3. Filter serum through column, proceed as with Method No. One, store at 4 C, stable for several months
7. Wash through with ½-PBS, collect 1 ml fractions, monitor at 280 nm, combine peak fractions and adjust to 1.4 OD units (about 1 mg/ml)	

ENZYME-CONJUGATION

1. Low speed 2 mg Sigma Type VII alkaline phosphatase for 5 min at 6,000 rpm (pellet will be soft)
2. Resuspend pellet in 1 ml (∿1mg) purified gammglobulin
3. Dialyze 3 times against 500 ml PBS
4. Add fresh (up to 2-wk-old) glutaraldehyde to 0.05% (v/v), leave 4 hr
5. Dialyze 3 times against 500 ml PBS+ 0.01% sodium azide
6. Add bovine serum albumin to 5 mg/ml, store at 4 C, stable up to several months

[a] PBS = 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM $NaHPO_4$, 2.7 mM KCl pH 7.4 with 0.02% NaN_3

Table 5-4. ELISA protocol for use with Gilford PR-50 EIA Automatic Analyzer system

1.	Add 300 ul immunogammaglobulin diluted 1:1000[a] (v/v) in coating buffer (0.05 M sodium carbonate, 0.02% NaN_3, pH 9.6) to each well
2.	Incubate plate 4 hr at 37 C, then overnight at 4 C (stable up to several weeks at 4 C)
3.	Rinse plates 3 times, 3 min each, with PBS[b] (Table 5-3) + 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate)
4.	Add 300 ul sample diluted in virus buffer (PBS-Tween + 0.2% egg albumin and 2.0% polyvinylpyrrolidone-PVP 10)
5.	Incubate overnight at 4 C
6.	Repeat step 4
7.	Add 300 ul enzyme-conjugate duluted 1:1000[a] (v/v) in virus buffer
8.	Incubate 4 hr at 37 C
9.	Repeat step 4
10.	Add 300 ul p-nitrophenyl phosphate substrate (0.6 mg/ml in 9.7% diethanolamine, 0.2% NaN_3, pH 9.8) to each well
11.	Incubate 5-60 min, measure OD at 405 nm

[a] Optimum dilutions determined by standardization trials may vary with each conjugate or globulin produced

[b] PBS = 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.0 mM $NaHPO_4$, 2.7 mM KCl pH 7.4 with 0.02% NaN_3

application and staining. Antiserum and antigen solutions were applied by floating grids on drops of the solutions for 5-20 minutes, followed by touching grids to blotter paper and rinsing with droplets of distilled water 20 times, removing wash water with blotter paper. Grids in sequence were coated with a 1:50, 1:100, or 1:500 (v/v) dilution of CMV-B_{32} antiserum (bleeding no. 4), rinsed in buffer (0.01 M phosphate pH 7.0), CMV-B antigen (0.22 mg/ml, 1% formaldehyde) applied, rinsed with distilled water, decorating antiserum applied at 1:50 dilution, rinsed, and 2% uranyl acetate stain applied for 1-2 minutes and blotted off. Controls included all combinations of treatments, alone and together.

RESULTS AND DISCUSSION

Purification. Among bean cultivars tested, 'Bountiful' was chosen as the most suitable propagation host based on high virus yields of up to 17 mg virus/50 g tissue. 'Red Kidney' and 'Limelight' were also high-yielding hosts but 'Black Turtle' and 'Columbia Pinto' generally yielded poorly. 'Lemon' and 'Improved Long Green' cucumbers were tested only once and yielded 2.9 and 0.34 mg virus/50 g tissue, respectively. 'Xanthi' tobacco was a suitable host but virus could not be purified from 'Samsun NN' tobacco plants inoculated with CMV-B, Le, or F. Purified virus remained stable when stored at -10C in EDTA buffer or after lyophilization.

Virus yields (Table 5-5) were approximately equal for both purification methods but Method No. Two was preferred because of increased stability of the virus purified by this procedure and stored in EDTA buffer and because fewer hours were required. Addition of 0.1% sodium

Table 5-5. Yield and $A_{260/280}$ ratio of cucumber mosaic virus (CMV) isolates purified from *Phaseolus vulgaris* 'Bountiful' (BNT) and *Nicotiana tabacum* 'Xanthi' (XAN) by Purification Method One or Two[a]

ISO-LATE	HOST	PURIF METHOD	TRIALS	INCUB[b] (DAYS)	YIELD[b] (mg/50g)	YIELD RANGE	$A_{\frac{260}{280}}$ nm ± s
B	BNT	1	9	8.2	3.9	2.0-7.1	1.51 ± .05
B	BNT	2	14	11.3	3.5	.26-8.6	1.56 ± .16
Le	BNT	1	3	12.0	5.8	.60-12	1.59 ± .02
Le	BNT	2	23	11.1	3.4	.30-9.6	1.60 ± .08
B_{32}	BNT	1	2	11.0	4.5	2.2-6.8	1.59
B_{32}	BNT	2	7	12.3	1.8	.02-3.9	1.55 ± .11
F	BNT	2	26	9.9	3.2	.08-17	1.60 ± .09
Pg	BNT	2	7	10.4	.82	.22-1.5	1.64 ± .07
B	XAN	2	2	6.0	2.8	1.6-4.1	1.66
Le	XAN	2	3	6.0	4.0	1.5-5.2	1.60
F	XAN	2	3	6.0	3.1	1.5-5.8	1.48
Pg	XAN	2	3	6.0	.59	.28-1.1	1.58

[a] See text and Table 5-1 for description of Purification Methods One and Two.

[b] Values represent averages of all replications.

[c] Not corrected for light scattering

thioglycollate to the extraction buffer of Method Two just before grinding tissue contributed to virus yield and particle integrity.

Yields of purified virus varied among isolates (Table 5-5). Average yield of isolates purified from 50 g of 'Bountiful' bean tissue by Method Two was 3.5, 3.4, 3.2, 1.8, and 0.82 mg for CMV isolates B, Le, F, B_{32}, and Pg, respectively. It was also observed that CMV-Pg produced the lowest yield in 'Xanthi' tobacco plants at 0.59 mg/50 g tissue compared to 4.0, 3.1, and 2.8 mg/50 g tissue for CMV-Le, F, and B, respectively. It is interesting but not unexpected that these isolates differed in yields of purified virus, one of the few characteristics distinguishing them.

The widest range in yields obtained in similar purifications conducted over a two-and-one-half year period was 0.08-17 mg/50 g tissue with CMV-F (Table 5-5), over 200-fold difference. This very large variation was probably due to fluatuations in greenhouse environment, condition of propagation-host plants, and length of incubation time. Variations in yield of virus purified from 'Xanthi' grown in the controlled conditions of the growth chamber were never more than 4-fold (Table 5-5).

An important principle is apparent in virus yield data, as with other biological systems. Variables that affect the results of an experiment may not be controllable by the experimenter, and this lack of control should be considered in interpretations of results. Hence, an experimenter could be falsely persuaded to reject a purification method or variation being tested if the replications are insufficient to account for this variability.

The ratios of absorbance at 260 nm to 280 nm light for these iso-

lates (Table 5-5) compares favorably with the value of 1.65 previously reported for this virus (28). Variations in this value probably reflect degree of particle integrity and relative quantities of host-derived impurities rather than inherent differences among isolates.

Antiserum titer. The titers of antisera from each bleeding were determined by dilution end point in microprecipitin tests (Table 5-2). A higher titer antiserum was obtained in CMV-B (1024) than in CMV-Le or CMV-B_{32} (256 each) injected rabbits, primarily because CMV-B was injected over a much longer time period with several small intravenous doses. All antisera, however, were readily useable. Purified CMV is generally considered a poor immunogen (28). Injection schemes for CMV-Le and CMV-B_{32} were identical and although differences in antigenic response are common among animals, these rabbits produced similar antisera titers.

Ouchterlony tests.

Limitations of crude extracts. Extracts from infected tobacco and cucumber plants reacted typically in gel double-diffusion (Ouchterlony) tests. However, reactions were not normally observed with extracts from infected bean or cowpea plants. Similar difficulties of testing bean tissues have been encountered by others (M. Silbernagel, personal communication). This phenomenon was pursued to gain an understanding of its basis.

Conversely, CMV which had been purified from infected bean plants reacted in gels. Samples were tested at each stage of the purification procedure and it was determined that the preparation became serologically reactive after the initial extraction in the 0.5 M citrate pH 6.5

buffer with 0.1% sodium thioglycollate and chloroform (1g:2ml:2ml). Interpretation of these results was that chloroform disrupted cell membranes, thioglycollate reduced polyphenols, and the high molarity citrate provided virus particle stability. Triton X-100 (0.1%, v/v), but not sodium dodecyl sulfate (0.1%, w/v), was an excellent substitution for chloroform during extraction of the virus from infected tissue prior to gel serology. Chloroform required phase separation and tended to inhibit serological reactions in trace amounts. Mercaptoethanol (0.1%) was found to be interchangeable with sodium thioglycollate. The citrate-thioglycollate (or mercaptoethanol)-Triton X-100 buffer was found to be superior to any other combination tested and was adopted as a standard Ouchterlony buffer (sap also treated with 1% formaldehyde, see below) for beans and cowpeas.

Formaldehyde fixation. Because antisera were produced to formaldehyde-fixed antigen (with an unknown effect on antigenic specificity), the effect of treatment of the crude bean sap with formaldehyde to a final concentration of 1% was investigated. It was found that serological reactions in gels were intensified and sometimes dependent on addition of the formaldehyde to the sap after straining through cheesecloth.

Reactions in gels using purified virus were also greatly enhanced by formaldehyde fixation, but differentially among antisera. The greatest increase in sensitivity to fixed antigen was noted with CMV-B antiserum which produced sharp precipitin bands at reciprocal dilutions of 32 when tested with fixed virus at 0.08 mg/ml, compared to a reciprocal dilution of 8 and virus concentration of 0.35 mg/ml with unfixed virus. Antisera to CMV-Le and CMV-B_{32} showed a similar but less dramatic response. The increased reactivity of formaldehyde-fixed

CMV antigens to antisera prepared to similarly fixed CMV has been noted by others (H. T. Hsu, personal communication).

These results suggest that formaldehyde fixation may not only stabilize but also alter the antigenic structure of virions, thus eliciting an antibody with a degree of specificity for fixed antigen. To determine whether fixed and unfixed antigen were serological distinguishable, the two preparations were reacted side by side against antisera produced to fixed antigen (CMV-B, Le, and B_{32}) and an antiserum produced to unfixed antigen (ATCC-CMV). All precipitin bands were conterminous, indicating that no gel-serological heterology was detectable between fixed and unfixed antigens. CMV-B antiserum collected after eight to ten antigen injections reacted more strongly to unfixed CMV than from earlier bleedings. I interpreted this as compliance with existing knowledge that during prolonged immunization of laboratory animals, the specificity of antibodies from subsequent bleedings commonly declines. Logically, formaldehyde-altered virions would have elicited antibodies which, after prolonged immunization, reacted progressively less specifically with fixed nucleoprotein.

Virus detection sensitivity. The lowest purified virus concentration detectable in gel double-diffusion tests was 0.05-0.1 mg/ml. Based on an average purification yield of about 3 mg/50 g bean tissue or 0.06 mg/g (probably a slightly low estimate due to loss of virus during purification), the average concentration of virus in the extract from grinding 1 g bean tissue in 2 ml buffer would be about 0.03 mg/ml. Low virus titer in the tissues tested, binding of virions to host tissues and/or inactivation during extraction are limitations presumably encountered in gel serology. Some workers have obtained

better results subculturing CMV from naturally infected plants to 'Xanthi' tobacco and concentrating the chloroform-clarified extract eight times with PEG before testing in gels (17). Diffulties in producing high-titered antisera add to this general problem of detection in Ouchterlony tests.

Isolate homology. Purified virus was used to directly compare isolates in gels for serological homology. In the first experiment CMV isolates B, Le, F, and B_{32}, were placed alongside each other in all possible combinations twice (in outer wells of a 6 well set) and found to be completely homologous by the lack of spurs formed between precipitin bands when tested with CMV-B or CMV-Le antisera in the center wells (Figure 5-1, A). In a similar experiment using the same antisera but a slightly different well pattern, CMV-Pg was compared alongside CMV-B, Le, F, and B_{32} and also found to be homologous to these isolates (Figure 5-1, B,C). Purified extracts of healthy tissue and normal serum were included as controls (Figure 5-1, B,C).

In these tests, CMV isolates F, B, Le, Pg, and B_{32} were serologically indistinquishable and appeared to comprise a single CMV serogroup. CMV-F was previously reported to belong to the DTL serogroup (61); therefore, all isolates homologous to CMV-F would also presumably belong to the DTL serogroup. As a further test of homology, antisera representing both serogroups (CMV-D antiserum of the DTL serogroup and CMV-S antiserum of the ToRS serogroup) were obtained from the American Type Culture Collection (ATCC). CMV-S infected tissue was also obtained from ATCC and cultured in tobacco. All isolates were tested alongside CMV-S using antisera to CMV-D, S, Le, B, and B_{32}. No spur formation was observed in tests using the standard 0.4%

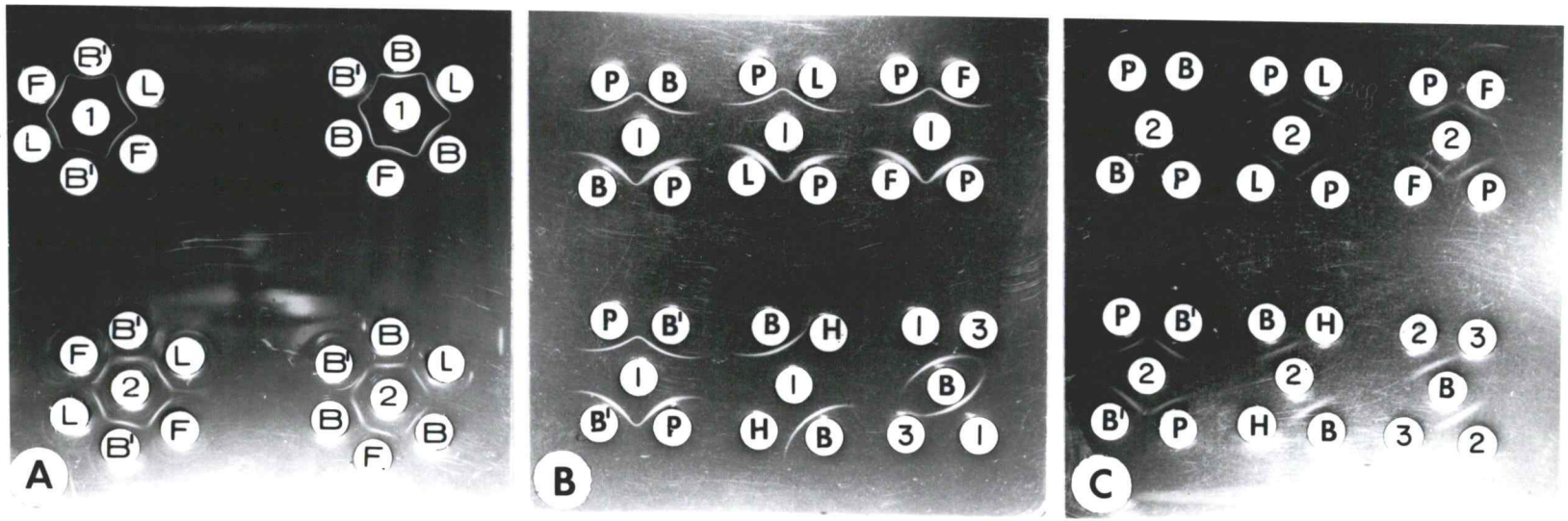


Figure 5-1, A-C. Gel double diffusion serology plates showing evidence of serological homology among cucumber mosaic virus (CMV) isolates. Antigens: B = CMV-B; L = CMV-Le; F = CMV-F; B' = CMV-B_{32}; P = CMV-Pg; H = purified extract of healthy 'Bountiful' bean plant. Antiserum: 1 = CMV-B antiserum; 2 = CMV-Le antiserum; 3 = normal serum.

gels or subsequently in tests with 0.25% gels. Limiting factors in the gel tests or CMV-S mis-labeling or mis-identification are possible causes.

Serological relatedness was also tested by cross-absorption (29). CMV-B antiserum was cross-absorbed with CMV-Le, or CMV-B_{32} antigens and tested in gels for reactions to all three antigens. Reactions were eliminated by cross-absorption with CMV-Le, but results of cross-absorbtion with CMV-B_{32} were inconclusive.

ELISA. The two methods of purifying immunogamma-globulin worked equally well. Method two, using AFFIGEL BLUE, was chosen for simplicity and speed.

Virus detection sensitivity. The ELISA test was found to be capable of detecting 6 ng/ml of purified CMV. CMV was also detected by ELISA in fresh 'Bountiful' bean tissue at a 1000-fold dilution with buffer (w/v) in desiccated 'Bountiful' bean tissue at a 100,000-fold dilution (w/v), and in one part of infected 'Bountiful' bean tissue mixed with 100 parts healthy tissue and diluted 10-fold (w/v). Assuming the level of virus might be lower in naturally infected plants, testing of up to 10 plants together was well within the limits of detection for this system. These results support the conclusions of Devergne et al. (17) that ELISA was greatly superior to gel double-diffusion serology for detecting CMV in naturally infected plants.

Quantitation. The quantitative use of ELISA in comparing serological relatedness of CMV isolates was attempted. A linear relationship between absorbance (A_{405} nm) of p-nitrophenol (test result) and concentration of purified virus was observed in some experiments and not in others. Therefore, the ELISA system was not employed quanti-

tatively to compare isolates, but such use of ELISA would seemingly be possible with proper internal controls and standardization to minimize test aberations. Burrows et al, (12) have determined the necessary experimental designs and controls for accomodating variation in the Dynatech microtiter plates so that precise and unbiased treatment comparisons can be made.

Procedural notes. Treatment of infected sap with formaldehyde generally reduced ELISA reactions and was not employed. Used plates, rinsed in 0.1 N HCl overnight, then rinsed with water, gave satisfactory results when reused, with no indication of carry-over response (3). Enzyme-conjugate worked well when reused and is often reused by others for up to a month (4). Enzyme-conjugate cannot be stored as long as gamma-globulin, two to six months appeared to be the practicable longevity for conjugate. Background levels for healthy controls were sometimes very high, possibly indicating deterioration of enzyme-conjugate. Another indication of conjugate quality loss may be a decrease in the reaction rate, which ranged from 5-60 minutes for full scale color development.

EM. CMV isolates B, Le, B_{32}, F, and Pg were found to contain spherical particles about 33-37 nm in diameter (Figure 5-2). However, precise internal standards were not used in electron microscopy and particle size is assumed to be about 30 nm. The reported size of CMV particles ranges from 20 to 30 nm (10,11,25,61,63,64,86), with average size generally given at 30 nm.

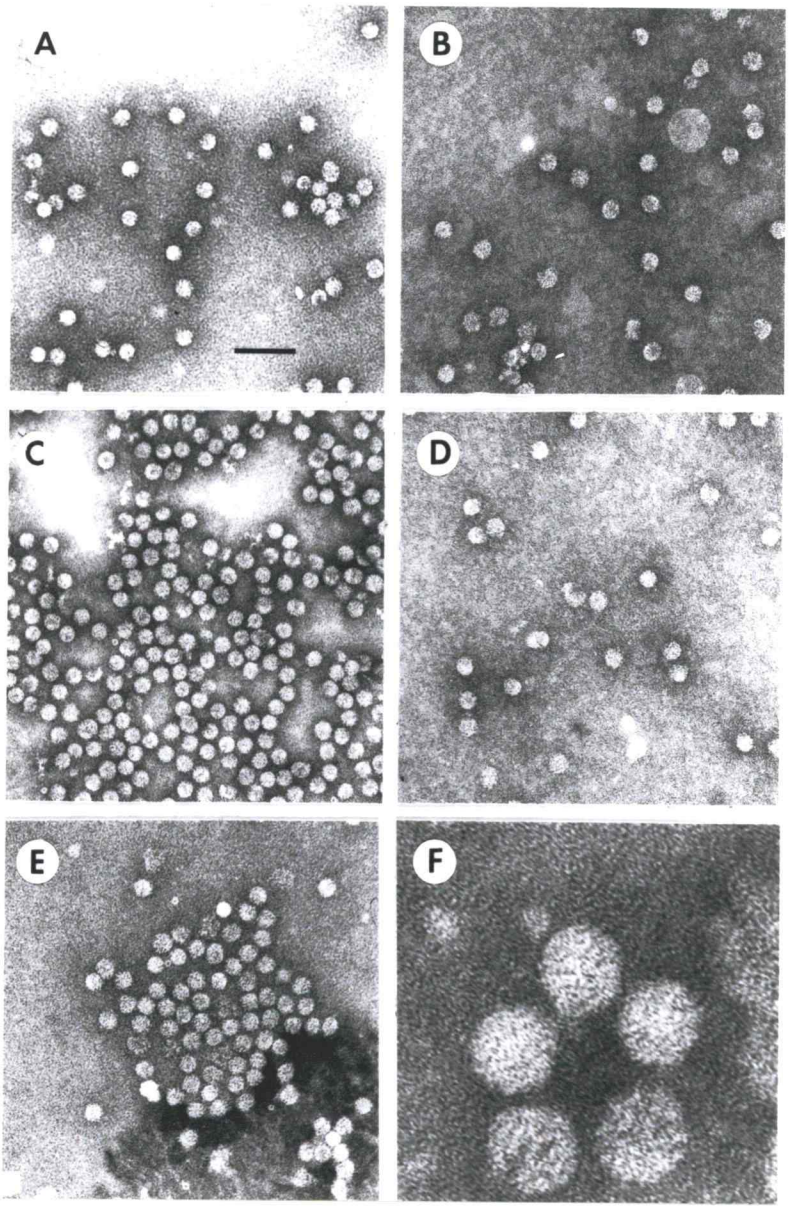


Figure 5-2, A-F. Electron micrographs of CMV particles. A-E, Virions of CMV-B, Le, B_{32}, F, and Pg_3 respectively, fixed in 1% formaldehyde and negatively stained with 2% phosphotungstic acid pH 3 (X∿80,600). F, CMV-Le virions treated as above (X∿510,000). A-E, bar = 100 nm; F, bar = 16 nm.

CMV particles are notoriously unstable. It was found that fixation of particles in 1% formaldehyde greatly reduced the amount of particle degradation observed by electron microscopy. Meiners et al. (63) reported a similar effect of 2% glutaraldehyde on CMV-Pr particle stability. The pH of PTA stain was also found to effect stability, as reported by others (64). Stability was greatly improved by the use of PTA at pH 3, versus pH 5 or pH 7. Particle degradation was monitored during storage at 4 C and -10 C in 5 mM borate, pH 9 versus 10 mM EDTA, pH 7 buffer. The EDTA buffer was selected because CMV particles remained stable and monodispersed during storage in this medium. EDTA has been noted for these properties by others as well (64,91). For storage of more than two weeks, -10 C was preferred. Stabilization by formaldehyde fixation has been discussed and fixed particles remained morphologically intact during the course of this work, but lost biological infectivity.

SSEM. Artifacts resembling virus particles appeared on stained grids both with and without antiserum (Figure 5-3, A,B). Coating of grids with a 100-fold dilution of antiserum prior to addition of virus resulted in greater number of particles attached in a more uniform distribution compared to uncoated grids (Figure 5-3, E,C), particularly when virus concentration was low. Antiserum dilutions of 50 or 500-fold (Figure 5-3, D) were not as effective. Decoration of virus particles by a second coating of antiserum at 50-fold dilution resulted in a coarser background that obscured most virus particles (Figure 5-3, F). Some single particles and aggregates of several particles were obvious but decoration was generally much less effective than staining virus particles on uncoated grids or antibody-coated grids.

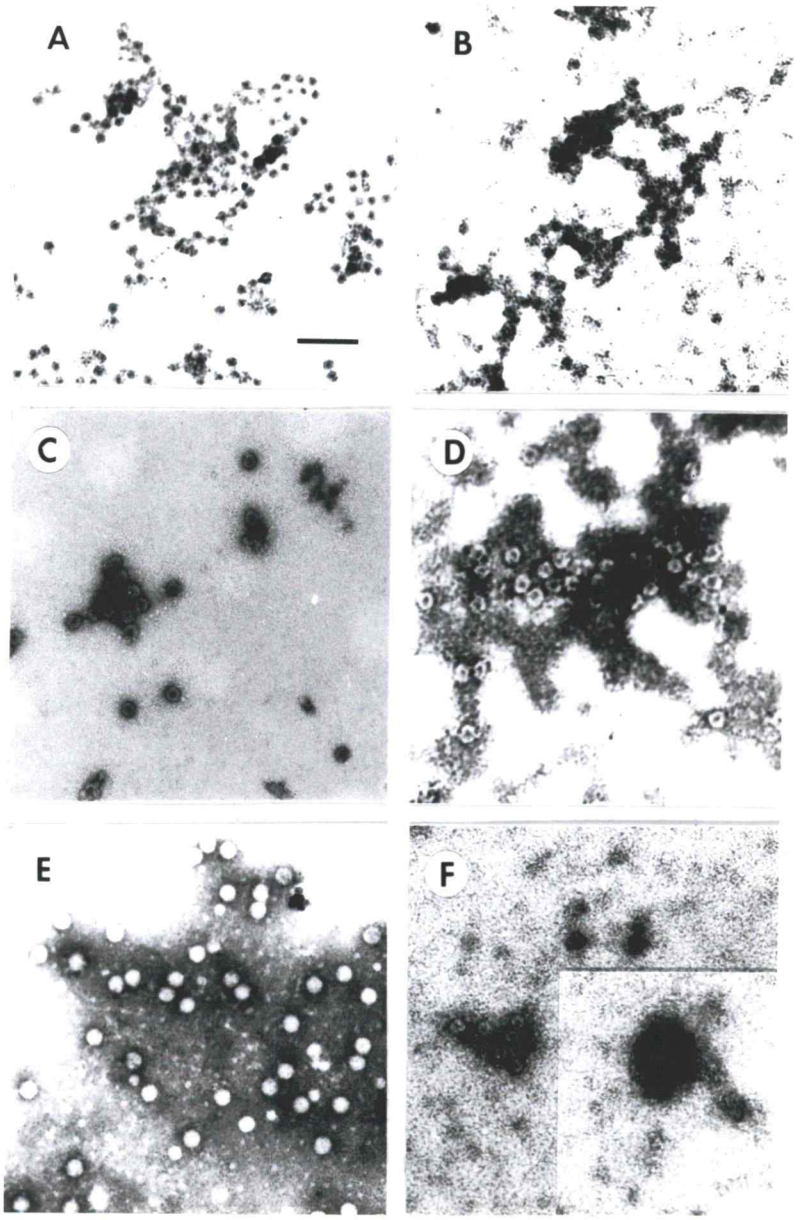


Figure 5-3, A-F. Serologically-specific electron microscopy (SSEM) of CMV-B particles with CMV-B_{32} antiserum. All grids stained with uranyl acetate (UA). A, UA only; B, coating antiserum (CA) diluted 1:50, decorative antiserum (DA) diluted 1:50, no virus; C, CMV-B, no antiserum; D, CA diluted 1:500, CMV-B; E, CA diluted 1:100, CMV-B; F, CA diluted 1:100, CMV-B, DA diluted 1:50. Insert in F shows another area of same grid illustrating aggregation of decorated particles. Magnification ∿80,600. Bar = ∿100 nm.

Chapter Six

RNA Component and Coat Protein Subunit Analysis of Cucumber Mosaic Virus Isolates Seedborne in *Phaseolus* Compared to a Non-Seedborne CMV Isolate

ABSTRACT

Ribonucleic acid (RNA) components of cucumber mosaic virus (CMV) isolates B, F, B_{32}, and Pg (seedborne in *Phaseolus vulgaris*) and CMV-Le (non-seedborne in *P. vulgaris*) were compared by polyacrylamide gel electrophoresis (PAGE). RNA's 1-4 were present in these isolates, but CARNA-5-like RNA species were absent. Molecular weights (MW) of RNA 1-4 were 1.22, 1.10, 0.82, and 0.37 million daltons, respectively, with no significant differences among isolates at the 5% level. Resolution of each RNA species into two components was observed, possibly representing different conformational states, and varied among isolates. Coat protein subunits of isolates B, F, B_{32}, Pg, and Le were compared by SDS-PAGE and found to be about 30,000 daltons each.

INTRODUCTION

Since 1974, six isolates of cucumber mosaic virus (CMV) have been reported to be seed-transmitted in beans (*Phaseolus vulgaris*). These are designated CMV-B (81), CMV-F (61), CMV-B_{32} (10), CMV-Pr (63), CMV-Za8 (1), and CMV-Pg (Chapter Two, p.15). Reported seed-transmission rates ranged from 0.3% to 54%. Knowledge of the intrinsic virus characteristics contributing to seed-transmissibility is herein pursued to develop an understanding of the process of seed-transmission and aid efforts in breeding for resistance. For this purpose, RNA species and

protein coat subunits of seedborne and non-seedborne isolates (CMV-Le, 41) were compared. Average molecular weights for RNA's 1-4 of CMV-Q have been reported to be 1.30, 1.13, 0.78, and 0.34 million daltons, respectively (74). Lower values have been reported for CMV-S: 1.01, 0.89, 0.68, and 0.33 million daltons for RNA's 1-4, respectively (52).

Several minor RNA species, smaller than major CMV RNA's, have been reported, among them two satellite RNA's (31,48,66). One of these satellites, named CMV-associated-RNA-5 (CARNA-5), caused increased severity of CMV infection in tomatoes, leading to a previously unrecognized necrosis-inducing disease (51). Thus, the possibility that additional RNA's might contribute to CMV seed-transmissibility in beans was investigated.

Because it is conceivable that the protein component of CMV might uniquely influence seed-transmissibility of the virus, a superficial attempt wasmade to discern conspicuous gel-electrophoretic differences among seedborne (CMV-B, F, B_{32}, and Pg) and non-seedborne (CMV-Le) isolates. CMV coat protein subunits were previously reported (34) to have a molecular weight of approximately 24,500 daltons.

In summary, CMV isolates B, F, B_{32}, Pg, and Le were compared by RNA component analysis and molecular weights in polyacrylamide gel electrophoresis and by electrophoresis of the coat protein subunits.

MATERIALS AND METHODS

Virus isolates. Isolate sources were described in Chapter Three, p.20. Isolates propagated in 'Bountiful' beans were preserved in desiccated infected tissue for use as standard inoculum sources.

Virus purification. Isolates were propagated in 'Bountiful' plants for 7-14 days and purified as previously described (Chapter 5, p.55). To facilitate the search for CARNA-5-like RNA components, isolates were propagated in tobacco (Nicotiana tabacum 'Xanthi' (51) and purified from this host after four passages. Purified isolates were lyophilized for electrophoresis and stored at -10 C.

RNA electrophoresis. Electrophoresis in 2.4% polyacrylamide tube gels was performed in a vertical tube-gel apparatus (BIO-RAD Model 150A) according to the method of Loening (55) with some modifications. Acrylamide and bis-acrylamide were not recrystalized. Gels (0.4 X 8 cm columns) were formed in 0.036 M Tris, 0.34 M NaH_2PO_4, 0.001 M disodium ethylenediaminetetraacetate (EDTA) pH 7.6 buffer (gel buffer) and cast in plexiglass tubes. Gels were preelectrophoresed for 1 hour and after sample loading were electrophoresed for 1-4 hours at 4 mA/gel in the gel buffer with 0.2% (w/v) sodium dodecyl sulfate (SDS). SDS was removed by soaking gels overnight in 0.04 M acetate pH 4.6. Gels were stained for 1 hour with 0.2% methylene blue in acetate, destained overnight in acetate, and scanned at 546 nm (ISCO Model 1310 gel scanner, UA-5 optical unit).

Electrophoresis in 2.8% polyacrylamide-0.5% agarose slab gels was performed in the same buffer system using a vertical plexiglass apparatus with attached buffer resevoirs. Composite gels were prepared by the method of Peacock and Dingman (73) and cast at 40 C between two glass plates (24 X 16.5 X 0.5 cm) separated by 1.5 mm spacers and sealed on the bottom with rubber tubing. Insertion of a slot former produced 10 slots, each 8 mm-wide. Duration of preelectrophoresis was one hour

and samples were electrophoresed 7-8 hours at 30 mA current or 5 hours at 30 mA for CARNA-5 determinations. Gels were stained in 0.05% methylene blue in acetate, but otherwise handled similarly to tube gels. Migration of bands from origin was measured directly.

Before tube or slab gel electrophoresis, purified, desiccated CMV was dissociated by resuspending in gel buffer with 0.2% SDS, 5% sucrose, and 0.004% bromophenol blue, to 20 mg/ml and 8 mg/ml, respectively. Approximately 120 ug of CMV was applied to tube gels,80 ug to single slots of slab gels. In search of CARNA-5-like RNA components, slots were loaded with 270-370 ug CMV. About 7-12 ug of yeast (Saccharomyces cerevisiae) whole cell 18 S and 25 S RNA or 8-20 ug Escherichia coli 16 S and 23 S ribosomal RNA (kindly provided by Dr. D. Mills and Dr. H. Schaup, respectively) were applied as internal standards to tube gels. For slab gel standards, 6 ug of E. coli rRNA were applied to each of two slots per slab.

Protein electrophoresis. Viral protein subunits were electrophoresed in 12% polyacrylamide resolving gels (0.5 X 10 cm) using the discontinuous SDS-gel (SDS-disc) system of Maizel (60). About 3-5 ul of virus at 1 mg/ml (diluted in electrode buffer) were mixed with 20 ul of sample buffer (8mM Tris pH 6.7, 10% glycerol, 1% SDS, 0.004% bromophenol blue, and 10% 2-mercaptoethanol added just before application to gels). For some experiments two CMV isolates were mixed. About 25 ul of a 1:20 dilution (v/v) of SDS-PAGE low molecular weight standards (BIO-RAD, see Figure 6-3) in electrode buffer were mixed with 20-25 ul of sample buffer with or without CMV. Samples were heated for 4 minutes at 100 C in a heating block, cooled, and layered onto 2-4 mm of

spacer gel after one hour of preelectrophoresis at 10-50 volts per gel. Electrophoresis continued at constant voltage until the dye front was about one cm from the bottom of the gel. Gels were soaked overnight in 10% trichloracetic acid, stained in coomassie Brilliant Blue R250, destained, and scanned as with RNA tube gels.

RESULTS AND DISCUSSION

RNA. Figure 6-1 shows a typical scan of CMV RNA electrophoresed in tube gels alone (Figure 6-1, A) and with E. coli rRNA markers (Figure 6-1, B). CMV RNA-2 and 23 S rRNA comigrated under these conditions. Migration of RNA species from the origin was measured and plotted against log molecular weight of the markers, to estimate molecular weights of CMV RNA molecules. Molecular weights (MW) of the four major CMV RNA species, (RNA 1-4, respectively) were determined to be: 1.26, 1.10, 0.80, and 0.38 million daltons for CMV-B_{32} (four trials); 1.30. 1.16, 0.78, and 0.34 for CMV-B (two trials); and 1.30, 1.14, 0.75, and 0.35 for CMV-Le (one trial). Problems of band streaking and other technical difficulties with this system, time requirement for producing gels, lack of resolution beween CMV RNA 2 and E. coli 23 S rRNA, and the need for internal standards with each gel prompted further evaluations of slab gel systems.

Typical slab gel results are shown in Figure 6-2, A. Isolates produced 4 major bands, RNA 2 again comigrating with 23 S rRNA. Uniform migration of bands was observed in all slots of these gels except bands in the outer slots which sometimes streaked. Average migration of the electrophoretic standards was plotted against molecular weight for each gel, and CMV-RNA molecular weights were determined as with tube gels.

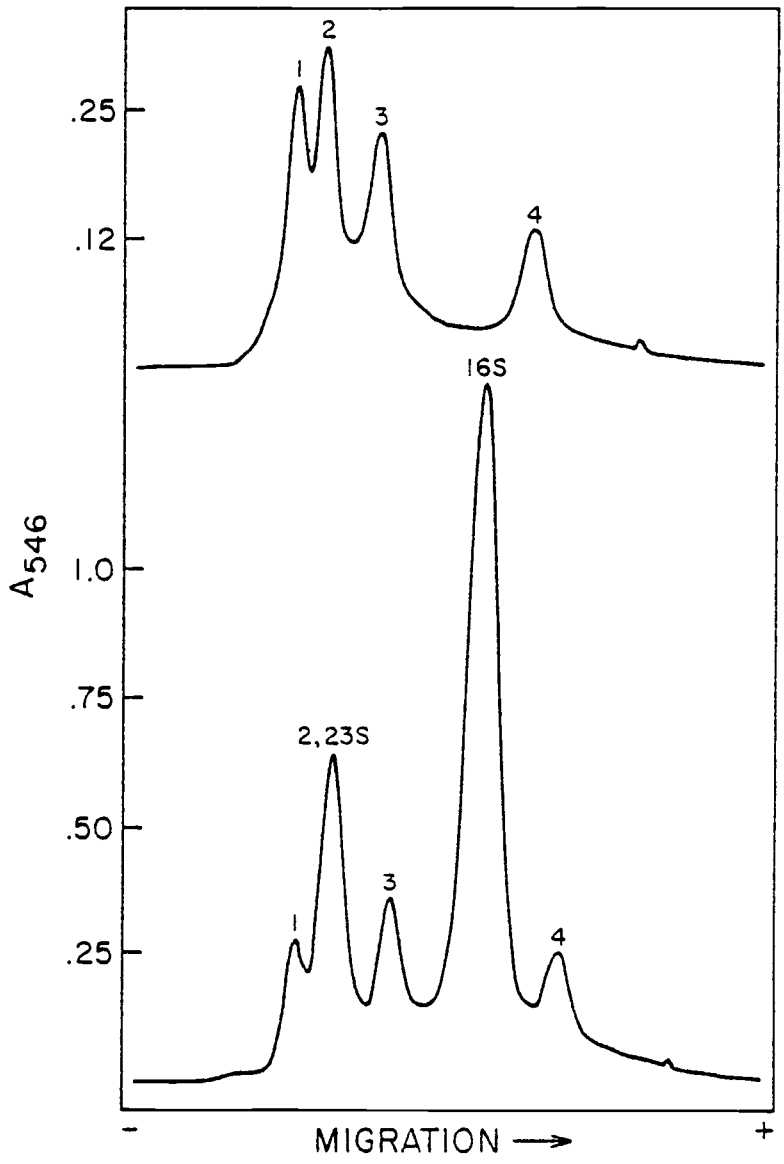


Figure 6-1. Migration versus absorbance (546 nm) profiles for RNA PAGE. Top, CMV-B_{32} RNA (15 ug). Bottom, CMV-B_{32} RNA (15 ug) plus 23 S and 16 S *E. coli* rRNA markers (20 ug). Numbers 1-4 refer to CMV-RNA 1-4, respectively. Note comigration of CMV-B_{32} RNA 2 and 23 S rRNA. Electrophoresis in 2.4% polyacrylamide tube-gels at 4 mA/gel for 2 hours. Migration is left to right.

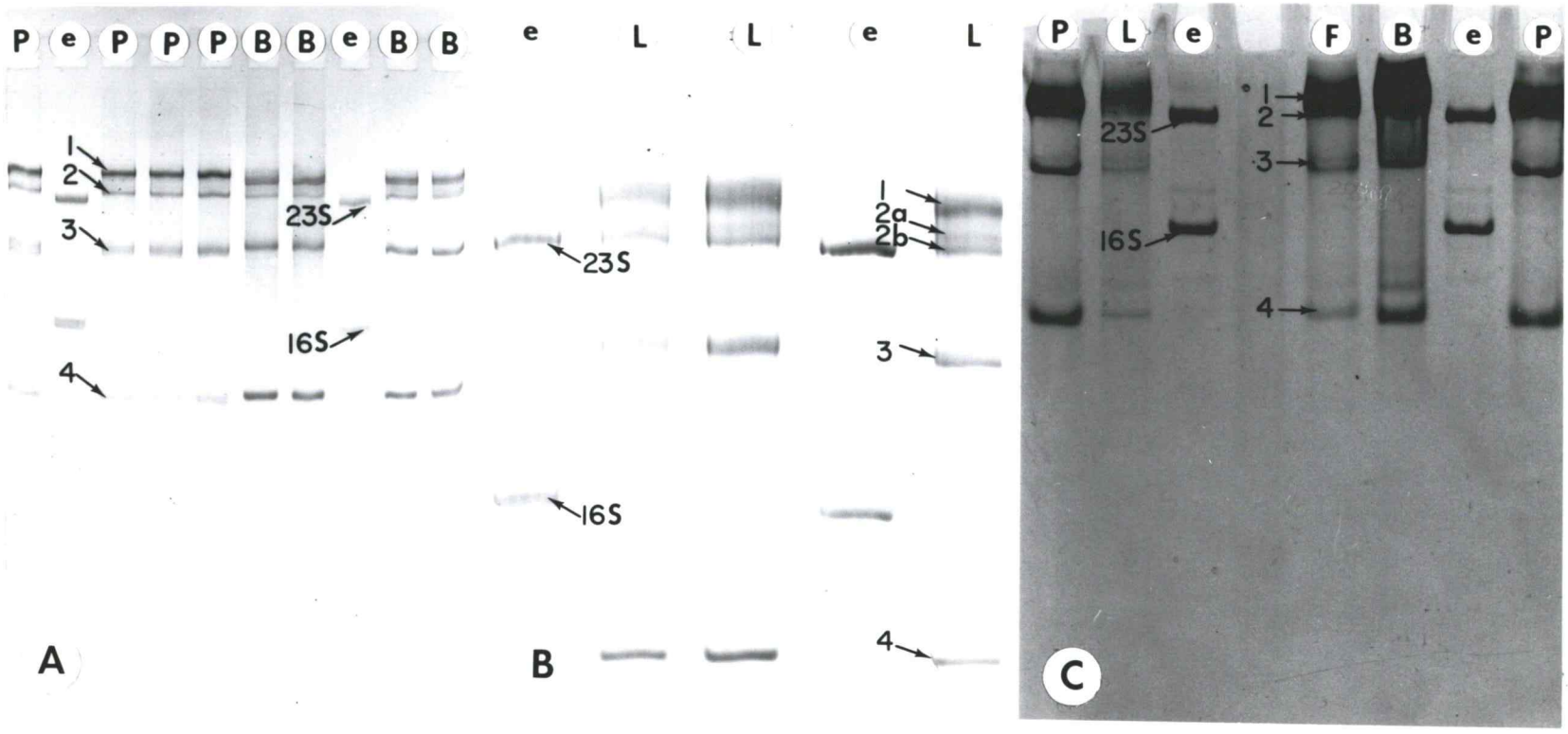


Figure 6-2, A-C. Slab gel electrophoresis of CMV RNA. A, typical separation of CMV RNA's 1-4 (indicated by numbers 1-4, respectively) in gels used to estimate molecular weight of RNA's. B, separation of RNA 2 into two electrophoretic components (2a and 2b). C, RNA component analysis of isolates after four passages in tobacco, showing complete absence of CARNA-5-like RNA molecules. B = CMV-B, F = CMV-F, P = CMV-Pg, L = CMV-Le, e = E. coli rRNA species 23 S and 16 S (molecular weights = 1.07 and 0.55 million daltons, respectively). Electrophoresis was for seven hours (A,B) and five hours (C) at 30 mA/gel. Migration from top (-) to bottom (+).

Table 6-1 shows the results of seven slab gels. There were no significant differences in molecular weight, at the 5% confidence level, of any RNA species among the isolates tested. Average molecular weight for RNA's 1-4 of CMV-B, F, B_{32}, Pg, and Le as determined by slab gel electrophoresis was 1.22, 1.10, 0.80, and 0.37 million daltons, respectively. The molecular weights calculated for the RNA's of the CMV isolates reported here agree closely with the values reported by Peden and Symons for CMV-Q (74). The lower estimates of CMV RNA's reported by Kaper (52) for CMV-S were not obtained with the isolates reported here. Determination of CMV-S RNA molecular weights in this system could help to resolve apparent differences.

Failure to detect significant differences in molecular weights among the RNA components of these seedborne (CMV-B, F, B_{32}, and Pg) and non-seedborne (CMV-Le) isolates indicate that the seed-transmissibility is not grossly manifest in RNA profiles or molecular properties. One would predict from biological, morphological, and serological similarities that differences in rate of seed-transmission in beans among these isolates result from subtle changes at the gene level. RNA differences might be detected much more sensitively by hybridization measurements of nucleotide homology.

It was interesting to note that each RNA species sometimes resolved into two distinct components in the gels (Figure 6-2, B - separation of RNA 2). When this occured, molecular weights of both components were calculated. Differences in RNA component separation among isolates, expressed as a proportion of the slots in which band separation occurred are shown in Table 6-2. Separation of RNA 3 into two components was reported by Mossop and Francki (67). These authors considered

Table 6-1. RNA molecular weight estimates of selected cucumber mosaic virus (CMV) isolates based on polyacrylamide-agarose slab gel electrophoresis[a]

CMV ISOLATE	TRIALS	RNA SPECIES 1	2	3	4
B	8	1.22 ± .03	1.10 ± .01	0.82 ± .02	0.38 ± .01
F	14	1.23 ± .04	1.11 ± .04	0.83 ± .03	0.38 ± .01
B_{32}	8	1.19 ± .04	1.09 ± .01	0.83 ± .02	0.38 ± .01
Pg	8	1.22 ± .02	1.10 ± .01	0.80 ± .02	0.37 ± .01
Le	15	1.21 ± .03	1.08 ± .03	0.80 ± .01	0.36 ± .01
AVERAGE[b]		1.22 ± .02	1.10 ± .01	0.82 ± .02	0.37 ± .01

[a] Total of seven gels in which E. coli rRNA species 23 S (1.07×10^6 daltons) and 16 S (0.55×10^6 daltons) were used as standards in two slots of each of the ten-slot gels. MW values determined by plotting migration against MW of rRNA standards as described in text.

[b] No significant differences in molecular weight of RNA species (at the 5% level) exists among isolates seedborne and non-seedborne (CMV-Le) in Phaseolus vulgaris.

Table 6-2. Differential separation of RNA components among cucumber mosaic virus (CMV) isolates in polyacrylamide-agarose slab gel electrophoresis: proportion of slots in which each RNA component separated into two distinct electrophoretic bands

CMV ISOLATE	No. SLOTS	RNA SPECIES			
		1	2	3	4
B	8	0.75	0	0	0.25
F	14	1.00	0.43	0.57	0
B_{32}	8	1.00	0	1.00	0.62
Pg	8	0	0	0.50	0.38
Le	15	0.53	0.40	0	0

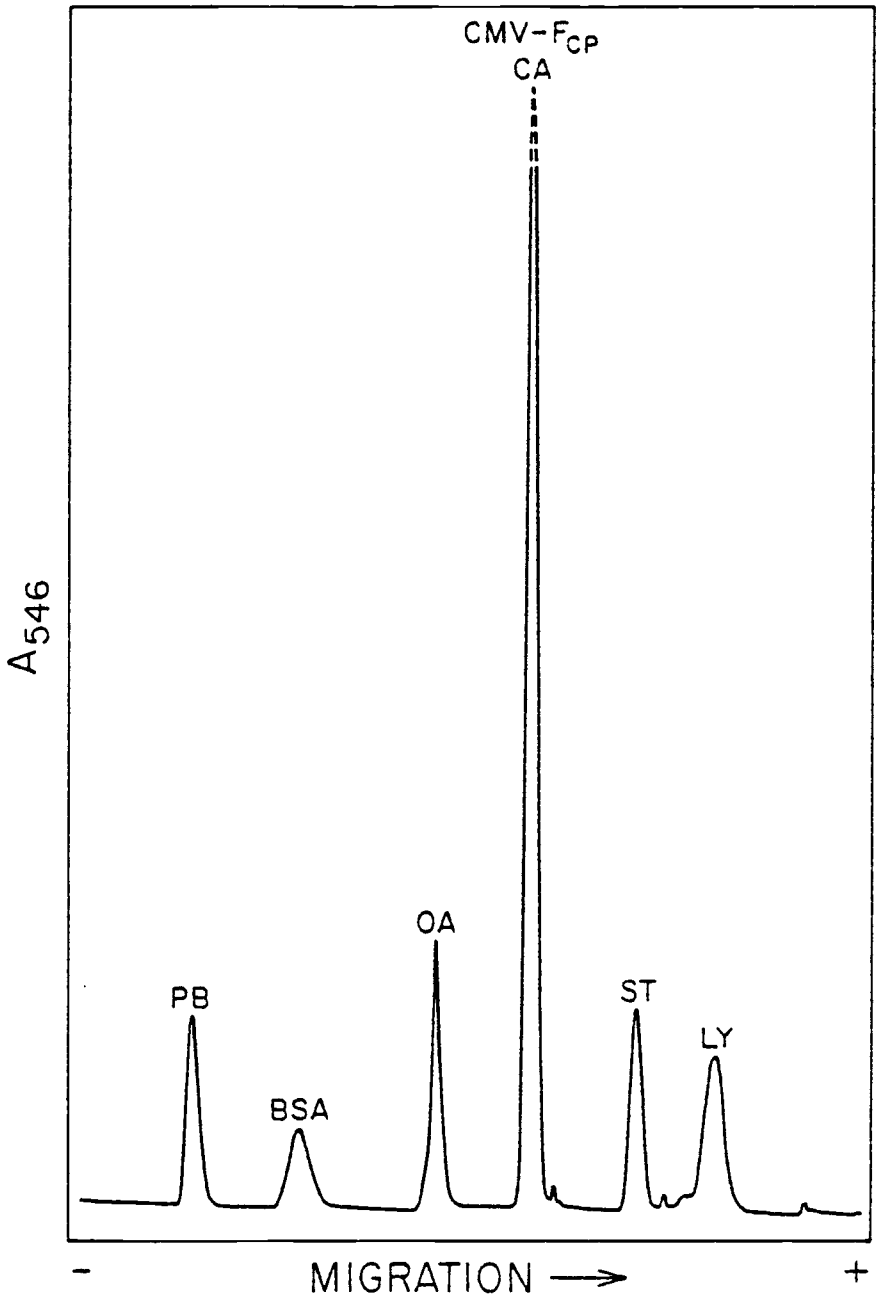
the components to represent different conformational states of the RNA species. If the multiple banding discerned in the current study is due to different conformational states, it would appear that each RNA can exist in one or both states, with different adaptations toward distinctive conformational states among isolates. Inherent differences in RNA structure among isolates, rather than artifacts, would seem most likely; otherwise all RNA's of a given species would have tended to respond similarly under uniform experimental conditions.

The polyacrylamide-agarose slab gels were easier to work with than tube gels, less time consuming, required less RNA standard for molecular weight determinations, and gave excellent, reproducible results once standardized.

CMV-associated-RNA-5 (CARNA-5). Figure 6-2,C shows a slab gel that had received large amounts (270-370 ug) of CMV isolates B, F, Pg, and Le, purified from tobacco after four passages. No CARNA-5 or any other minor RNA species was detected. Based on migration and molecular weight of RNA species, CARNA-5 or similar sized RNA would have migrated only about half the length of the gel. Other slab-gel electrophoresis experiments testing CMV RNA after one or two passages in tobacco (an excellent host for production of CARNA-5, 51), and tube-gel experiments testing isolates purified from beans, also failed to indicate presence of minor RNA's.

Protein. A typical absorbance profile of 12% polyacrylamide SDS-disc gels loaded with CMV-F protein and electrophoretic standards is shown in Figure 6-3. Similar gels with standards or CMV-F viral coat protein alone, indicated that CMV-F protein was comigrating with carbonic anhy-

Figure 6-3. Relative absorbance of 546 nm wavelength light versus migration (left to right) during electrophoresis of CMV-F coat protein and protein molecular weight (MW) standards. Note CMV-F coat protein (CMV-FCP) comigrating with carbonic anhydrase (CA, MW = 30,000 daltons). Other protein standards (BIO-RAD) and their MW's are: PB = phosphorylase B, 94,000 d; BSA = blood serum albumin, 68,000 d; OA = ovalbumin, 43,000 d; ST = soybean trypsin inhibitor, 21,000 d; and LY = lysozyme, 14,300 d. Electrophoresis was for 4.5 hours at 50 volts in 12% polyacrylamide SDS-DISC tube gels.


CMV-F_{CP}
CA
A_{546}
PB
BSA
OA
ST
LY
-
MIGRATION →
+

drase (30,000 daltons molecular weight). A linear relationship was obtained when migration of the standards was plotted against molecular weight, omitting largest and smallest standards (Figure 6-4). In this way the molecular weight of CMV-B, F, B_{32}, and Pg coat protein subunits was determined to be 30,000 daltons. CMV coat protein and carbonic anhydrase were not resolved in this system, regardless of run length, indicating ostensibly identical molecular weights. Figure 6-5 shows a typical absorbance scan of a gel in which CMV-F and CMV-Le were electrophoresed together. Protein preparations of CMV-F and CMV-Le formed a single peak with no resolution in four such gels; thus molecular weight of CMV-Le coat protein is identical to other CMV isolates tested, i.e., 30,000 daltons as estimated in this system.

Neither the extent to which CMV coat protein participates in the seed-transmission phenomenon, nor certainly the nature of such involvement, were elucidated in this study. Rather, an attempt was made to discern any conspicuous gross-molecular difference between the coat protein of seed-transmitted and non-seed-transmitted isolates. No such difference was manifest in comparative gel electrophoresis of these two CMV types. Investigations into a possible carbohydrate component of CMV coat protein, as implicated in seed-transmissibility of barley stripe mosaic virus (71,72), may represent a viable approach to elucidating CMV seed-transmissibility.

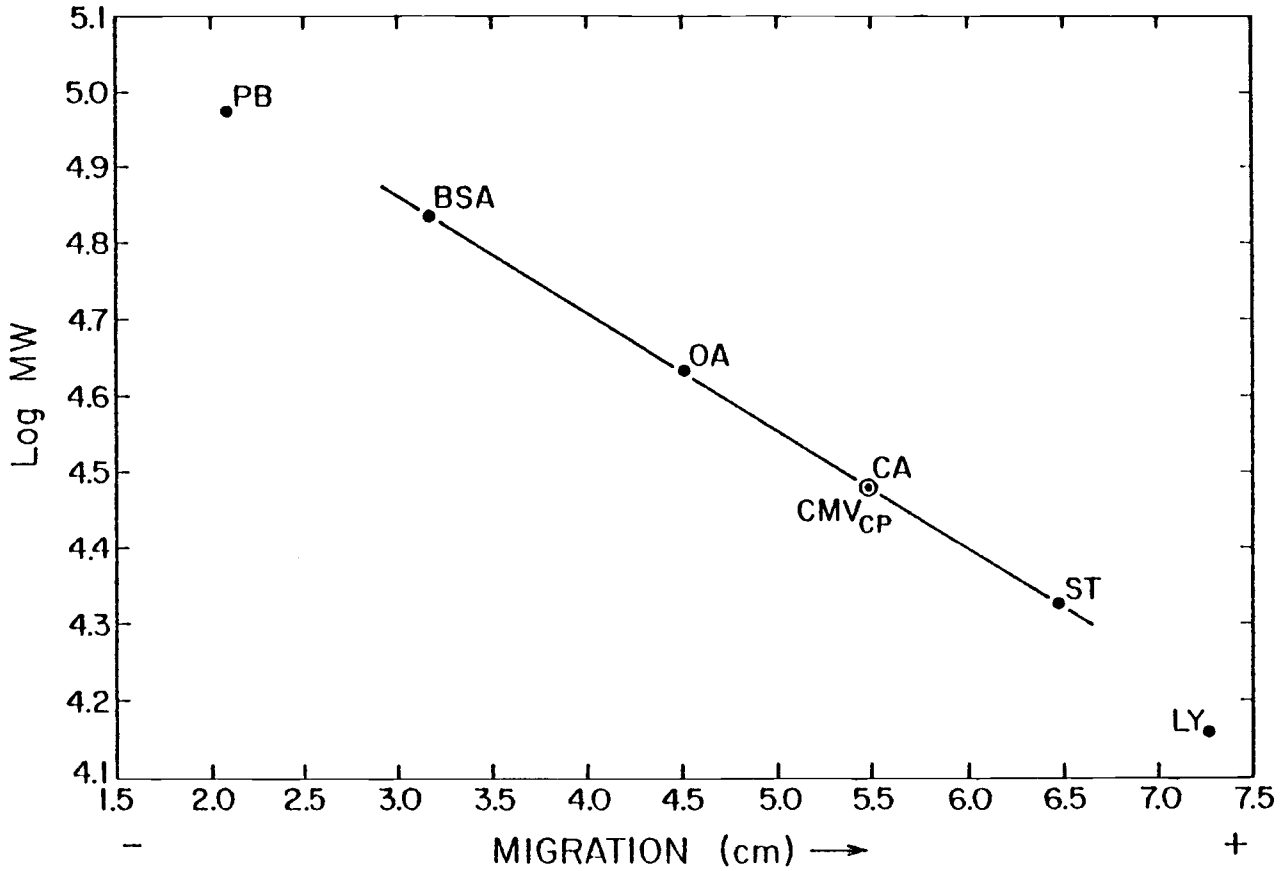


Figure 6-4. Plot of migration versus logarithm molecular weight for proteins shown in Figure 6-3. Note linearity of protein standards in range of 68,000 (BSA) to 21,000 (ST) daltons. Line of best fit does not include PB and LY. CMV-F coat protein (CMV_{CP}) comigrated with 30,000 dalton protein standard, carbonic anhydrase (CA). See Figure 6-3 legend for explanation of other symbols.

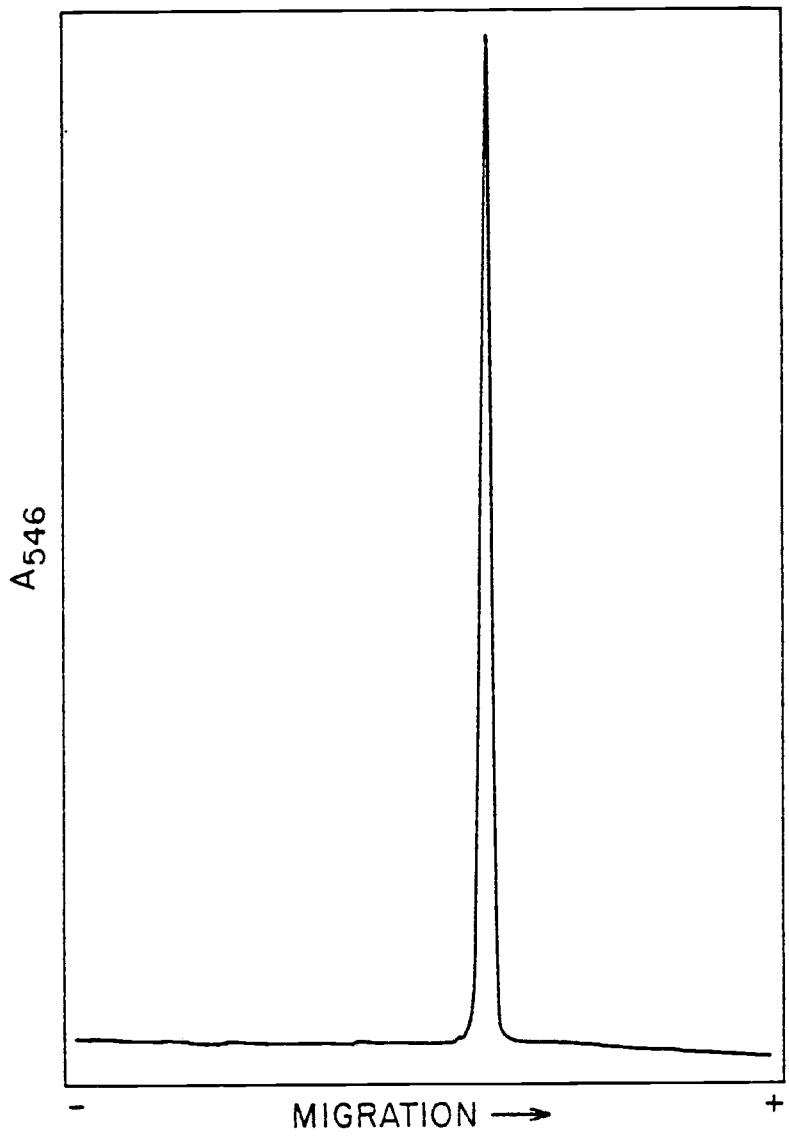


Figure 6-5. Absorbance (546 nm) profile for electrophoresis of CMV-F and CMV-Le coat proteins. Note comigration of proteins, indicating identical electrophoretic mobility in this system. Electrophoresis was for 4.5 hours at 50 volts in 12% polyacrylamide SDS-DISC tube-gels. Migration is left to right.

Chapter Seven

Production of Pseudorecombinants between the Viral Genomes of two Cucumber Mosaic Virus Isolates

ABSTRACT

RNA components 1&2, 3, and 4 of a cucumber mosaic virus isolate (CMV-Pg) that is seedborne in beans (Phaseolus vulgaris 'Topcrop') and a non-seedborne isolate (CMV-Le) were separated by three cycles of sucrose density gradient centrifugation. Large yields of RNA were recovered by this technique while RNA infectivity was maintained, in contrast to RNA derived by electrophoretic separation, attempted initially. RNA's 3 and 4 were essentially pure after three cycles of separation, whereas RNA 1&2 still contained approximately 10% of RNA 3. RNA pseudorecombinations prescribed to determine the RNA component(s) instrumental in CMV seed-transmissibility were prepared. 'Topcrop' bean plants were inoculated with these pseudorecombinants. Analysis of the seed-transmissibility and RNA determinants, will be conducted as part of a continuing study.

INTRODUCTION

Cucumber mosaic virus (CMV, cryptogram: R/1;1.3/18 + 1.1/18 +.8+.3/18:S/S:S/C,Ve/Ap) contains a functionally divided genome of four major RNA species designated RNA 1-4, in order of decreasing molecular weight (25,28). The three largest RNA's are required for infectivity (58,74). RNA's 1 and 2 are unique sequences, while each has a region of about 300 nucleotides in common with RNA 3, and the sequence of RNA 4 is completely contained in RNA 3 (32). RNA 4 is derived from

RNA 3 *in vivo* (74) and probably not from any RNA 4 added in inoculum (8,32,93). The coat protein gene, contained in RNA 3 and RNA 4, is translated *in vitro* from RNA 4 (85). RNA 1 and RNA 2 seemed to be monocistronic messengers for 105,000 and 120,000 daltons products, respectively, *in vitro*, whereas RNA 3 codes for three major products in the range of 34,000-39,000 daltons. RNA 4 directed minor products *in vitro* in addition to the major coat protein

Pseudorecombination experiments have provided evidence that genes determining host range of CMV and virus concentration in infected plants are located on RNA 1 and/or RNA 2, and that gene(s) for coat protein are on RNA 3 (35,36). The primary role of raspberry ringspot virus RNA 1 in determining seed-transmissibility was shown by production of pseudorecombinants with the RNA of tomato black ring virus (39).

The intent of this chapter is to describe 1) the separation of RNA species from CMV-Pg, seedborne in beans (*Phaseolus vulgaris* 'Topcrop') and CMV-Le, non-seedborne in beans, and 2) the subsequent production of pseudorecombinants between these isolates. In continuing studies of these pseudorecombinants, identification of genomic determinant(s) of CMV seed-transmissibility in beans will be attempted.

MATERIALS AND METHODS

Virus isolates. CMV isolates F, Pg, and Le were included in this study. Sources of these isolates, propagation in 'Bountiful' beans, and purification were as previously described (Chapters Three and Five).

Electrophoretic separation and elution. Electrophoresis of CMV-F and

CMV-Le RNA in 2.8% polyacrylamide-0.5% agarose slab gels was performed as described in Chapter Six. RNA components 1, 2, and 3 were eluted separately from gels by the phenol method of Schwinghamer and Symons (85). Numbers of gels and total RNA applied are shown in Table 7-1. RNA's 1 and 2 were further purified by a second cycle of electrophoresis.

Sucrose density gradient separation. A total of 1.42 mg CMV-Le RNA and 0.61 mg CMV-Pg RNA were separated into components by centrifugation in 7-27% linear sucrose gradients prepared with 0.02 M phosphate, 1 mM disodium ethylenediaminetetraacetate (EDTA), 0.1 M sodium chloride pH 7.0 buffer (PES), for 16 hours at 32,000 rpm in a Beckman SW 40 rotor (49). Gradients were monitored by light absorbance at 254 nm and fractionated with an ISCO density gradient fractionator and UA-5 optical unit. Peak fractions were collected and combined where appropriate. RNA was ethanol precipitated from sucrose fractions and resuspended in PES buffer.

For the first cycle of separation, lyophilized CMV preparations were dissociated by resuspension in PES buffer containing 0.2% sodium dodecyl sulfate (SDS). About 0.3 ml aliquots containing 0.2-0.4 mg CMV (37-74 ug RNA) were layered onto sucrose gradients. For the second and third cycle separations, ethanol precipitated RNA was resuspended in PES buffer without SDS, heated to 100 C for 2 minutes, cooled on ice, and 0.3 ml aliquots containing 20-60 ug RNA were layered onto each gradient.

Biological assay for purity. RNA molecules separated by electrophoresis or sucrose density gradient centrifugation were tested by bioassay in

various combinations to determine degree of purity. RNA was diluted in 0.035 M phosphate, 0.05 M glycine pH 9.2 buffer (70) with 100-200 ug/ml bentonite purified according to the method of Fraenkel-Conrat (23). Replicate leaves of Chenopodium amaranticolor (Corvallis strain) assay plants were rub-inoculated with RNA at 0.25-15 ug/ml, depending on the assay, using ground glass stoppers. Lesions were recorded in 5-10 days.

Production of pseudorecombinants. CMV-Pg and CMV-Le RNA species were inoculated into 'Xanthi' tobacco (Nicotiana tabacum) plants in the following combinations to produce seven recombinants (Rc): Rc 1 = Le 1&2&3; Rc 2 = Pg 1&2&3; Rc 3 = Pg 1&2/Le 3; Rc 4 = Le 1&2/Pg 3; Rc 5 = Le 1&2&3/Pg 4; Rc 6 = Le 1&2; Rc 7 = Pg 1&2. Infected tobacco tissue was harvested after about seven days and desiccated. Desiccated tissue of each recombinant was used to inoculate about 40 'Topcrop' bean plants. Beans were grown in a greenhouse with supplemental artificial lighting.

RESULTS AND DISCUSSION

Electrophoretic separation. The degree of separation of RNA species by electrophoresis was similar to that shown in Figure 6-2,A (p.82). Yield of RNA after elution from gels was very low (Table 7-1). Starting with 1.8 and 2.0 mg of CMV-F and CMV-Le, respectively, only about 3 ug each of purified RNA's 1 and 2 were available after two cycles of separation. Less than one percent of the RNA originally applied was recovered as RNA 3 and RNA 4 after two cycles of electrophoresis.

After one cycle of electrophoretic separation of CMV-Le RNA, combinations of any two of the three major RNA's required for infectivity caused local lesion development on the assay host, whereas

Table 7-1. Yield of CMV-F and CMV-Le RNA after one and two cycles of polyacrylamide-agarose gel electrophoresis and elution

CYCLE[a]	CMV ISOLATE/RNA	No. GELS	TOTAL RNA APPLIED(mg)	TOTAL RNA ELUTED(mg)	YIELD(%)
1	F/1-4	13	1.76	0.168[b]	10[b]
2	F/1	1	0.061	0.003	5
2	F/2	1	0.060	0.003	5
TOTAL[c]					0.7
1	Le/1-4	16	2.04	0.153[b]	8
2	Le/1	1	0.086	0.002	2
2	Le/2	1	0.040	0.003	7
TOTAL[d]					0.5

[a] Slab gel electrophoresis in 2.8% polyacrylamide-0.5% agarose gels and phenol elution as described in text.

[b] RNA 4 not included.

[c] Total yield of CMV-F RNA after 2 electrophoretic-elution cycles, estimated by doubling the sum of RNA 1 and RNA 2 yields after 2 cycles and dividing by total RNA originally applied.

[d] Same as footnote [c] for CMV-Le RNA.

RNA 3 alone did not cause infection (Table 7-2). These results indicated that RNA 1 and RNA 2 were contaminated with each other and with RNA 3. Electrophoresis confirmed this conclusion (results not shown). RNA 3 was apparently 'pure' after one cycle of separation and was not purified further.

Infectivity assay of RNA 1 and RNA 2 after the second cycle of separation indicated the RNA's were 'pure', except for CMV-F RNA 1 and RNA 2, one or both of which was apparently contaminated with RNA 3 (Table 7-2), since one local lesion was observed. This single lesion might represent true lack of purity or it might have been caused by the unfractionated RNA applied on the opposite half of the leaf. Interpretation of this datum was complicated by the observation that only two lesions were produced by recombining fractionated RNA's 1&2&3.

Electrophoretic separation reduced the infectivity of the RNA, as evidenced by the low numbers of lesions (9%) produced when RNA was recombined after one cycle, compared to lesions produced by unfractionated RNA at the same concentration (Table 7-2). After two separation cycles, recombined RNA, when applied at three times the concentration of unfractionated RNA, caused only 4% as many lesions (Table 7-2, average of CMV-F and CMV-Le RNA 1&2&3). Lowered infectivity of RNA following elution has been reported by others (74). Electrophoretic separation and phenol elution may effect other properties in addition to infectivity. Because of effect on virus integrity and low yields with this technique, sucrose density gradient separation was evaluated as an alternative method for isolating RNA species.

Sucrose density gradient separation. Separation of RNA's by sucrose

Table 7-2. Local lesions produced on replicate half-leaves of Chenopodium amaranticolor plants rub-inoculated with CMV-F or CMV-Le RNA after one or two cycles of electrophoresis-elution

RNA SPECIES	CMV-Le 1 CYCLE[a]	CMV-Le 2 CYCLES[b]	CMV-F 2 CYCLES[b]
1&2	5/175[c]	0/67	1/60
1&3	13/245	0/53	0/62
2&3	8/386	0/46	0/52
3	0/111	0/104	0/69
1&2&3	10/115	5/87	2/84

[a] RNA at 5 ug/ml, 3 half-leaves on separate plants.

[b] Treatment RNA at 15 ug/ml, unfractionated RNA at 5 ug/ml, 2 half-leaves on separate plants.

[c] Total lesions produced by treatment RNA/total lesions produced by unfractionated RNA inoculated onto opposite half-leaves.

density gradient centrifugation offered many advantages including high recovery rates and gentle treatment of RNA. However, because separation of RNA 1 and RNA 2 has been refractory, they were treated as a unit (RNA 1&2) to be compared with RNA 3 and RNA 4 for effects on seed-transmission. After it's discovery, CMV-Pg was substituted for CMV-F as the seedborne isolate by virtue of much higher seed-transmission rates in experimental tests (Chapter Four).

After three cycles of sucrose density gradient centrifugation, about 50 ug of each RNA was recovered, for a total of 25% of CMV-Pg RNA and 10% of CMV-Le RNA originally applied. Percent yields of individual RNA's after one, two, and three cycles of sucrose density gradient separation ranged from 26-98% (Table 7-3), much higher than yields of RNA after electrophoretic separation.

Contamination of the separated RNA species with each other was monitored directly as a result of absorbance profiles (254 nm) generated in the process of gradient fractionation after one, two, and three cycles of sucrose density gradient separation (Figure 7-1). Heat denaturation of the RNA after the first separation cycle was necessary, as previously reported (84), to release pieces of RNA co-sedimenting with the RNA of a particular size. The effect of heat denaturation was especially prominent with RNA 1&2 (Figure 7-1,A:middle) in which substantial amounts of RNA molecules were released with sedimentation peaks close to RNA 3 and RNA 4. RNA 3 and RNA 4 were apparently less contaminated with other RNA molecules after one cycle of separation (Figure 7-1,B,C:Middle). After the second separation (third gradient centrifugation), the RNA's appeared as 'pure' as could be achieved by this technique (Figure 7-1,A-C:bottom). Fractionation of these third

Table 7-3. Yield of CMV RNA after one, two, and three cycles of separation by sucrose density gradient centrifugation

CYCLE[a]	CMV RNA	GRADIENTS (No.)	TOTAL RNA APPLIED (mg)	TOTAL RNA RECOVERED (mg)	AVERAGE YIELD (%)
1	Pg 1-4	10	0.611	0.524	86
2	Pg 1&2	4	0.239	0.092	38
2	Pg 3	3	0.138	0.083	60
2	Pg 4	3	0.147	0.092	63
3	Pg 1&2	2	0.092	0.049	53
3	Pg 3	2	0.083	0.044	53
3	Pg 4	2	0.092	0.058	52
TOTAL[b]	Pg 1-4	26	0.611	0.151	25
1	Le 1-4	20	1.42	0.520	51
2	Le 1&2	2	0.191	0.068	26
2	Le 3	2	0.144	0.059	30
2	Le 4	2	0.185	0.056	26
3	Le 1&2	2	0.068	0.045	66
3	Le 3	2	0.059	0.042	71
3	Le 4	2	0.056	0.055	98
TOTAL[c]	Le 1-4	32	1.42	0.142	10

[a] Centrifugation in 7-27% linear sucrose gradients for 16 hours at 32,000 rpm in a Beckman SW 40 rotor. Fractions were collected with an ISCO fractionator and RNA recovered by ethanol precipitation.

[b] Total RNA recovered after third cycle compared to RNA originally applied at first cycle, CMV-Pg.

[c] Same as footnote [b] for CMV-Le.

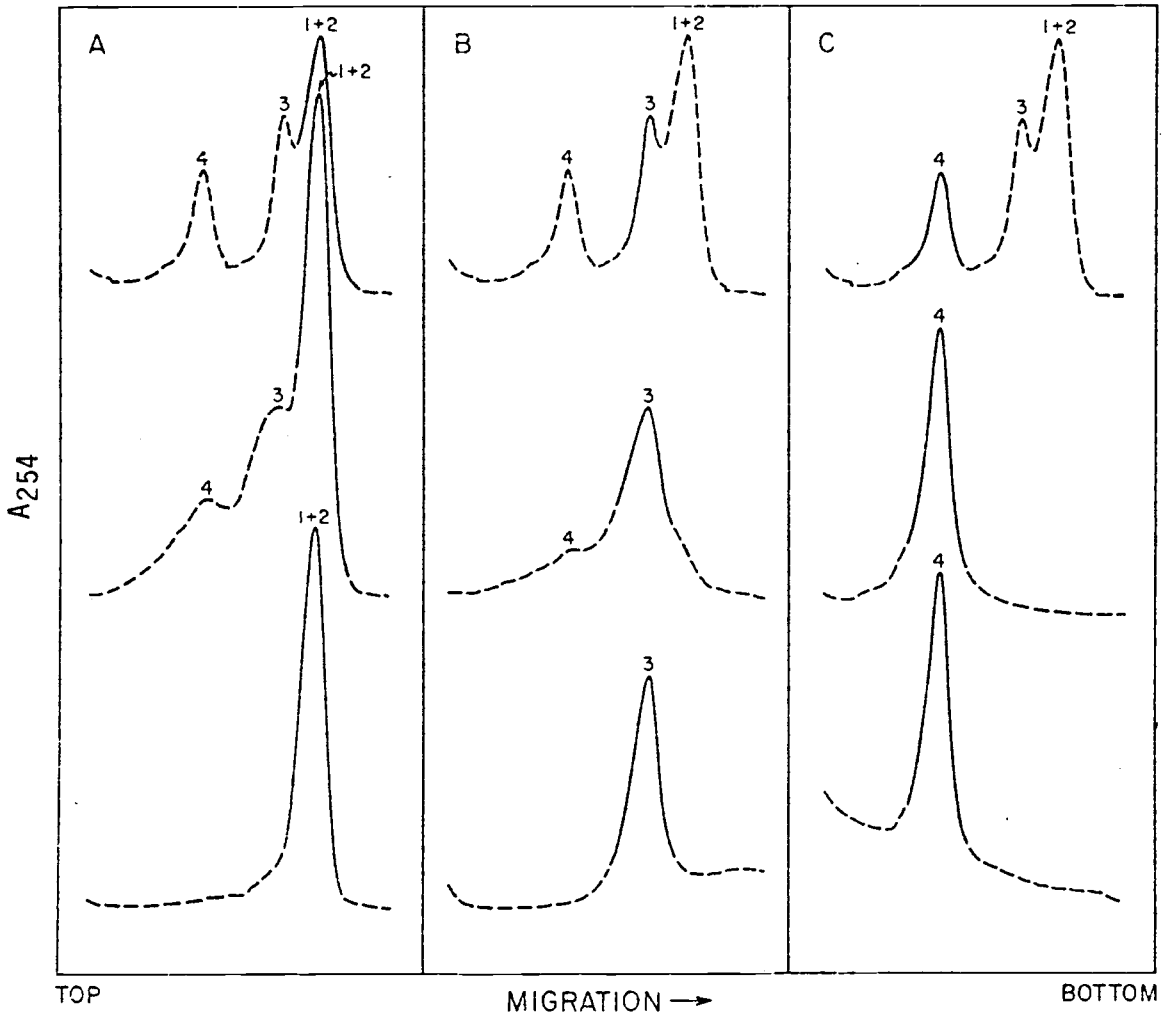


Figure 7-1. Absorbance (254 nm) profiles of cucumber mosaic virus (CMV-Pg) RNA after one (TOP), two (MIDDLE), and three (BOTTOM) successive cycles of sucrose density gradient centrifugation. A, RNA 1&2, B, RNA 3, and C, RNA 4. Numbers indicate RNA species. Solid portion of line indicates fractions collected for subsequent separation in the next centrifugation. Centrifugation for 16 hours at 32,000 rpm in 7-27% linear sucrose gradients as described in text. Relative migration from left to right; peaks of successive scans aligned manually.

cycle gradients constituted the third and final separation.

Purity of RNA's was determined indirectly by inoculating indicator assay plants with RNA purified by three cycles of gradient separation. In one experiment, Table 7-4, in which RNA was applied at 10 ug/ml, CMV-Pg RNA 1&2 produced 6% as many lesions as Pg RNA 1&2&3, and CMV-Le RNA 1&2 produced 14.5% as many lesions as Le RNA 1&2&3. High degrees of purity of RNA 3 and RNA 4 from CMV-Pg and CMV-Le were indicated by C. amaranticolor lesion production of less than 1% compared to RNA 1&2&3. These results were substantiated in another experiment, Table 7-5, in which RNA was similarly inoculated at 1.25, 2.5, and 5.0 ug/ml to test RNA purity as well as linearity of the assay system. Contamination of CMV-Pg RNA species 1&2, 3, and 4 (average of the three concentrations) was estimated to be 11.4, 2.4, and 1.8% of recombined RNA 1&2&3, respectively, and contamination of CMV-Le RNA species 1&2, 3, and 4 was 8.7, 1.0, and 0.3%, respectively. Lesion production was not linear with RNA concentration. The lowest concentration of RNA fractions yielded estimates of purity divergent from the two higher RNA concentrations. The average estimate of purity, eliminating the lowest RNA concentration (Table 7-5, last column), approximated the results of the first experiment (Table 7-4, last column). A greater degree of contamination of RNA 1&2 might have been expected from results shown in sucrose gradient profiles (Figure 7-1) and previously published reports (84,85).

RNA's separated by three cycles of sucrose density gradient centrifugation seemed as fully infectious, when recombined, as unfractionated RNA (results not shown). This indicates that separation by this method probably effects RNA structure and integrity minimally, if at all

Table 7-4. Local lesions observed on replicate leaves of four Chenopodium amaranticolor plants rub-inoculated using ground-glass stoppers with CMV-Pg and CMV-Le RNA's at 10 ug/ml, purified by three cycles of sucrose density gradient centrifugation . Experiment I

CMV ISOLATE	RNA SPECIES	PLANT NUMBER 1	2	3	4	TOTAL LESIONS	INFECTIVITY (%)
Pg	1&2	6	37	48	90	181	6.0[a]
Pg	3	2	1	4	3	10	0.3
Pg	4	3	0	3	2	8	0.3
Pg	1-3	11	888[b] (64,56)	975[b] (68,73)	1146[b] (93,87)	3020	100.0
Le	1&2	112	13	90	163	378	14.5
Le	3	0	0	1	0	1	0.4
Le	4	0	0	0	0	0	0
Le	1-3	785[b] (99,64)	107	1061[b] (73,99)	662[b] (98,53)	2615	100.0

[a] 100 X Total lesions for RNA species 1&2, 3, or 4 / Total lesions for RNA 1-3.

[b] Total lesions per leaf as estimated by averaging the lesions in each of two one-cm-square leaf areas (lesions for those areas given in parentheses) and multiplying by the leaf area in square-cm.

Table 7-5. Local lesions observed on replicate leaves of three Chenopodium amaranticolor plants rub-inoculated using a ground-glass stopper with 20 ul of CMV-Pg or CMV-Le RNA purified by three cycles of sucrose density gradient centrifugation. Experiment II

CMV RNA	CONC (ug/ml)	PLANT No.			AVERAGE			
		1	2	3	LESIONS	(%)[a]	(%)[b]	(%)[c]
Pg 1&2	1.25	29	21	5	18.3	21.4	11.4	6.4
Pg 1&2	2.5	7	3	12	7.3	3.7		
Pg 1&2	5.0	9	15	7	10.3	9.1		
Pg 3	1.25	4	9	3	5.3	6.2	2.4	0.5
Pg 3	2.5	0	2	0	0.7	0.4		
Pg 3	5.0	0	0	2	0.7	0.6		
Pg 4	1.25	0	11	0	3.7	4.2	1.8	0.5
Pg 4	2.5	0	3	0	1.0	0.5		
Pg 4	5.0	2	0	0	0.7	0.6		
Pg 1-3	1.25	85	120	52	85.7	100.0		
Pg 1-3	2.5	95	94	-	95.0	100.0		
Pg 1-3	5.0	-	210	130	113.3	100.0		
Le 1&2	1.25	1	4	0	1.7	1.4	8.7	12.4
Le 1&2	2.5	2	17	20	13.0	8.7		
Le 1&2	5.0	34	20	17	23.7	16.0		
Le 3	1.25	0	0	0	0	0	1.0	1.4
Le 3	2.5	0	1	0	0.3	0.2		
Le 3	5.0	6	5	1	4.0	2.7		
Le 4	1.25	0	0	0	0	0	0.3	0.4
Le 4	2.5	0	0	0	0	0		
Le 4	5.0	0	3	1	1.3	0.9		
Le 1-3	1.25	170	130	53	117.7	100.0		
Le 1-3	2.5	160	155	135	150.0	100.0		
Le 1-3	5.0	120	240	85	148.3	100.0		

[a] Percent of RNA 1-3 infection at same concentration.

[b] Average percent infection of all dilutions.

[c] Average percent infectection of 2.5 and 5.0 ug/ml concentrations.

Pseudorecombinant production. Pseudorecombinants were produced as described in the Materials and Methods section. All pseudorecombinants were infectious and seed production by infected beans is in progress.

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APPENDICES

APPENDIX I

Table 2-1a. Results of sampling Phaseolus vulgaris germplasm in Idaho, Washington, and Oregon for seedborne cucumber mosaic virus

YEAR	LOCATION	SOURCE[a]	No. PLANTS SAMPLED	ASSAY METHOD[b]	RESULTS[c]
1978	So. Idaho	Seed Co. No. 1	9	B_1	0/9
		2	4		0/4
		3	3		0/3
		4	3		0/3
		5	4		0/4
		6	3		0/3
	E. Washington	5	1	B_2,M,S	0/1
		7	1		0/1
	Prosser	Breeding Plots	59		0/59
	Corvallis	Breeding Plots	3	B_3	0/3
1979	So. Idaho	Seed Co. No. 1	14	B_4/E	0/14
		3	5		0/5
		4	7		0/7
		5	17		0/17
	W. Oregon	1	4		0/4
	E. Washington	7	10	B_2,M,S	0/10
		8	1		0/1
		9	14		0/14
	Grandview	10	1		0/1
	Prosser	Breeding Plots	56		0/56
	Corvallis	Breeding Plots	40	B_4/E	0/40

[a] Seed Companies represent commercial bean seed-producing companies. Corvallis breeding plots represent Dr. J. Baggett's bean breeding program and Prosser plots represent Dr. M. Silbernagel's and Dr. D. W. Burke's breeding programs.

[b] B_1 = Bioassay, hosts: Phaseolus vulgaris 'Bountiful','Black Turtle', and 'Limelight'; and Cucumis sativus 'Improved Long Green'. B_2 = Bioassay, hosts: P. vulgaris 'Bountiful' and 'Monroe'; Pisum sativum 'Alaska' and'Dark Skin Perfection'; Vigna unguiculata 'Ramshorn'; and C. sativus 'Boston Pickling'. B_3 = Bioassay, hosts: Chenopodium amaranticolor; 'Improved Long Green' cucumber; and 'Ramshorn' cowpea. M = Electron microscopy on selected hosts. S = Gel double-diffusion serology. E = Enzyme-linked immunosorbent assay (ELISA).

[c] No. samples in which CMV was detected/no. samples tested. Virus was detected in CMV-infected control plants but not CMV-free plants with all assay methods.

APPENDIX II

Table 2-1b. Plant Introduction (PI) lines tested for seedborne cucumber mosaic virus (CMV)

GEOGRAPHIC[a] ORIGIN	PI LINE	No. PLANTS TESTED	ASSAY[b] METHOD	RESULTS[c]
New York	267762	27	B	0/27
	278668	18	B	0/18
	278670	29	B	0/29
	278675	24	B	0/24
	278676	44	B	0/44
	278678	46	E	0/46
Spain	226856	54	E	0/54
	249919	31	E	0/31
	271998	56	E	** 2/56
France	226895	51	E	0/51
	226929	69	E	0/69
Iran	140301	24	E	0/24
	226521	28	E	0/28
	226522	23	E	0/23
	226523	36	E	0/36
Turkey	167399	21	E	0/21
	171794	36	E	0/36
	173024	54	E	0/54
	173042	30	E	0/30
	176683	36	E	0/36
Venezuela	109859	55	E	0/55
Canada	136739	14	E	0/14
El Salvador	150414	22	E	0/22
Mexico	165426	46	E	0/46
	165435	82	E	0/82
Netherlands	165616	28	E	0/28
S. Africa	172029	34	E	0/34
Syria	179426	39	E	0/39
Fed. Rep. Germany	180752	42	E	0/42
Puerto Rico	209052	37	E	0/37
	209053	74	E	0/74
Poland	285695	48	E	0/48

[a] Obtained from W-6 Regional Plant Introduction Station, Pullman, Wa.

[b] B = Bioassay, hosts: C. amaranticolor; C. sativus 'Improved Long Green'. E = Enzyme-linked immunosorbent assay (ELISA).

[c] No. samples in which CMV was detected/no. samples tested. Virus was detected in CMV-infected control plants but not CMV-free plants.

** Note positive results with PI 271998, isolate designated CMV-Pg.