SUNN-HEMP MOSAIC VIRUS AS A HELPER IN THE INTERCELLULAR SPREAD OF SOUTHERN BEAN MOSAIC VIRUS IN A RESISTANT HOST

by

Ana Lucía Fuentes

B.Sc., University of Costa Rica, 1981

M.Sc., University of Costa Rica, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF PLANT SCIENCE

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1992

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Department of Plant Science

The University of British Columbia
Vancouver, Canada

Date Sept 24, 1992

Abstract

Four bean cultivars ("Bountiful", "Pinto", "Top Crop", and "Tendergreen") allowed only subliminal replication of the cowpea strain of southern bean mosaic virus (SBMV-C). Bean protoplasts, on the other hand, sustained replication of SBMV-C upon *in vitro* inoculation. Antigen accumulation of the bean strain of southern bean mosaic virus (SBMV-B) and SBMV-C in bean protoplasts was similar, indicating that the replicating capacity of both viruses does not differ in bean cells.

When the four bean cultivars were inoculated with a mixture of sunn-hemp mosaic virus (SHMV), a tobamovirus, and SBMV-C, the latter was readily detected in the inoculated primary leaves. The rate of spread of SBMV-C in the presence of SHMV was compared to the rate of spread of SBMV-B in bean co-inoculated with SHMV. Virus accumulation in leaf blades, lateral veins, mid-ribs, petioles, stems and roots was similar for both strains in the non-vascular tissue of the inoculated leaf; a sharp decline in SBMV-C accumulation was observed starting from the lateral veins towards the mid and distal parts of the petiole, where virtually no virus could be found. These results contrasted with the uniform presence of SBMV-B throughout infected bean plants.

Leaf strips blotted on nitrocellulose paper and developed as for Western blotting confirmed these results, with SBMV-C antigen being detected in mesophyll tissue and in epidermal cells of the lateral veins of the inoculated

primary leaves. Electron micrographs of immunogold-labelled sections revealed the absence of uniform SBMV-C particles in the mesophyll cells; instead, heavily labelled, amorphous protein clumps in the vacuole were found. SBMV-C coat protein from infected cowpea and bean plants showed no difference in its mobility during electrophoresis in denaturing polyacrylamide gels.

These results indicate that SHMV facilitates cell to cell spread of SBMV-C in inoculated bean leaves but does not allow for the movement of the latter through the vascular system. Lack of efficient assembly of SBMV-C does not impede cell-to-cell movement of the virus in the doubly-infected leaves, yet it is probably an important factor involved in determining the inability of SBMV-C to move into and/or through the vascular system.

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List of Abbreviations

A_{260} - absorbance at 260 nm

A_{280} - absorbance at 280 nm

A_{405} - absorbance at 405 nm

AlMV - alfalfa mosaic virus

ATP - adenosine triphosphate

BCIP - 5-bromo-4-chloroindoxyl phosphate

BGMV - bean golden mosaic virus

BRMV - bean rugose mosaic virus

BSA - bovine serum albumin

BSMV - barley stripe mosaic virus

BYMV - bean yellow mosaic virus

C - Celsius

$CaCl_2$ - calcium chloride

cAMP - cyclic adenosine mono phosphate

cDNA - complementary DNA

cm (mm, nm, *u*m)- centimeter (millimeter, nanometer, micrometer)

CPMV - cowpea mosaic virus

DAS-ELISA - double antibody sandwich enzyme-linked immunosorbent assay

DDSA - dodecenyl succinic anhydride

DIECA - sodium diethyldithio-carbamate

DMP - 2,4,6-tri dimethyl-aurinomethyl phenol

DNA - deoxyribonucleic acid

ds - double-stranded

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

EM - electron microscopy

FITC - fluorescein iso thio cyanate

g (mg, *u*g) - gram (milligram, microgram)

g - gravity

h - hour

$HAuCL_4$ - hydrochloroauric acid

HCl - hydrochloric acid

H_2O - water

IgG - immunoglobulin G

ISEM - immunosorbent electron microscopy

K - potassium

kDa (Da) - kilodalton (dalton)

l (ml, *u*l) - liter (milliliter, microliter)

LiCl - lithium chloride

M (mM, *u*M) - molar (millimolar, micromolar)

$MgCl_2$ - magnesium chloride

min - minute

N - nitrogen

NaAc - sodium acetate

NaCl - sodium chloride

NaOH - sodium hydroxide

NBT - nitroblue tetrazolium

NH_4Cl - ammonium chloride

NMA - nadic methyl anhydride

ORF - open reading frame

OsO_4 - osmium tetroxide

P - phosphorus

PBS - phosphate-buffered saline (127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4)

PEG - polyethylene glycol

PLRV - potato leaf roll virus

PVP - polyvinylpyrrolidone

PVX - potato virus X

PVY - potato virus Y

RCMV - red clover mottle virus

s - second

RNA - ribonucleic acid

SBMV - southern bean mosaic virus

SBMV-B - bean strain of southern bean mosaic virus

SBMV-C - cowpea strain of southern bean mosaic virus

SDS - sodium dodecyl sulfate

SHMV - sunn-hemp mosaic virus

ss - single-stranded

SSC - a buffer containing 0.15 M sodium chloride and 0.015 M trisodium citrate

TE - a buffer containing 10 mM Tris-HCl pH 7.5 and 1 mM EDTA

TMV - tobacco mosaic virus

TMV-L - TMV strain which induces hypersensitive reactions

TNE - a buffer containing 10 mM Tris-HCL pH 7.5, 10 mM NaCl and 1 mM EDTA

TomRsV - tomato ringspot virus

TRV - tobacco rattle virus

UA - uranyl acetate

UV - ultraviolet

v - volume

vRNP - virus-specific ribonucleoprotein

Acknowledgements

I wish to thank my supervisor, Dr. R.I. Hamilton for his guidance and patience during the course of this work. I also wish to express my appreciation and admiration for him as a scientist who not only cares about, but also works towards, solving plant pathology problems in the developing countries.

I thank the members of my supervisory committee, Dr. R.J. Copeman, Dr. C.J. Douglas, Dr. D.M. Rochon, Dr. V.C. Runeckles and Dr. M. Weintraub, for their help and valuable advice throughout my work on this project.

I am indebted also to Dr. M. Weintraub, former Director, Vancouver Research Station, Dr. D.L. Struble, Director, and the Research Station staff for providing the facilities for this work. Special thanks to Dr. Fran Leggett for her help, excellent advice and patience in the electron microscopy lab.

I thank M. Weis and Dr. L. Samuels for their assistance in the electron microscopy lab at the University of British Columbia.

I thank my husband John; my children Irene, Lorena and Alexander and my mother for their understanding and support during the writing of this thesis.

Chapter 1

Introduction

1.1. General Introduction

When a plant is inoculated with a virus, its ability to establish an infection depends upon two processes: 1) the capacity of its genome to replicate in the cells of the inoculated plant and 2) the movement of the virus from cell to cell and to other parts of the plant. The capacity of a virus to perform these functions in a particular plant species depends on the genome of both plant and virus (Atabekov and Dorokhov, 1984; Zaitlin and Hull, 1987). Thus, both virus and host have an active role in determining the course of the infection process.

Responses by plants to inoculation with a virus have been categorized by Matthews (1991) as immune and infectible. In an immune plant, the virus cannot replicate in protoplasts or in cells of the intact plant and no virus progeny are produced. An infectible plant is one in which the virus can infect and replicate in protoplasts. Several kinds of infectible plants or hosts have been defined: 1) resistant: virus multiplication is limited to initially infected cells, resulting in a subliminal infection; 2) hypersensitive: infection is limited to an area of cells around the initially infected cells, usually with the formation of

visible necrotic local lesions; and 3) susceptible: virus replicates and moves systemically. The host can be sensitive, i.e., exhibit more or less severe symptoms of disease, or tolerant, when there is little or no apparent effect on the plant (Matthews, 1991).

The basis for the different responses of plants to viruses is not yet fully understood. There are few examples in which plants are a source of resistant protoplasts (immune plants). Beier et al. (1977, 1979) found that out of 50 lines of cowpea that were operationally immune to cowpea mosaic virus (CPMV), "Arlington" was the only one that was a source of resistant protoplasts. The resistance of "Arlington" protoplasts is thought to be mediated by an inhibitor of the proteolytic processing of CPMV polyproteins (Ponz and Bruening, 1986). This kind of extreme resistance seems to be an exception, most plant cells being capable of replicating essentially any plant virus (van Loon, 1983).

Three types of response have been defined above for infectible hosts. In the case of a subliminal infection, virus replication in the initially infected cells is not blocked, yet the virus cannot move beyond these cells. This type of response could be due to the lack of a functional transport factor that would allow the virus to move away from the initially infected cells, and/or to an antiviral defence reaction operating in the plant tissue but not in protoplasts or individual cells (Atabekov and Dorokhov, 1984). Limitation of viral spread also occurs in the

hypersensitive host reaction, which is usually accompanied by the development of a necrotic lesion, localized at the site of virus infection.

The hypersensitive host reaction is a generalized response to different pathogens and is thought to depend on the interaction between a host resistance gene and a pathogen avirulence gene (Flor, 1971). Most of the genetic studies on viral-induced hypersensitivity have been done with tobacco mosaic virus (TMV) interacting with plants in the genus *Nicotiana*. The N' gene in *N. sylvestris* has been identified as the gene controlling the hypersensitive response against most tobamoviruses, except the common strain (reviewed by van Loon, 1987; Culver et al., 1991). Coat protein substitutions and alterations in nucleotide sequence in the coat-protein open reading frame of the hypersensitive reaction-inducing TMV-L strain, demonstrated that coat protein gene sequences are involved in the induction of the N' gene (Saito et al., 1987; Knorr and Dawson, 1988). However, the N gene of *N. glutinosa,* which controls the hypersensitive reaction against almost all strains of TMV, is not induced by the coat protein (Dawson et al., 1988), while the Tm-2 and TM-2^2 hypersensitive resistance factors in tomato seem to be triggered by the TMV 30 kDa movement protein (Meshi et al., 1989).

Recognition of infecting viruses by plants does not involve a single viral component, as demonstrated by the results of studies on induction of the hypersensitive response. Thus, plants seem to have evolved to recognize any

viral-specific product presented to them (Culver et al., 1991). Viral genomes, on the other hand, are completely dependent on the host's replicating machinery for successful replication and must specifically interact with host macromolecules to move into other cells. A direct implication of this phenomenon is that evolution of viral genomes is closely tied to the evolution of the genome of the host plant.

Most of the recent molecular studies undertaken to understand the interactions between plant viruses and their hosts have focused on the viruses, given the relative simplicity of their genomes. After infection and replication in the initially infected cells, efficient establishment of a viral infection in a plant depends on the virus spreading systemically. Viral factors responsible for translocation have been identified for several viruses and some models on the interaction of these factors with host factors have arisen.

Plant viruses move in their hosts by slow, cell-to-cell transport within the parenchyma cells (i.e., short distance movement) and by rapid spread through the conducting system (i.e., long distance movement) (reviewed by Atabekov and Dorokhov, 1984; Zaitlin and Hull, 1987; Hull, 1989). Cell-to-cell spread is believed to occur through plasmodesmata, which are plasma membrane-lined channels that traverse plant cell walls, thus connecting adjacent cells (reviewed by Robards and Lucas, 1990). Long-distance movement, on the other hand, occurs in either the phloem or xylem (reviewed by Matthews, 1991).

In some cases, whole virions have been seen in the plasmodesmata (Esau et al., 1967; Allison and Shalla, 1974; Weintraub et al., 1976;), yet proper encapsidation does not seem to be a requirement for cell-to-cell movement of all plant viruses. Examples of cell-to-cell movement of viruses after deletions or point mutations in the capsid protein are tobacco mosaic virus (TMV) (Dorokhov et al, 1983, Takamatsu et al., 1987, Dawson et al, 1988), tomato golden mosaic virus (Gardiner et al., 1988), turnip crinkle virus (Hacker et al., 1992) and cucumber necrosis virus (M.A. McLean, personal communication).

Thus, there is no uniform evidence to suggest that all viruses move across plasmodesmata as one entity, be it virus particles, virus nucleic acid or virus-specific ribonucleoprotein (vRNP). The same is true for long distance movement through the conducting elements. In fact, with some viruses it appears that local and long distance spread have different requirements for coat protein. Using mutants with modifications in the coat protein gene or in the origin of assembly on the genomic RNA of TMV, Saito et al. (1990) showed that both coat protein, with a capacity to assemble into virus particles, and the assembly origin in the genomic RNA were involved in efficient long-distance movement. However, coat protein is entirely dispensable for systemic movement of some viruses, for example, barley stripe mosaic virus (BSMV) (Petty and Jackson, 1990), and tomato golden mosaic virus (Gardiner et al, 1988).

These differences in the requirements for coat proteins could indicate differences in the mechanisms and principles controlling viral movement, not only in terms of cell-to-cell and long distance movement, but also among different viruses. In spite of this, understanding of the mechanisms involved in viral cell-to-cell movement was greatly increased after it was established that a specific virus-encoded protein, the 30-KDa product of TMV, was required for cell-to-cell transport of that virus (Nishiguchi et al., 1978; Leonard and Zaitlin, 1982; Meshi and Okada, 1987; Deom et al., 1987).

Several studies, in which the migration of fluorescent molecules of defined sizes between cells was recorded, showed that plasmodesmata have a molecular size exclusion limit or "gating" capacity of approximately 800 Da (Madore et al., 1986, Wolf et al., 1989). Since mature virions and viral genomes are considerably greater in size than the molecular exclusion limit of plasmodesmata (Gibbs, 1976), it is generally accepted that viruses must modify plasmodesmata to migrate into adjacent cells. The 30-KDa TMV movement protein has been localized within plasmodesmatal connections by immunogold cytochemical studies (Tomenius et al., 1987). It has also been shown to modify the size-exclusion limits of plasmodesmata in leaf tissue of transgenic tobacco plants transformed with the 30-KDa movement protein gene and expressing the gene product (Wolf et al., 1989; Deom et al., 1990,1991). In such plants, plasmodesmata allowed the movement of fluorescein isothiocyanate-labelled

dextrans with average molecular masses of 9.4 KDa (approximately ten times as large as those which moved between control cells) (Wolf et al., 1989).

Recently, the movement protein of TMV was identified as a single stranded (ss) nucleic acid-binding protein (Citovsky et al., 1990). Based on this property, the TMV movement protein-RNA complex has been suggested as an intermediate in cell-to-cell movement (Citovsky and Zambryski, 1991). According to this model, only RNA molecules bound to the movement protein can be transported through the plasmodesmata. The movement protein would have two functions; it would act on the plasmodesmata and increase their permeability, and it would "unfold" the RNA and shape it into a transferable form (Citovsky et al., 1990). The nature of the interaction of the movement protein-nucleic acid complex with the plasmodesmatal subunits is unknown, but by analogy with gap junctions in animals, could involve cAMP-dependent phosphorylation of the plasmodesmatal subunits. This would be supported by the finding that cell-to-cell spread of TMV is dependent on intracellular levels of cAMP (Atabekov and Taliansky, 1990). After modification of the plasmodesmatal channels, the entire movement protein-nucleic acid complex or the nucleic acid alone would be translocated across the plasmodesmatal channel (Citovsky and Zambryski, 1991).

Regardless of the mechanism underlying cell-to-cell movement, there is evidence that in many cases the capacity of a given virus to be transported in

a plant determines whether that plant is a potential host for the virus (Taliansky et al., 1982a). Thus, although a virus may be able to replicate in the initially infected cells of a plant, if it cannot be transported beyond these, the plant can be regarded as resistant to that virus (Matthews, 1991). In certain cases, resistance of a plant to a virus can be overcome by co-infecting that plant with another virus. One virus, the "helper", normally replicates and spreads in the host when inoculated alone; the other, the "dependent" virus, may replicate in the inoculated cells but does not spread unless the helper virus is present.

Complementation of virus spread has been described for both related and unrelated viruses (for review, see Atabekov and Taliansky, 1990). For example, members of the tobamovirus group in different combinations and in different plants complemented one another's systemic spread (Malyshenko et al., 1989). Spread of phloem-limited viruses, such as potato leaf roll virus (PLRV), a luteovirus, into mesophyll cells, was brought about by mixed infection with the potato virus X potexvirus (PVX) (Atabekov et al., 1984) or the potato virus Y potyvirus (PVY) (Barker, 1987). This phenomenon is not surprising if most viruses code for movement proteins which operate in a similar fashion to alter plasmodesmata and facilitate viral movement. However, complementation does not occur with all viral combinations, nor does it occur for the same virus combinations in all hosts. For example, complementation of PLRV movement by PVY is effective in *N. clevelandii* but not in potato; no complementation was

observed between tobacco rattle virus (TRV) and barley stripe mosaic virus (BSMV) in tobacco or BSMV and alfalfa mosaic virus (ALMV) in wheat (Malyshenko et al., 1989).

Thus far there is no clear functional or structural classification of movement proteins that explains and predicts transport function complementation. Several mechanisms to explain the occurrence of complementation, which are not mutually exclusive, include: 1) non-homologous movement proteins coded by unrelated viruses which function in a similar fashion, yet the degree to which the modifications of the plasmodesmata can be utilized depends upon the timing and location of the "dependent" virus in relation to the replication and spread of the "helper" (Maule, 1991); 2) since complementation seems to be rather host-specific, it is possible that the host genotype can influence and modify the phenomenon of complementation of transport function (Barker, 1987; Atabekov and Taliansky, 1990); and 3) movement proteins of different viruses (or of taxonomic groups of viruses), use different mechanisms, involving other viral products and/or plant factors, for the expression of the transport function (Atabekov and Taliansky, 1990). In the third model, in order for complementation to occur, "dependent" virus-specific products should be compatible with the plant and/or to the "helper" movement protein.

One mechanism for transport which is compatible with the first

possibility is that proposed by Citovsky and Zambrysky (1991). Movement proteins bind ss-nucleic acids in a non-specific manner (Citovsky et al., 1990, 1991). The occurrence of complementation between two viruses would depend on whether the RNA of the "dependent" virus is in sufficient quantity and in close proximity to the movement protein of the "helper". If these two conditions are met, the movement protein could then bind the "dependent" RNA and effect its transport, thus allowing it to move beyond the initially infected cells. If the "dependent" viral RNA does not meet these requirements, however, the movement protein would not bind to the RNA and complementation would not occur.

The second model is supported by the fact that complementation of movement between certain viruses occurs in some hosts and not others. An example, mentioned above, is the helper effect induced by PVY that effects PLRV movement into non-phloem tissue in *N. clevelandii* but not in potato (Barker, 1987).

Finally, the third model is supported by recent findings on the requirements for complementation of red clover mottle virus (RCMV) by TMV (Taliansky et al, 1992). Previously it was shown that tobamoviruses enabled the transport of RCMV in tobacco plants normally resistant to RCMV (Malyshenko et al., 1988). However, RCMV transport does not take place in transgenic tobacco plants that produce the movement protein of TMV, whereas the transport of TMV

Ls1 mutant (a temperature sensitive mutant in cell-to-cell movement) is complemented in these plants. Nevertheless, complementation of RCMV transport does occur when the transgenic plants are infected with both RCMV and TMV Ls1 at the non-permissive temperature (33 C) (Taliansky et al., 1992). From these results, the authors suggest that the presence of the full-length TMV genome or a certain TMV-encoded product(s) in addition to the 30-KDa movement protein is necessary for complementation of the RCMV transport function.

Understanding how plant and viral genes control viral movement is necessary if control of such movement could effect management of virus disease. One approach , taken in this study, is to examine the course of events which occur in a mixed infection in which one virus acts as a "helper" and another as a "dependent".

In previous studies, Molefe et al (1983), showed that bean (*Phaseolus vulgaris* L. "Pinto"), singly inoculated with SBMV-C sustained only subliminal viral replication in inoculated primary leaves; no symptoms developed and very low amounts of virus were recovered. However, plants co-infected with SHMV, which infects bean systemically, showed local lesions in the inoculated primary leaves due to the replication of SBMV-C. Furthermore, SBMV-C replicated to levels detectable by ELISA and bioassay in the inoculated primary leaves, but not in the trifoliate leaves, of three other bean cultivars co-infected with SHMV

(Fuentes and Hamilton, 1988, 1991).

The "helper" virus, SHMV, is a member of the tobamovirus group and it occurs naturally in leguminous plants in several continents (Kassanis and Varma, 1975; Varma, 1985). The virus is rod-shaped; both full length (300 nm long and 17 nm wide), and shorter particles are produced in infected plants (Whitfeld and Higgins, 1976). Full length particles contain infectious single-stranded RNA of molecular weight 2 X 10^6 and the shorter particles contain subgenomic RNAs which serve as templates for the viral coat and movement proteins. SHMV has also been reported to complement the transport function of brome mosaic virus (BMV) in "Pinto" beans, which BMV normally does not infect (Taliansky et al., 1982b) and of the RNA of the B component of RCMV, (which is not transported unless it is co-inoculated with the M component) in *V. unguiculata* (Malyshenko et al, 1988).

The "dependent" virus, SBMV-C, is one of four strains described for SBMV. The cowpea and bean strains of SBMV share similar chemical and physical properties (Ghabrial et al., 1967), but they differ in their host ranges. SBMV-B infects most common bean cultivars but does not infect cowpea, whereas SBMV-C is restricted to *Vigna* cultivars but cannot infect bean (Tremaine and Hamilton, 1983). SBMV is an icosahedral virus, 30 nm in diameter, its capsid consisting of 180 polypeptides arranged with T=3 icosahedral quasi-symmetry

surrounding the single-stranded viral RNA (Abad-Zapatero et al., 1980). Sequence comparison of 400 bases from the 3' end of both SBMV-B and SBMV-C showed very little homology in the non-coding region yet extensive homology in the coding region (Mang et al., 1982).

Both the genomes of SHMV and SBMV have been sequenced. The complete nucleotide sequence of the transport gene of SHMV has been determined and the amino acid sequence of its 30-kDa protein is known (Meshi et al., 1982). On the basis of its similarity with other putative viral movement proteins, SHMV movement protein has been grouped into a "transport" (movement) group together with other tobamoviruses and with tobra-, caulimo-, nepo-, como-, and potyviruses (Atabekov and Taliansky, 1990). For SBMV, however, no transport gene has been definitively described, even though the complete nucleotide sequence of its genome has been determined (Wu et al., 1987).

In this study, host and viral responses were compared in single and double virus infections. Inoculation of isolated bean protoplasts with SBMV-C, SBMV-B and SBMV-C plus SHMV showed that SBMV-C is capable of replicating in isolated bean cells to the same extent as SBMV-B, and that its replication is not affected by the presence of SHMV. These results would indicate that SHMV facilitates the spread of SBMV-C in bean, rather than its replication.

The "helper effect" of SHMV on SBMV-C movement in mixed infections of bean was shown to be limited to short distance (cell-to-cell) movement in the inoculated primary leaves, with no virus (SBMV-C) moving into the vascular system. Relatively few SBMV-C virions were found in doubly infected parenchyma cells, yet amorphous masses of what appeared to be SBMV-C viral coat protein were found in these cells, probably indicating a lack of proper assembly of SBMV-C coat protein in bean, which might be associated with the lack of systemic movement of the virus.

1.2 Objectives

In spite of many recent advances that have taken place in plant viral research, the mechanism by which the transport gene(s) facilitates cell-to-cell and long distance movement in plants is still unknown. Mixed infections with a "helper" and a "dependent" virus, allow comparisons to be made between single infections of either virus, thus providing an insight into the host-virus interaction. This study was undertaken to determine the effects of a mixed infection in which one virus, SHMV, acted as a "helper", and another, SBMV-C, as a "dependent" in bean, a host of SHMV but not of SBMV-C

The objectives of this research were to:

1. Compare the general pattern of symptom development and antigen accumulation in single infections with SBMV-C and mixed infections with SBMV-C and SHMV in four bean cultivars.

2. Determine whether the increase in the accumulation of SBMV-C in bean co-inoculated with SHMV is due to complementation of a replicative or a movement function.

3. Establish the pattern of accumulation, throughout the plant, of SBMV-C and SBMV-B in mixed infections with SHMV.

4. Study the distribution of SBMV-C in cells of doubly infected bean tissue, and compare and determine differences with that of SBMV-B in bean and that of SBMV-C in cowpea.

Chapter 2

Biological characterization of the interaction between SBMV-C and SHMV

2.1 Introduction

Visible or otherwise detectable abnormalities in plants, termed symptoms of a disease, can be caused by viruses. When viruses cause symptoms which result in a significant deviation from normal growth of a crop plant, they become economically important. In mixed infections, viruses may interact to produce a disease which is not caused by either virus on its own, or may increase the severity of the symptoms (Reviewed by Matthews, 1991).

In this chapter, symptom development during single and mixed infections with SHMV and SBMV is described for four bean cultivars. The effect of SHMV on the amount of SBMV produced in these cultivars was monitored since, in many instances, the helper effect of a virus will be reflected in an increase in the amount of the "dependent" virus (for examples see: Dodds and Hamilton, 1972; Hamilton and Nichols, 1977; Goodman and Ross, 1974; Ishimoto et al., 1990).

Other factors such as time of inoculation, increase in temperature of

incubation and the effect of multiple passage on the interaction between SBMV-C and SHMV were also studied. Finally, the influence of other viruses on the replication of SBMV-C in bean was determined.

2.2 Materials and methods

2.2.1 Virus propagation and purification

The different virus isolates used were obtained from the dried, infected tissue collection of the Agriculture Canada Research Station, Vancouver. SBMV-B and SBMV-C were maintained in *Phaseolus vulgaris* "Bountiful" and *Vigna unguiculata* L., respectively, grown under greenhouse conditions at 20-26 C. Both virus strains were purified from infected leaf tissue 12-18 days after inoculation according to established protocols (Tremaine et al., 1981). Tissue was sprinkled with sodium diethyldithiocarbamate (quantity corresponding to 0.02 M in final extraction volume) and then ground in 0.2 M sodium acetate buffer (pH 5.0) with 0.1% mercaptoethanol. The pulp was sieved through cheese cloth, the extract was adjusted to pH 4.8 by addition of 10% acetic acid, left at 5° C for 4 hours and then clarified by centrifugation at 12,000 x *g* for 15 min. Polyethylene glycol (PEG) and NaCl were added to the supernatant to give 8% (w/v) and 0.01 M respectively, and the mixture was stirred for 1 hr at 4° C. The precipitate was recovered by centrifugation at 12,000 x *g* for 15 min. and the pellet was

resuspended in 0.1 M sodium acetate buffer pH 5. The precipitation procedure was repeated, after which virus was further purified in 10-40% linear sucrose density gradients, prepared by the method of Davis and Pearson (1978), by centrifugation in a Beckman SW 40 rotor at 26,000 x *g* for 3.5 hrs at 4 C. The virus, which appeared as an opalescent band near the centre of each gradient, was recovered with a needle and syringe and dialysed extensively against 0.1 M sodium phosphate buffer pH 7. Virus concentration was determined spectrophotometrically (E= 5.85 [mg/ml]$^{-1}$ cm^{-1} at 260 nm).

SHMV was propagated in *P. vulgaris* "Bountiful" and purified by the method of Kassanis and Varma (1975). Leaves were harvested 15-18 days after inoculation and ground in a blender with 2 volumes of 0.2 M K phosphate buffer pH 5.2. Sap was squeezed through cheese cloth and concentrated by two or three cycles of differential centrifugation (10 min at 10,000 X *g*; 1.5 h at 100,000 X *g*) and the pellets were resuspended in water or 0.01 phosphate buffer pH 7.0. Virus concentration was determined spectrophotometrically (E= 3.2 [mg/ml]$^{-1}$ cm^{-1} at 260 nm).

Other viruses that infect bean systemically were tested for their effect on the replication of SBMV-C in bean. SBMV-C was paired with alfalfa mosaic virus (AlMV), bean yellow mosaic potyvirus (BYMV), tomato ringspot nepovirus (ToRsV), bean golden mosaic geminivirus (BGMV) and bean

rugose mosaic comovirus (BRMV), and propagated in "Bountiful". Infected tissue was harvested and kept at -70° C until needed.

2.2.2 Virus inoculation

Fully expanded, Carborundum-dusted primary leaves of four cultivars of *P. vulgaris* ("Bountiful", "Pinto", "Top Crop" and "Tendergreen") were mechanically inoculated (foam pad) with purified SBMV-C or SHMV (0.1 mg/ml) in 0.01 phosphate buffer (pH 7.0), or with a 1:1 mixture of both virus preparations (SBMV-C/SHMV both at 0.1 mg/ml). To test the effect of other viruses on the replication of SBMV-C, infected leaf tissue was homogenized in 0.01 M phosphate buffer (pH 7.0) and primary leaves were inoculated with each virus alone or together with SBMV-C.

2.2.3 Collection and storage of samples

Inoculated primary leaves and trifoliate leaves from 4 to 6 different plants were harvested at different times post-inoculation and washed under running tap water for 2-4 h in order to remove any viral inoculum from the leaf surface. Leaves were dried, weighed and stored in plastic bags at -70° C until tested.

2.2.4 Bioassay

Presence of SBMV-C infectivity was determined by bioassay on a cowpea local lesion host (*V. unguiculata* 399419 or Georgia 21). Samples were ground in 0.01 M potassium phosphate buffer pH 7.0 and the homogenate was rubbed onto primary leaves of the cowpea plants. Local lesions appearing on leaves were counted 5-7 days post-inoculation.

A systemic host was used to assess the presence of the virus in SBMV-C singly inoculated bean leaves because of the minimal amount of local lesions produced in local lesion hosts. Primary bean leaves were harvested 7 and 15 days after inoculation with SBMV-C and tissue homogenates were rubbed onto a cowpea cultivar which supported systemic infection of SBMV-C.

2.2.5 Production of antibodies and detection of antigen by ELISA

The amount of SBMV-C antigen was determined by the double antibody sandwich enzyme-linked immunoassay (DAS-ELISA) (Clark and Adams, 1977). Antibodies used for ELISA were raised in rabbits following five weekly intramuscular injections, 1 mg each, of purified SBMV-C. Rabbit blood was collected when titres reached 1:512 (rabbit H2) and 1:1024 (rabbit H1). Immunoglobulins were precipitated from antisera with ammonium sulphate and

subsequently purified by DEAE-cellulose column chromatography as described by Clark, Lister and Bar-Joseph (1986). Concentration of immunoglobulins was adjusted to 1 mg/ml (A^{280}= 1.4) and stored at -20° C.

Flat-bottomed, 96-well, Linbro microtiter plates (Flow Laboratories, Mississauga, Ontario) were used for ELISA tests. Wells were coated overnight at 4 C with SBMV antibodies by the addition to each well of 200 µl of polyclonal anti-SBMV immunoglobulin diluted to 1 µg/ml in phosphate-buffered saline (PBS)(127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4). They were then blocked with 0.2% BLOTTO (Johnson et al., 1984) in PBS (BLOTTO is 10 g of nonfat, dried milk made up to 100 ml with distilled water and 0.02% sodium azide as a preservative), for 30 min at room temperature. Leaf tissue from inoculated and healthy plants was ground with a mortar and pestle in 1 ml sample buffer (0.05% Tween 20, 2% polyvinylpyrrolidone [PVP] and 0.2% ovalbumin in PBS)/0.1 g of leaf tissue, and 200 µl samples were added to each well. The plates were incubated for 1 h at 37 C and then washed with tap water three times. SBMV-immunoglobulin-enzyme conjugate was diluted in freshly prepared sample buffer (200 µl conjugate/20 ml buffer), 200 µl was added to each well and plate was incubated for 45 min at 37 C. Plates were washed as above, substrate (p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml in 10% diethanolamine pH 9.8 was added to the wells and the plates were incubated for 1 h at 37 C. The absorbance of each well was recorded at 405 nm

(A_{405}) in a Titertek Multiscan MCC plate reader (Flow Laboratories).

2.2.6 Temperature study

The effect of temperature on symptom development and virus concentration was determined by inoculating "Pinto" and "Bountiful" primary leaves with a mixture of SBMV-C and SHMV and incubating the plants at 32° C. Symptom development was recorded at 7, 14 and 21 days post-inoculation. Samples were collected and assessed for virus as described above.

2.2.7 Multiple passage study

Twelve plants each of "Bountiful" and "Pinto" were inoculated with a mixture of SBMV-C and SHMV as described in section 2.2.2. After 15-20 days, primary leaves were homogenized and re-inoculated to another set of plants. This procedure was repeated 15 X; the experiment lasted from April 28 1988 until April 7, 1989. SBMV-C accumulation and infectivity on cowpea was tested every three passages. After the seventh passage, SBMV-C was purified on a sucrose gradient (section 2.2.1) and its infectivity was tested on "Pinto".

2.3 Results

2.3.1 Symptoms in singly and doubly inoculated plants

Symptoms in primary and trifoliate leaves of four bean cultivars ("Pinto", "Bountiful", "Top Crop" and "Tendergreen") were recorded 10, 15 and 20 days after inoculation with SBMV-C alone, SHMV alone and a mixture of the two viruses.

None of the cultivars, singly inoculated with SBMV-C, presented symptoms (Fig. 1A, B). Primary and trifoliate leaves had the same appearance and developed at the same rate as those in mock-inoculated plants. Symptoms of SBMV-C/SHMV mixed infections varied between the seasons in three of the four cultivars ("Bountiful", "Tendergreen" and "Top Crop"), yet were qualitatively similar to those in plants inoculated with SHMV alone. During the months of June, July and August, primary leaves infected with both viruses showed severe symptoms (Fig. 2A) while primary leaves inoculated with SHMV alone showed no symptoms or very slight chlorotic symptoms (Fig. 2B). During the rest of the year, primary leaves of SBMV-C/SHMV- and SHMV-inoculated plants presented chlorosis and ring-like lesions extending from the mid-rib outwards, while trifoliate leaves developed a rugose yellow and dark green mosaic (Fig. 3). The relative severity of symptoms in doubly inoculated plants was not correlated with variations in SBMV-C concentration.

Fig. 1. "Pinto" bean singly inoculated with SBMV-C. A) Inoculated primary leaf showing no visible symptoms 7 days post-inoculation; and B) Whole plant presenting no symptoms 20 days post-inoculation. The same results were obtained with "Bountiful", "Top Crop" and "Tendergreen".

A

B

Fig. 1

Fig. 2. Symptoms, during the months of June, July and August, in "Bountiful" primary leaves inoculated with: A) Mixture of SBMV-C and SHMV; and B) SHMV alone. Doubly-infected leaves presented severe symtoms, while singly-infected leaves presented only slight chlorosis or no visible symptoms.

A

B

Fig. 2

Fig. 3. "Bountiful" plant, doubly-inoculated with SHMV and SBMV-C. Primary leaves presented chlorosis and ring-like lesions while trifoliate leaves developed yellow and dark green mosaic and blistering.

In "Pinto", mixed infections resulted in pinpoint local lesions in the inoculated primary leaves (Fig. 4A) which were not induced upon single infection with either virus (not shown) but were similar, although smaller, to those produced by single infection with SBMV-B (Fig. 4B)

2.3.2 SBMV-C accumulation in singly and doubly inoculated plants

Upon inoculation with SBMV-C, the four bean cultivars proved to be non-permissive hosts, allowing only subliminal replication in inoculated primary leaves. Replication at this level was not detected on the local lesion host *V. unguiculata* Georgia 21, and A_{405} values in ELISA were in the range of values found for mock-inoculated tissue. Sap from leaves of the four cultivars inoculated with SBMV-C was infective in a systemic host for the virus.

When these same bean cultivars were inoculated with a mixture of SHMV and SBMV-C, SBMV-C was readily detected in the inoculated primary leaves by bioassay (Fig. 5) and by ELISA (Fig. 6). Homogenates of "Bountiful" primary leaves showed the greatest infectivity followed by "Tendergreen" and "Top Crop", while "Pinto" showed the lowest. These results were correlated with the amount of SBMV-C capsid protein detected by ELISA in the four different cultivars. Low infectivity and ELISA values found for "Pinto"

Fig. 4. Symptoms in "Pinto" primary leaf inoculated with: A) SHMV and SBMV-C; and B) SBMV-B. Small necrotic local lesions developed in the mixed infection (A), while similar, but larger, necrotic local lesions, which later turned light brown, developed in "Pinto" primary leaves inoculated with SBMV-B.

A

B

Fig. 4

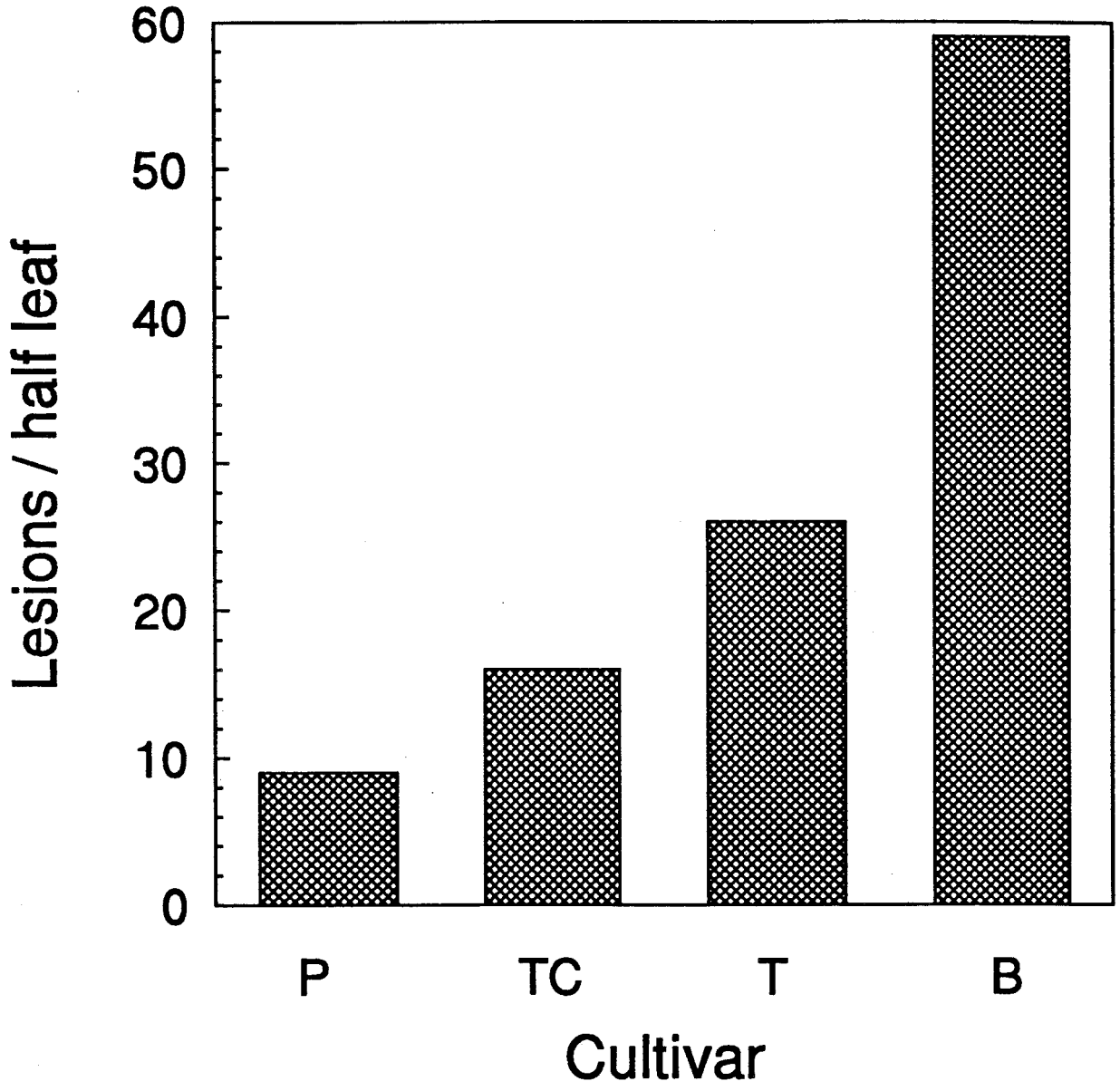


Fig. 5. Detection of SBMV-C by bioassay on cowpea "Georgia 21" in primary leaves of different bean cultivars doubly inoculated with SHMV and SBMV-C. (P, "Pinto"; TC, "Top Crop"; T, "Tendergreen"; B, "Bountiful").

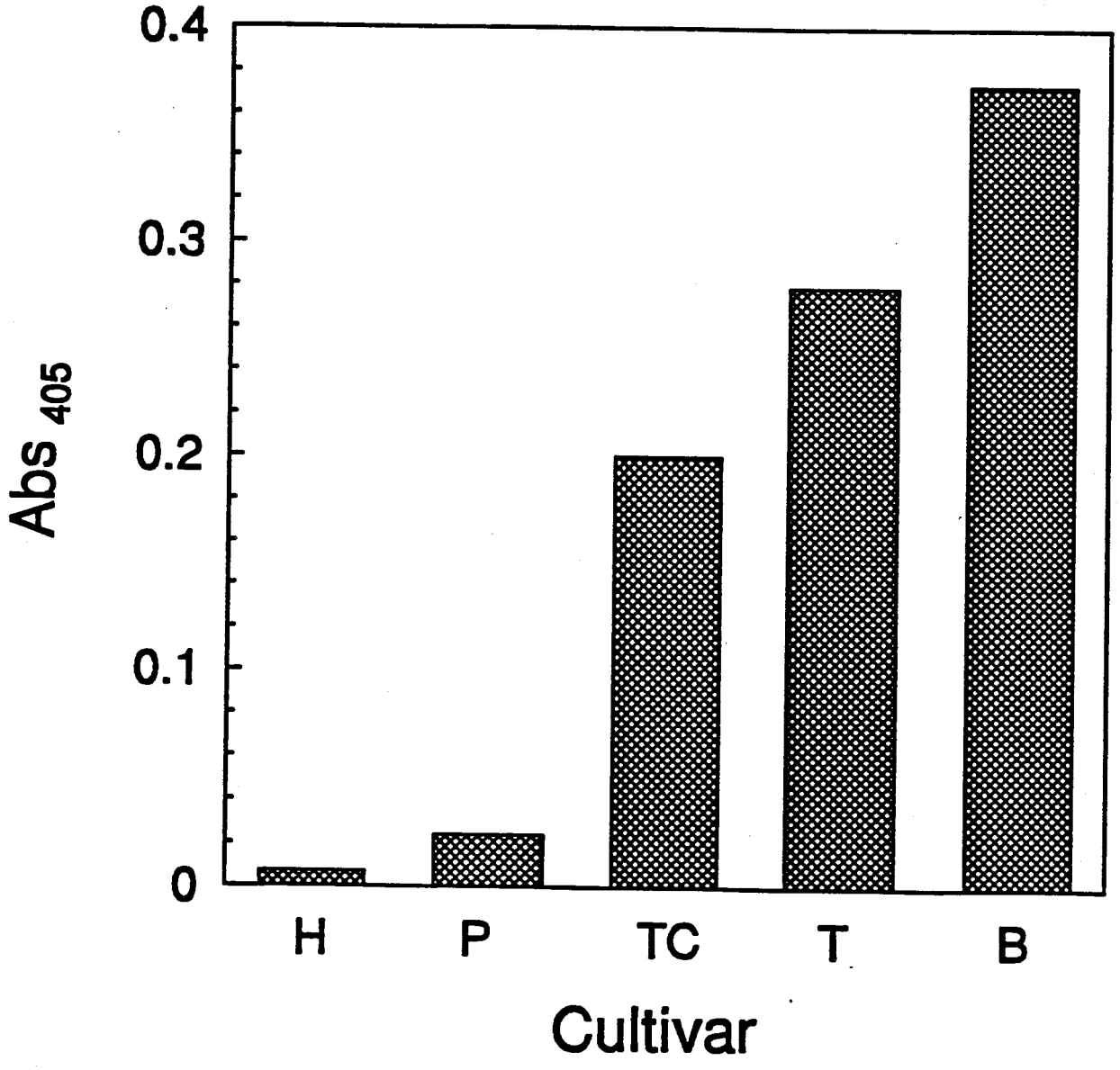


Fig. 6. Detection of SBMV-C antigen, by ELISA, in primary leaves of four different bean cultivars doubly-inoculated with SBMV-C and SHMV. (H, Healthy; P, "Pinto"; TC, "Top Crop"; T, "Tendergreen"; B, "Bountiful".)

can be explained by the fact that the virus is localized in necrotic local lesions.

Trifoliate leaves of the four doubly inoculated cultivars were harvested at different intervals up to 60 days after inoculation and tested for the presence of SBMV-C by bioassay (on both systemic and local lesion hosts) and ELISA. No virus was detected in the trifoliate leaves of any of the cultivars at any time.

The effect of SHMV on the accumulation of SBMV-B was tested in cowpea but there was no measurable increase in the accumulation of the latter, therefore the helper effect was not reciprocal in cowpea.

2.3.3 Effect of sequential inoculation

Replication of SBMV-C in primary leaves of the four bean cultivars was detected when SBMV-C was inoculated up to 72 h after SHMV inoculation. When SBMV-C was rubbed onto primary leaves prior to SHMV, its replication could be detected when the interval between inoculations was shorter than 24 h.

No SBMV-C replication could be detected after inoculation of each half of a primary leaf with the individual viruses, nor was SBMV-C detected

in any part of the plants after simultaneous or sequential inoculation of trifoliate leaves with SBMV-C and SHMV.

2.3.4 Effect of temperature shifts

Two of the four cultivars described above ("Bountiful" and "Pinto") were inoculated and maintained at 32 C. Results of several experiments demonstrated that the amount of SBMV-C antigen, measured by ELISA, in primary leaves was greater and symptoms were more severe although qualitatively similar. Despite these results, SBMV-C was not found in trifoliate leaves of these plants when tested by bioassay and ELISA.

2.3.5 Effect of multiple passage

After sequential passage of SBMV-C and SHMV mixed infections through 15 cycles of "Bountiful" plants, SBMV-C was virtually lost. The decline in SBMV-C was gradual and was accompanied by an increase of the severity in symptoms produced by SHMV on the trifoliates. After seven passages, the viruses were purified from the primary leaves of "Bountiful", yet no SBMV-C was recovered from the sucrose gradient, although the initial homogenate was infectious on a local lesion host for SBMV-C. When fractions from the sucrose gradient were tested on Pinto, samples from one visible band, found in the

position expected for SHMV, induced symptoms typical of those of SHMV in single infection.

2.3.6 Effect of other viruses that replicate in bean on the replication of SBMV-C

Five viruses from different taxonomic groups were used to test their effect on the replication of SBMV-C. The common characteristic shared by all five viruses was their ability to infect bean systemically. Results of these mixed infections are presented in Table I. Bean yellow mosaic virus (a potyvirus) and bean rugose mosaic virus (a comovirus) both acted as helpers for SBMV-C.

Trifoliates of SBMV-C/BYMV- and SBMV-C/BRMV-inoculated plants were also tested by infectivity assay on cowpea. No local lesions were produced, indicating that the helper effect of these two viruses was similar to that of SHMV in that it was limited to the inoculated primary leaves.

Table I. Effect on the replication of SBMV-C by other viruses that infect bean systemically

SBMV +	Symptoms	SBMV-C infection[a]	Helper
AlMV[b]	same as single[c]	none	no
BYMV	same as single	30 lesions	yes
TomRsV	same as single	none	no
BGMV	same as single	1 +/- 0.5 lesions	no
BRMV	same as single	50 lesions	yes

[a] Infectivity was assayed on half leaves of *V.unguiculata* 399419, a local lesion host for SBMV-C.
[b] AlMV, alfalfa mosaic virus; BYMV, bean yellow mosaic virus; ToRsV, tomato ringspot virus; BGMV, bean golden mosaic virus; BRMV, bean rugose mosaic virus.
[c] Symptoms were indistinguishable from those of single infection by the tested virus.

Chapter 3

Replication of viruses in singly and doubly inoculated bean protoplasts

3.1 Introduction

The introduction of techniques for the inoculation of plant protoplasts with viruses (Cocking, 1966; Takebe and Otsuki, 1969) has allowed the study of some aspects of virus behaviour that are difficult to examine in whole plants. The ability or inability of a virus to replicate in protoplasts can give some insight into the level at which plant resistance is operating. It also allows the study of the interaction of two viruses in the same host plant at a cellular level (Barker, 1980).

The aim of this work was to determine whether SBMV-C was able to replicate in isolated bean protoplasts and, if so, to compare the amount of virus produced by SBMV-C with that of SBMV-B. Replication of SBMV-C alone was also compared to its replication in mixed infections with SHMV, in order to establish whether SHMV had any effect on the amount of SBMV-C produced in isolated bean protoplasts.

3.2 Materials and methods

3.2.1 Protoplast isolation

All manipulations were performed under sterile conditions in a laminar flow hood. Protoplasts were isolated from fully expanded leaves of "Bountiful" bean by the one-step method (Power and Cocking, 1969). The tissue was surface-sterilized with 70% ethanol and rinsed three times in distilled water. Leaves then were cut into 1 mm^2 strips which were incubated overnight, at room temperature, in a solution of 0.6 M mannitol, pH 5.4, 0.2% cellulase Onozuka R-10 and 0.025% Macerozyme (Yakult Honsha Co., Minato-ku, Tokyo, Japan). The suspension was filtered through Miracloth (Calbiochem Corp., La Jolla, CA), centrifuged at 1,000 X *g* for 3 min, and the pelleted protoplasts were washed twice with 0.6 M mannitol. Viability was assessed in a 10% solution of Evans blue (Larkin, 1976). Preparations with 80% or higher viability were used for viral inoculation.

3.2.2 Protoplast inoculation and incubation

Freshly prepared protoplasts were inoculated with SBMV-C, SBMV-B, or SHMV in a 30% PEG solution (Maule, 1983). Briefly, 10 or 20 μg of virus was added to approximately 2 X 10^6 protoplasts. The cells were resuspended in a minimal volume of 30% PEG in 0.6 M mannitol and 3 mM $CaCl_2$, added within 30 s while gently swirling to avoid protoplast disruption. The suspension was immediately diluted 10-fold with a solution of 0.6 M mannitol and 1 mM $CaCl_2$ and

was allowed to stand at room temperature for 5 min. The inoculated protoplasts were washed three times with 0.6 M mannitol and 1 mM $CaCl_2$ at pH 5.6 and resuspended to a final concentration of 5-10 x 10^5 cells/ml in a modified minimal salt medium (Takebe,1977) (0.2 mM KH_2PO_4, 1.0 mM KNO_3, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 1 μM KI, 0.01 μM $CuSO_4$ and 50 μg/ml gentamycin) at pH 5.4 (adjusted with HCl or KOH). The protoplast suspension was incubated at 25° C under constant illumination of about 10,000 lux. At selected intervals, 1 ml samples were collected, centrifuged and stored at -70 C until further use.

3.2.3 Assays for virus in protoplasts

Presence of SBMV-C, SBMV-B and SHMV in protoplasts was assayed by ELISA. Frozen, inoculated protoplasts were thawed, resuspended in 1 ml of 1 X PBS (20 mM phosphate buffer, pH 7.4, and 150 mM NaCl), sonicated and placed in pre-coated wells as described in section 2.2.5. SBMV-C infectivity was tested on a cowpea local lesion host (*V. unguiculata* 399419). Inoculated protoplasts were resuspended in 1 ml of 0.01 M potassium phosphate buffer, pH 7.0, sonicated and the extract was rubbed on eight half leaves of 1 week old cowpea plants. Numbers of lesions were recorded 7 days after inoculation.

3.2.4 Protoplast fixation and fluorescent antibody staining

Protoplasts from inoculated "Bountiful" and "Pinto" leaves were isolated as described in section 3.2.1. After enzyme digestion, cells were washed with 0.6 M mannitol and a drop of the protoplast suspension was smeared onto a microscope slide, previously coated with a layer of Mayer's albumin (Cassels and Gatebny, 1975), and dried in a stream of warm air.

Fixation and staining were done essentially as described by Wood (1985). The protoplasts in the smear were fixed in acetone for 30-45 min, gently rinsed with distilled water to remove mannitol, then equilibrated in PBS. SBMV antiserum was diluted 1/100 in PBS, a drop was placed on top of the smear and the slide was incubated at 37 C for 1 h in a moist chamber. After washing for 30 min with PBS, fluorescein-labelled, sheep anti-rabbit immunoglobulin (Sigma Chemical Co., St Louis, MO), diluted 1/20 with PBS, was added and incubated for 30 min. The slide was washed thoroughly in PBS, mounted in 10 mM sodium carbonate buffer in 90% glycerol, pH8-9, and examined under a fluorescence microscope fitted with an excitation filter (KP500) and barrier filters (LP520 and LP540).

3.3 Results

3.3.1 Proportion of SBMV-C infected cells in singly and doubly infected leaves

Fluorescent antibody staining of protoplasts isolated from primary leaves of "Pinto" and "Bountiful" doubly inoculated with SBMV-C and SHMV resulted in the detection of some stained protoplasts. In "Pinto" only 0.1% of the cells fluoresced while in Bountiful 5% of the cells fluoresced. Protoplasts from plants that were singly inoculated with SBMV-C failed to fluoresce, probably due to the low amount of antigen present in subliminal infections.

3.3.2 Replication of SBMV-C in singly inoculated protoplasts

To confirm that bean cells can support viral replication, Bountiful protoplasts were inoculated with SBMV-C. Viral antigen in infected protoplasts could be detected using ELISA, in most experiments, 10 h after inoculation. It increased steadily until 42-58 h and remained constant thereafter. Bioassays of virus infectivity were positive about 30 h after inoculation, and lesion numbers increased steadily until 68 h (Fig.7). Mock-inoculated protoplasts showed no increase in absorbance values which were, on average, 0.015 at A_{405}.

Protoplast viability was assessed every time a sample was taken. The greatest decline was observed immediately after inoculation (viability decreased from 80-90% to 60-70%) and at 48-53 h after placing in minimal salt medium (viability varied with different protoplast preparations).

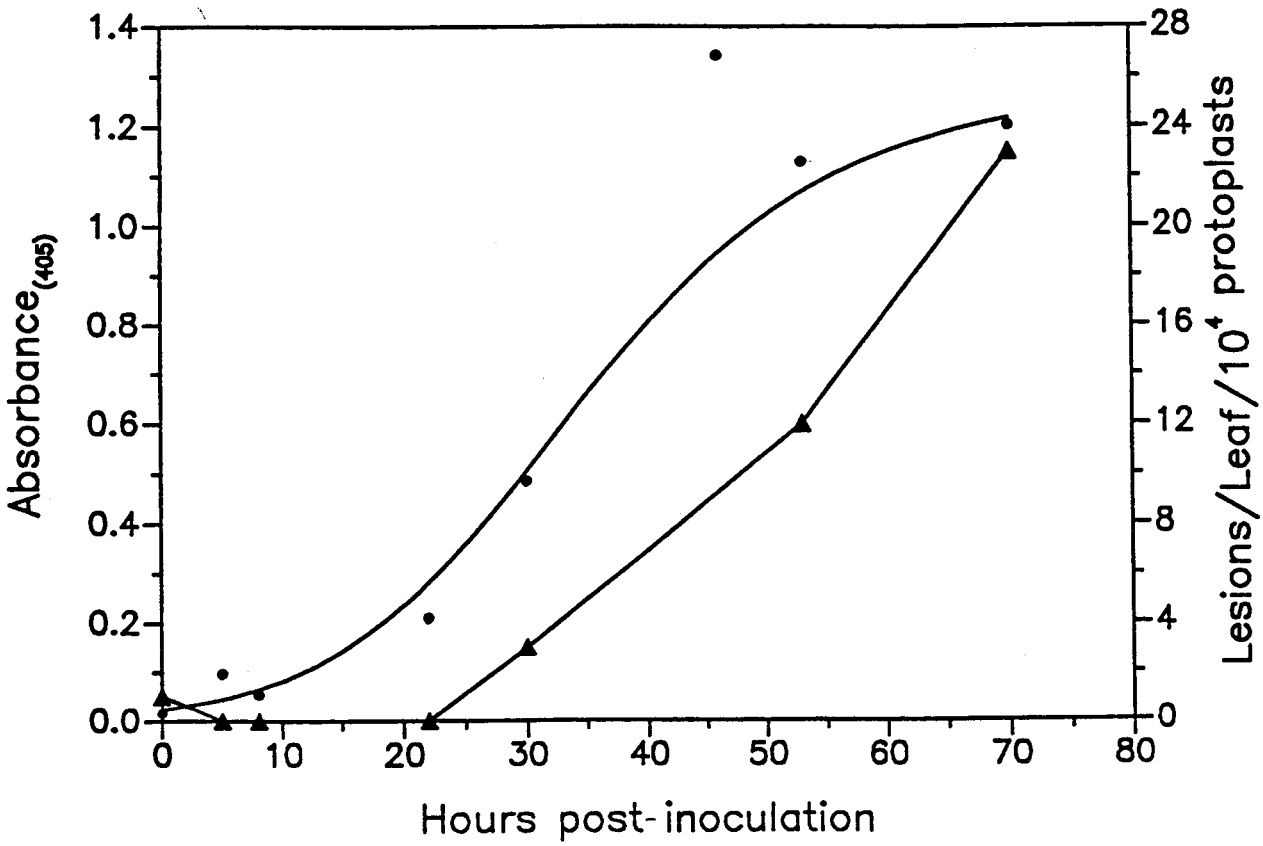


Fig.7. Time course of the replication of SBMV-C in "Bountiful" protoplasts detected by ELISA (•) and bioassay (▲) on Vigna unguiculata L. var 399414.

3.3.3 Replication of SBMV-C in doubly inoculated protoplasts in comparison to SBMV-B-inoculated protoplasts

A single batch of protoplasts was divided into two equal parts; one was inoculated with SBMV-C alone and the other with a mixture of SBMV-C and SHMV. This was done to determine whether the presence of SHMV would affect SBMV-C replication in bean protoplasts. Accumulation of SBMV-C antigen after double infection of protoplasts with SHMV and SBMV-C was similar to that attained when protoplasts were singly inoculated with SBMV-C (Table II).

To determine the replicating efficiency of the two strains of SBMV, two equal parts of a batch of protoplasts were infected with either SBMV-C or SBMV-B. The infection courses and antigen accumulation for both strains were similar (Table II). Thus, individual bean protoplasts sustained replication of SBMV-C and SBMV-B with the same efficiency, and the level of replication of SBMV-C in the isolated protoplasts was unaffected by co-inoculation with SHMV.

TABLE II. Replication of SBMV-C and SBMV-B and replication of SBMV-C in single and mixed infections with SHMV in Bountiful bean protoplasts detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

	Absorbance ($A_{405}/10^4$ protoplasts)			
Time post inoculation (hours)-	SBMV-C[a]	SBMV-B	SBMV-C	SBMV-C + SHMV
0	0.089	0.031	0.314	0.438
5	0.044	0.008	0.272	0.371
12	0.022	0.003	n.d	n.d.
24	0.377	0.414	0.275	0.345
46	n.d.[b]	n.d.	0.749	0.867

[a] Experiment on first two columns was done simultaneously with the same preparation of Bountiful bean protoplasts that was divided into two equal parts, one for each treatment and the last two columns with another lot of bountiful bean protoplasts also divided into two equal parts, one for each treatment.

[b] n.d. indicates not determined.

Chapter 4

Distribution of SBMV-C within inoculated leaves and throughout the plant

4.1 Introduction

One means of controlling the pathological effects of viruses is by blocking their systemic spread throughout the plant. In order to achieve this goal, it is necessary to understand how viruses move through plants and the way virus distribution varies within the tissues of the infected plants.

In this study, the spread of SBMV-C in bean, in mixed infections with SHMV, was followed using a combination of ELISA, dot-blot hybridization, and light and electron microscopy. A parallel study, using another strain of the virus which infects bean systemically (SBMV-B), allowed comparison of the distribution of a virus in its host (SBMV-B in bean) with a "dependent" virus in a non-host (SBMV-C in bean with SHMV acting as the "helper").

4.2 Materials and methods

4.2.1 Collection of samples

Various tissues from "Bountiful" inoculated with SHMV and SBMV-C or SHMV and SBMV-B were harvested from 6 different plants 5,7 and 11 days post-inoculation. Inoculated primary leaves were washed under tap water for 2 h, and interveinal tissue was separated from the lateral veins, main vein and petiole with the aid of a scalpel. Stems, roots and trifoliate leaves were also separated. All tissues were weighed and used immediately in assays for infectivity of viral particles and viral RNA or stored at -20 C until future use.

4.2.2 ELISA and bioassay

Equal weights of different separated plant parts were ground in 1 X PBS (for ELISA) or 0.01 M potassium phosphate buffer, pH 7.4 (for bioassay) and tested for the presence of SBMV-C or SBMV-B as described in sections 2.2.4 and 2.2.5.

4.2.3 Dot blot hybridization assay for viral RNA

Total leaf RNA from different leaf sections of inoculated and healthy plants was extracted following the method of Siegel et al. (1976). Leaf material (100-500 mg) was ground to a fine powder in liquid nitrogen using a mortar and pestle and transferred to a microfuge tube to which was added 400 *ul* phenol/chloroform/ octanol (25/24/1) and 400 ul 10X TNE (100 mM Tris-HCl pH

7.5, 100 mM NaCl and 10 mM EDTA), 0.1% SDS and 5% 2-mercaptoethanol. After mixing vigorously, the suspension was centrifuged (10,000 X g) for 2 min and the aqueous phase was removed and re-extracted two times (second time with chloroform/octanol only). One tenth volume of 2 M sodium-acetate pH 5.8 and 2-2.5 volumes of absolute alcohol were then added to the aqueous phase and the RNA was precipitated at -70 C for 10 min. The precipitate was pelleted by centrifugation, washed with 70% ethanol and resuspended in sterile, deionized water.

For further separation of high molecular weight ssRNA, instead of washing and resuspending the pellet in water, it was resuspended in TE (10 mM Tris-HCL pH 7.5 and 1mM EDTA), 100 µl of 8 M LiCl was added and the solution was incubated on ice for 1-3 h. After spinning for 10 min, the pellet was resuspended in autoclaved water, re-precipitated with NaAc and ethanol as described above, and resuspended in sterile, deionized water. The supernatant fluid remaining after LiCl precipitation was removed and 2.5 volumes of absolute ethanol were added and the suspension was centrifuged in a microfuge for 10 min. The pellet containing dsDNA, dsRNA and low molecular weight ssRNA was washed with 70% ethanol and resuspended in sterile H_2O. All samples were stored at -70 C.

Presence of SBMV-C RNA was assessed by dot- blotting using

a nick-translated (Rigby *et al.*, 1977) ^{32}P-labeled probe (10^7-10^8 cpm/μg) of SBMV cloned DNA designated "slorf 18" (kindly provided by Dr. Claire A Rinehart, University of Wisconsin, Madison, WI).

Dot-blot hybridization analysis was done on nitrocellulose membranes (Schleicher and Schuell, Keene, NH), which were cut to 8 x 10 cm, soaked sequentially in deionized H_2O for 5 min, and in 20X SSC (20X SSC is 3 M NaCl, 0.3 M sodium citrate), and dried. Each sample of total nucleic acid or ssRNA was thawed and 2 μl or a volume equivalent to approximately 20 ng and 40 ng of nucleic acid was deposited on the filters. Membranes were allowed to dry and were then baked under vacuum for 1 h at 80 C. The filters were then transferred to a Seal-a-Meal plastic bag and were prehybridized at 42 C, for 1 h, in hybridization buffer (50% [v/v] deionized formamide, 50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.2% bovine serum albumin [BSA], 0.2% PVP, 0.2% Ficoll, 0.1% sodium pyrophosphate, 10% sodium dextran sulphate and 250 μg/ml sheared salmon sperm DNA).

Hybridizations were carried out in the same solution used for pre-hybridization. The labelled probe was denatured by adjusting to 0.1 M NaOH and then boiling for 5 min. After quickly cooling the probe, it was added to the solution in the bag and allowed to hybridize with the RNA immobilized on the filters overnight, at 42 C with continuous agitation.

Following hybridization, the filters were rinsed with 2 X SSC plus 0.1% SDS, then washed for 15 min, at 55-60 C successively in each of the following three solutions: 2 X SSC plus 0.1% SDS; 0.5 X SSC plus 0.1% SDS and finally in 0.2 X SSC. Membranes were blotted lightly to remove excess moisture, wrapped in plastic film and exposed to X-OMAT film (Kodak) for 2, 4 and 12 hr at -70 in an X-ray cassette with Lightning Plus (Dupont) intensifying screens.

4.2.4 Tissue print-immunoblotting

Tissue prints were performed essentially as described by Cassab and Varner (1987). The nitrocellulose paper (Schleicher & Schuell, #BA85, Mandel Scientific, Edmonton, AB) was pre-treated with 0.2 M $CaCl_2$ for 30 min, dried on paper towels and placed on Whatman filter paper on a flat glass plate.

Infected and healthy leaves, petioles and stems were cut with a razor blade, rinsed briefly in distilled H_2O and dried on Kimwipes facial tissue. The freshly exposed surfaces were blotted onto the nitrocellulose paper by applying light pressure on the membrane for 10-20 s. The tissue print was then dried with warm air and either processed immediately or kept at 4 C. The remaining free binding sites, on the nitrocellulose paper, were blocked by submerging the sheet in 2% BSA and 1% Triton X-100 in 0.01 M Tris pH 7.4, 0.85% NaCl (TBS) for 2 h at 37 C or overnight at 4 C.

Total leaf and viral proteins transferred by blotting were detected by staining the blot with India ink (Hancock and Tsang, 1983). Drawing India ink for fountain pens (Pelikan AG, D-300, Hanover 1, Germany) was diluted to 1 *ul*/ml of PBS-Tween and the blot was left overnight in stain. They were rinsed in deionized water for 5 min and then dried.

Virus antigens adsorbed on the blots were detected using alkaline phosphatase-conjugated antibody as described by Blake et al (1984). Primary antibody against SBMV-C was diluted 1/1000, 1/2000 or 1/4000 in antibody solution (1% BSA, 0.3% Triton-X100, 0.05% Tween in TBS) and incubated for 1 h at 37 C in a shaker. The membrane was washed at room temperature, 10 times, 5 min each time, in TTBS (TBS, 0.05% Tween).

Anti-rabbit IgG (Fc) alkaline-phosphatase conjugate was diluted 1/10,000 in antibody solution and the membrane was incubated for 1.5 h at 37 C. Membranes were washed as above except that the last two washes were in substrate buffer (0.1 M NaCl, 5 mM $MgCl_2$ in 0.1 M Tris, pH 9.5). Substrate solutions, BCIP (5-bromo-4-chloroindoxyl phosphate) and NBT (nitroblue tetrazolium), (both from GIBCO BRL Immunoselect Cat. No. 8280 SA), were prepared just prior to use. To 10 ml of substrate buffer, 22 µl of NBT were added and gently mixed by inversion of the tube, then 17 µl of BCIP were added and mixed. The membranes were incubated in substrate solution until purple prints

appeared. The reaction was stopped by addition of 5 mM EDTA in 20 mM Tris pH 7.5 for 10 min, membranes were washed in distilled H_2O and dried between several layers of Whatman filter paper.

4.2.5 Cryosectioning and immunofluorescent labelling of thick sections

Samples from mixed infected and healthy tissue were prepared according to Griffiths (1984) post-embedding immunocytochemical technique for high resolution immunofluorescence.

Tissue was fixed in 2 or 4% formaldehyde in 0.05 M phosphate buffer, pH 7.0, for 1 h on ice. After rinsing with buffer, sections were infiltrated into sucrose through a series of 20,40,60,80 and 100% sucrose (in phosphate buffer, v/v). Samples were mounted on small aluminum stubs and immediately frozen in liquid N_2. At this point, samples could be stored at -70 C for further processing. Samples were sectioned on tungsten-coated glass knives with a Reichert Ultramicrotome E in a cryobox (FC4E). Sections were picked up on drops of sucrose and placed on Poly L Lysine (Sigma P1274 mw 100,500)-coated glass slides. Slides were rinsed in TBS and stored overnight at 4 C. All of the following procedures were done at room temperature.

Free aldehydes were blocked with NH_4Cl, 50 mM, for 10 min., then slides were left in BLOTTO for 30 min to block nonspecific protein binding sites. Following blocking, SBMV antibody was added at different concentrations (1:100, 1:500 and 1:1000, diluted in BLOTTO) and samples were incubated for 3 h. Slides were rinsed in TBS-Tween several times, anti-rabbit IgG-FITC conjugate was added and samples were incubated for 1 h. After thorough rinsing, the backs of slides were washed and samples were mounted in glycerol/phosphate buffer (80:20 v/v) and examined with a fluorescence microscope.

4.2.6 Immunosorbent electron microscopy

To facilitate visualization of viral particles from homogenates of infected plants, grids were pre-treated with protein-A and SBMV antiserum. Copper grids, 400 mesh, covered with Formvar and coated with a carbon layer were floated on 15 µl drops of Protein A (10 *u*g/ml of distilled water) for 10 min. Grids were washed once in PBS, floated for 10 min in 15 *u*l drops of SBMV antiserum diluted 1/10 in PBS and washed one more time in PBS.

Samples of inoculated and healthy plants were homogenized in PBS with a mortar and pestle and pre-treated grids were floated on droplets of sap and incubated at room temperature for 1 h. After incubation, grids were washed by floating in five drops of PBS, 2 min on each drop, and finally on one

drop of Bacitracin for 1 min. Samples were stained with a 2% solution of uranyl acetate (UA) for 1 min and dried. Preparations were observed with a Hitachi 600 transmission electron microscope.

4.2.7 Tissue fixation and embedding for electron microscopy

Samples from inoculated and healthy bean and cowpea were fixed according to standard glutaraldehyde and osmium tetroxide embedding procedure, and two other procedures: Hatta and Francki's (1981) procedure to digest ribosomes, and an enhanced method for fixation and gold labelling (Berryman and Rodewald, 1990).

For the standard fixation procedure, leaves and petioles taken at different times post-inoculation were cut into 1 mm^2 pieces and placed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, for 12-18 h (first hour at room temperature and the remaining time at 4 C). Samples were washed twice for 15 min in 0.1 M cacodylate buffer pH 7.0. Specimens were post-fixed in 1% (or 0.5, or 0.25%) OsO_4 in cacodylate buffer for 1 to 3 h, rinsed in distilled water and then washed twice, 15 min each time, in distilled water.

Fixed tissue was then dehydrated by washing twice, 15 min each time, in each of 30%, 50%, 70%, 95% and absolute ethanol and then three

times (15 min each time) in propylene oxide. A solution of 50/50 propylene oxide and Epon 812 mixture (10 g Epon 812, 0.36 g of 2,4,6-tri dimethyl-aurinomethyl phenol [DMP], 4.46 g of nadic methyl anhydride [NMA] and 3.76 g of dodecenyl succinic anhydride [DDSA]), was used to begin infiltration. Samples were left overnight in uncovered vials, then transferred to flat embedding molds filled with fresh Epon 812 mixture. Blocks were left to dry at 55 C for 48 h.

Alternatively, fixed tissue was dehydrated by washing once for 45 min at 4 C in each of 50%, 70% and 90% acetone. It was then placed, for 60 min at 4C in vials with 1:1 LR White medium/acetone, followed by 7:3 LR White medium/acetone. Tissue was finally infiltrated with 3 changes of 100% LR White medium for 60 min each, then transferred into flat embedding molds which were left at -20C under UV light to allow for polymerization of the medium.

To avoid interference from ribosomes when observing infected tissue, these were digested with RNase according to Hatta and Francki (1981). Tissue was cut in pieces as described above and then fixed for 16 hours, at 4 C in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.13 M phosphate buffer pH 7.3. Sections were rinsed in 2 X SSC (SSC: 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and then washed in several changes of 2 X SSC for 6 h at room temperature. Samples were then digested with pancreatic RNase (2 μg/ml) (type III, Sigma Chemical Co. St Louis, MO) in 2 X SSC for 16 h at 25

C. After digestion, specimens were post-fixed in 0.4% osmic acid for 1-2 h, washed repeatedly in distilled H_2O and then dehydrated and embedded as described for samples above.

To avoid problems of osmium interference with immunolabelling of antigenic sites, an alternative fixative without this substance was used (Berryman and Rodewald, 1990). Samples were cut and fixed, at room temperature for 2-3 h, in 1% glutaraldehyde, 0.2% picric acid, 4% formaldehyde, and 0.05 mM $CaCl_2$ in 0.1 M potassium phosphate buffer pH 7.4. A solution of 3.5% sucrose in 0.1 M phosphate buffer with 0.5 M $CaCl_2$ was used as a wash following fixation. After washing for 2 h in several changes of the sucrose solution, free aldehydes were quenched with 50 mM ammonium chloride in sucrose:phosphate buffer for 1 h at 0 C. Phosphate ions were removed by rinsing the samples in cold 0.1 M maleate buffer, pH 6.5 containing 3.5% sucrose. Specimens were then dehydrated and infiltrated in Epon and LR White medium following the procedures described above.

Ultrathin sections were cut with a Reichert OMU2 ultramicrotome using glass knives (made with an LKB Knifemaker, type 7801B). Sections were collected on 100 mesh copper or nickel grids, covered with Formvar and carbon. Sections were stained with a 4% aqueous uranyl acetate solution.

4.2.8 Immunogold labelling of antigens

Colloidal gold was prepared by reduction of hydrochloroauric acid ($HAuCl_4$) with sodium citrate (Frens, 1973; Slot and Geuze, 1981) which resulted in suspensions of colloidal gold particles with average diameters of 5, 10 and 17 nm. The colloidal gold suspensions were then complexed with Protein A as described by Horisberger and Rosset (1977).

The affinity of the gold-Protein A complex for viral antibody was tested on grids which had been floated on drops of tissue homogenate from infected plants. Grids, which had been incubated on drops for 10 min, were then sequentially washed with six drops, 5 min each, of BSA-Tween (0.5%-0.05%) in PBS. Antiserum or purified IgG was diluted 1/100 and 1/500 in PBS-BSA-Tween (as above) and grids were floated on the drops for 1 h after which they were washed as before. For gold labelling, drops of 5, 10 and 17 nm gold-Protein A diluted in PBS-BSA-Tween were placed on parafilm and grids were incubated on them for 30-60 min. Grids were washed with 6 drops of PBS-BSA-Tween for 5 min, rinsed in PBS and incubated in 2% glutaraldehyde for another 5 min. Before staining, grids were washed twice for 10 min. All reactions were carried out at room temperature.

Labelling of antigens on ultrathin sections mounted on grids was essentially as described above except that grids were not floated on sap from infected plants but were pre-treated with PBS-lysine (0.1%) for 5 min, before the first PBS-BSA-Tween wash. To stain sections, grids were submerged in a 1:1 dilution of lead citrate in 0.01 M NaOH for 3 min, washed in distilled water, then stained with UA for 5 min., washed again and incubated one more time in lead citrate for 3 min. Grids were rinsed in distilled water and examined in an Hitachi 600 transmission electron microscope.

4.2.9 Electrophoresis and Western blotting of viral proteins

Viral proteins from purified virus preparations (section 2.2.1) and from soluble protein preparations from infected plants, were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) through 12% gels using a discontinuous Laemmli buffer system (Laemmli, 1970) as described by the manufacturer (BioRad). Following electrophoresis, gels were transferred to 3 changes of fixative (30% methanol/10% acetic acid), and agitated gently for 30 min in each change. Gels were stained with Coomassie blue staining solution (50 ml methanol, 50 ml H_2O, 10 ml acetic acid and 0.2% Coomassie blue G250) for 1 h and destained overnight with destaining solution (40 ml methanol, 50 ml H_2O and 10 ml acetic acid). Alternatively, proteins were transferred to teflon membranes (Millipore Immobilon P, Mississauga, Ont.) using

a wet blot apparatus (TE series transphor electrophoresis unit, Hoefer Sci., Minnesota). SBMV-C proteins were detected using both polyclonal anti-SBMV-C and monoclonal (kindly provided by Dr. D. Mackenzie, Ag. Can. Res. St.) anti SBMV-C antibodies, following the method described in section 4.2.4 , except anti-mouse IgG (Fc) alkaline-phosphatase conjugate was used for monoclonal antibody detection.

Soluble proteins from infected and healthy plants were isolated according to Gegenheimer (1990). Fresh plant material (500 µg) was homogenized in 1:5 (w/v) ice-cold extraction buffer (50 mM Tris pH 8.0, 5 mM borate, 1.5% insoluble PVP, 5% glycerol, 20 mM DIECA and 0.01% mercaptoethanol). The homogenate was centrifuged in a microfuge for 5 min at 4 C, the supernatant fluid was decanted and 1:1 (v/v) 2 X SDS sample buffer (0.08 M Tris HCl pH 6.8, 0.13% glycerol, 2.6% SDS, 0.06% 2-beta-mercaptoethanol and 0.016% bromophenol blue) was added. Samples were stored at -20 C until needed.

4.3 Results

4.3.1 Distribution of SBMV-C capsid protein compared to that of SBMV-B in double infections with SHMV

To establish the course of infection and distribution of SBMV-

C, in the presence of SHMV, in bean plants, antigen accumulation in the interveinal tissue, lateral veins, midrib, petiole, stem, roots and trifoliate leaves was assessed at different times after inoculation. Parallel tests were done with SBMV-B in the presence of SHMV, and accumulation of viral antigen was compared for both strains (Fig.8). In the early stages of infection, estimates of virus concentration in the interveinal tissue were similar for both strains, although SBMV-C was slightly higher in most of the repeated experiments. These results are likely accounted for by the fact that primary leaves infected with SBMV-B and SHMV suffered severe wilting and yellowing as early as 4 days post-inoculation.

Further dissection of the leaf and petiole revealed that the concentration of SBMV-C decreased from the lateral veins toward the mid- and distal parts of the petiole, where virtually no virus could be found. In later stages of infection, the bean strain of SBMV was present in the roots and trifoliate leaves, whereas the cowpea strain remained confined to the inoculated primary leaves, and no antigen was detected in stem, roots, or trifoliate leaves even at 2 months post-inoculation. The absence of infective SBMV-C outside the inoculated leaves was confirmed by bioassay of extracts of plant parts on a local lesion host.

Thus, in the presence of SHMV, the cowpea strain of SBMV appeared to move through the epidermal and mesophyll cells of inoculated

Fig. 8. Accumulation of SBMV-C (□) and SBMV-B (■) capsid, detected by ELISA, in mixed infections with SHMV in : a) roots, b) stem, c) petiole, d) main vein, e) lateral vein, f) mesophyll and g) trifoliates of bean (P. vulgaris cv. Bountiful). Graphs A, B and C show accumulation profiles 5,7 and 11 days post inoculation, respectively. The symbol "X" indicates the tissue was necrotic at sampling time and "*" indicates that values for these samples were < 0.025.

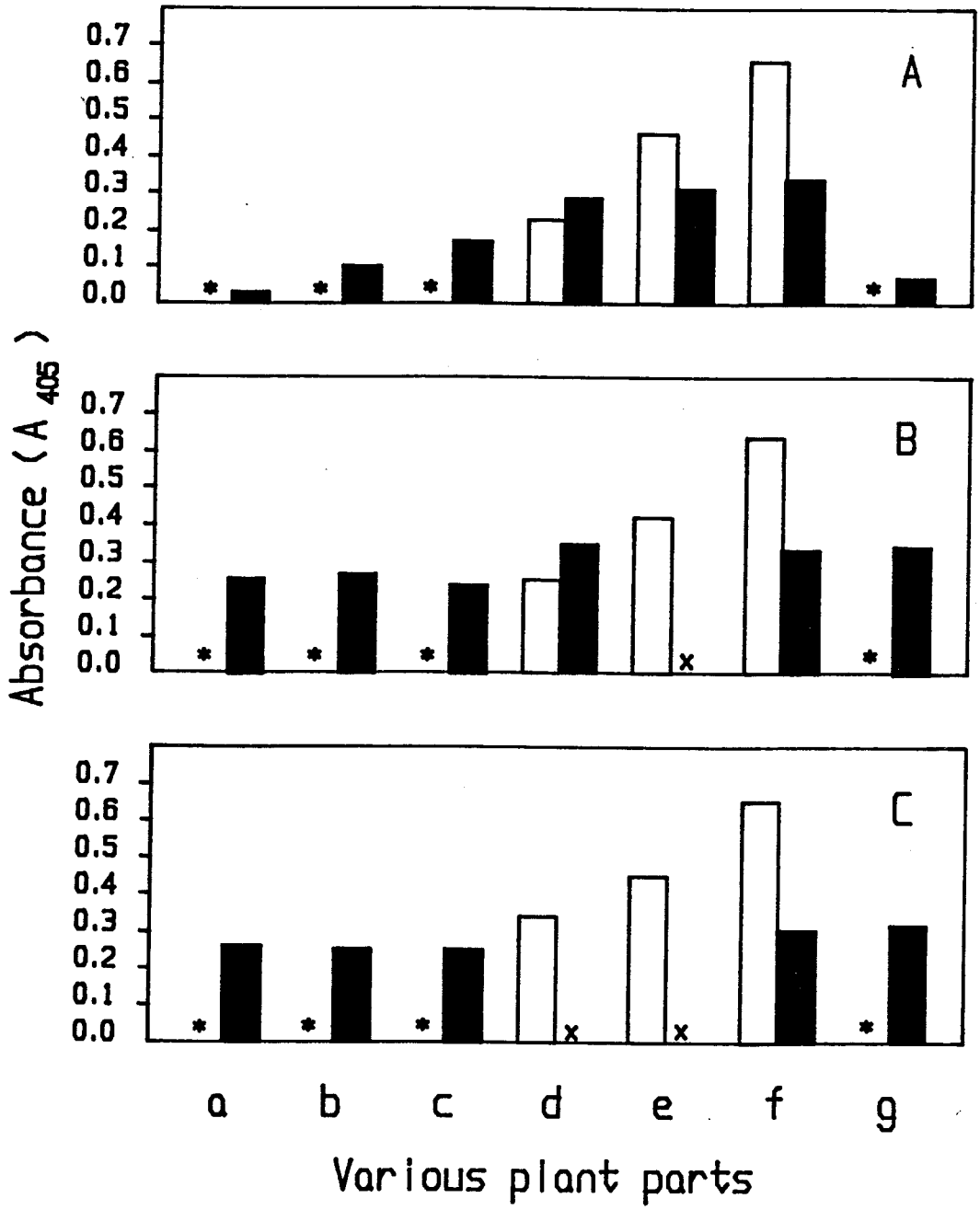

A
B
C
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance (A 405)
a
b
c
d
e
f
g
Various plant parts

Fig. 8

primary leaves at the same rate as the bean strain. However, movement of SBMV-C into the vascular tissue was limited or nonexistent. The antigen detected in lateral veins and midrib could be due to the presence of SBMV-C-infected mesophyll tissue that could not be completely eliminated during dissection and also to its presence in parenchyma cells bordering the conducting system.

4.3.2 Distribution of SBMV-C RNA in doubly inoculated plants

Although the absence of infective SBMV-C outside the inoculated leaves was determined by bioassay, it was further confirmed by determining the distribution of SBMV-C RNA. Dot blot hybridization analysis revealed a greater amount of SBMV-C RNA in the interveinal tissue, smaller amounts in the lateral veins and main vein, and no SBMV-RNA in the petiole or stem (Fig.9). No signal in these two plant parts was observed even after a 32 h exposure of the filter. However, the presence of very small amounts of SBMV-C RNA cannot be completely excluded by these detection methods.

4.3.3 Viral antigen distribution in thick sections of leaf and petiole by tissue blot analysis

Efficient transfer of leaf and viral proteins was achieved by

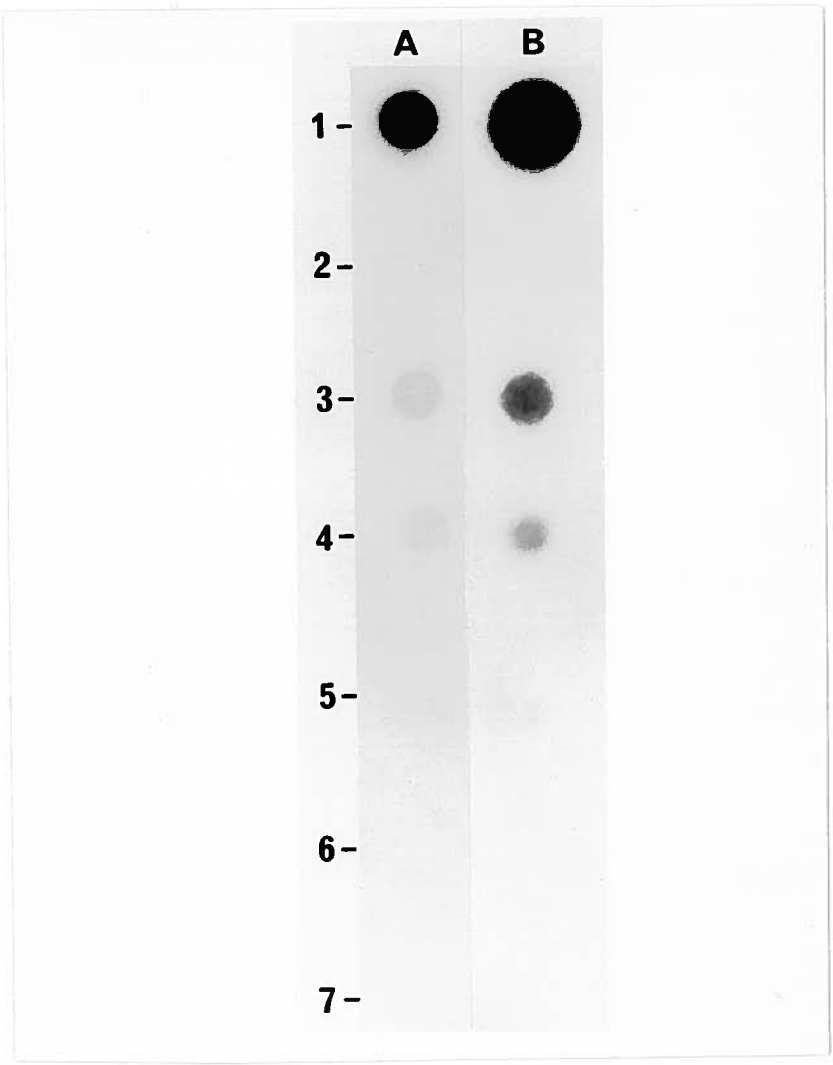


Fig. 9. Detection of the cowpea strain of southern bean mosaic virus (SBMV-C) RNA in different parts of bean plants (*Phaseolus vulgaris* "Bountiful") by dot-blot hybridization using a nick-translated ^{32}P c-DNA probe of SBMV-C. Total RNA was extracted from each plant part and 100 ng added for each sample. 1, mesophyll; 2, extraction buffer; 3, lateral veins; 4, main vein; 5, healthy tissue; 6, petiole; 7, stem. A and B, 4 and 12 h exposure, respectively.

pressing freshly sectioned material onto nitrocellulose membranes, as shown by results of India ink staining (Fig.10).

Samples were taken from cowpea leaves inoculated with SBMV-C and Bountiful bean leaves inoculated with a mixture of SBMV-C and SHMV. Healthy bean tissue was used as negative control. A dilution series of purified SBMV-C was blotted on one side of the filter to be used as a guide for colour change when substrate was added.

Of approximately 90 prints of each sample, only 14 of those from bean leaves inoculated with SBMV-C + SHMV showed a positive signal. These positive strips belonged to leaves that had been inoculated 7 and 12 days prior to sampling; none of the strips from leaves collected 21 or more days after inoculation showed a positive signal. This contrasted with approximately 60 positive signals for SBMV-C in cowpea, from samples collected 7, 12 and 21 days post-inoculation.

Positive controls of SBMV-C in cowpea primary leaves showed a deep purple stain throughout the entire section (Fig. 11A, B), or in certain areas of the mesophyll and the vascular tissue (Fig. 12A, B). Sections from bean primary leaves infected with SBMV-C + SHMV, on the other hand, presented a deep purple colour in mesophyll and epidermal cells of the leaf but no positive

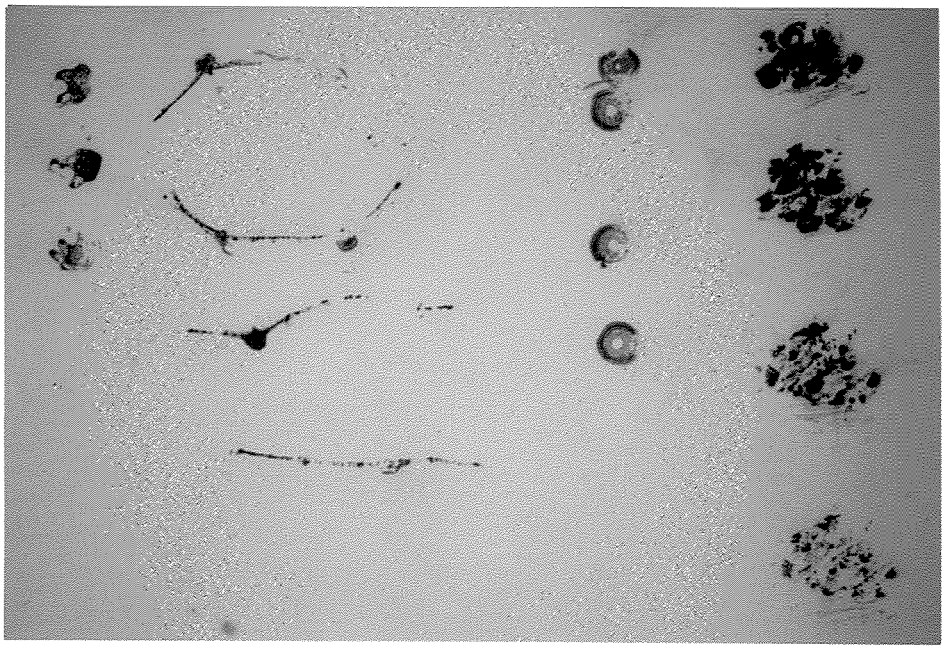


Fig. 10. Transfer of proteins from leaf sections and tissue pressed onto nitrocellulose membranes. Proteins were stained with India ink as described in Materials and Methods. A) Cross section of petiole from a primary bean leaf. B) Cross section of leaf blade and lateral veins of a primary bean leaf. C) Cross section of stem of a bean plant. D) Sap from primary leaf.

Fig. 11. SBMV-C antigen detected in SBMV-C-infected cowpea primary leaf blade and vein tissue by tissue print immunoblotting. A. Cross section of leaf blade and lateral vein, (magnification, 100X). B. Cross section of leaf and lateral vein, (magnification, 200X). Deep purple coloring represents areas where antigen is present.

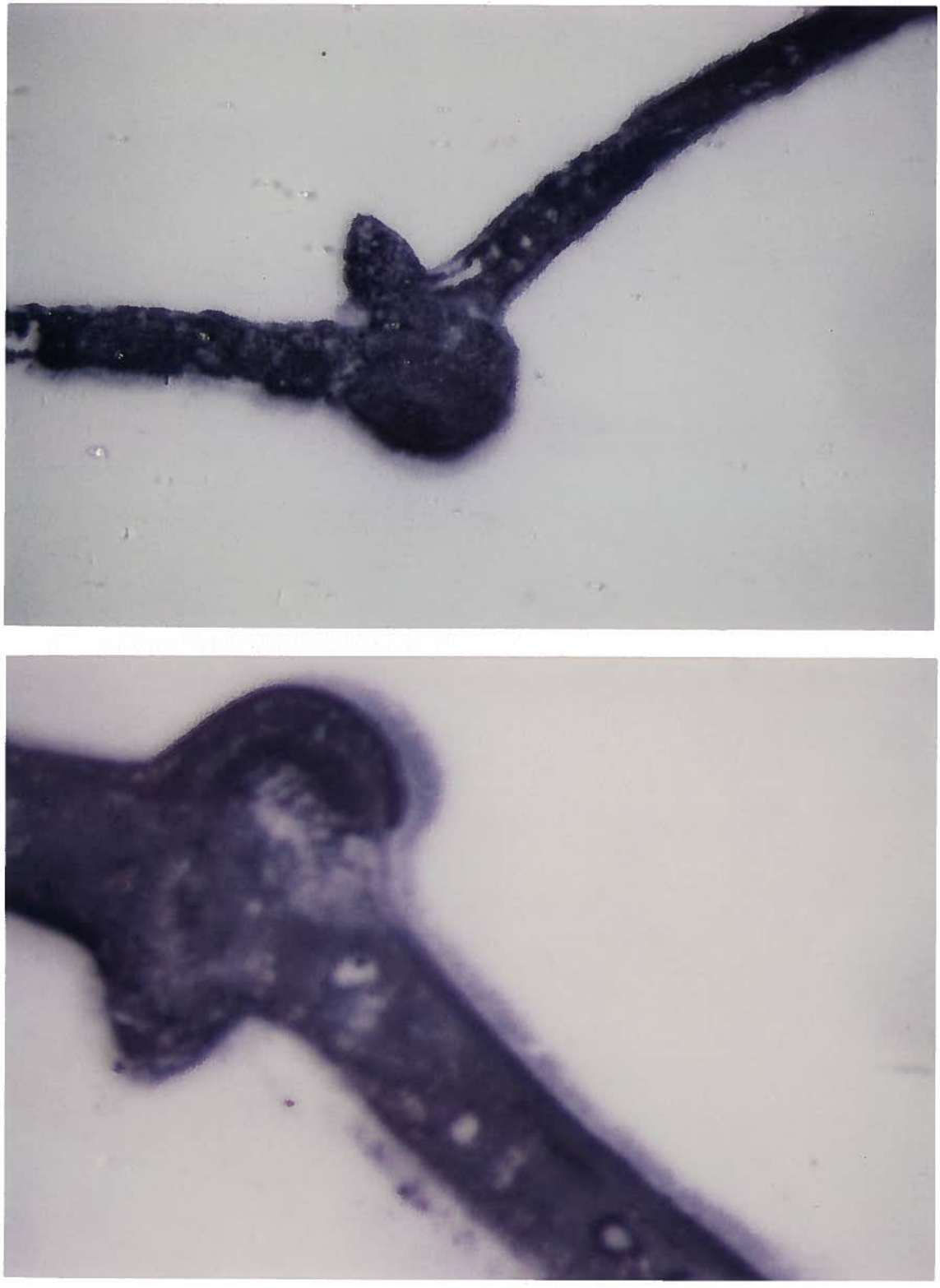
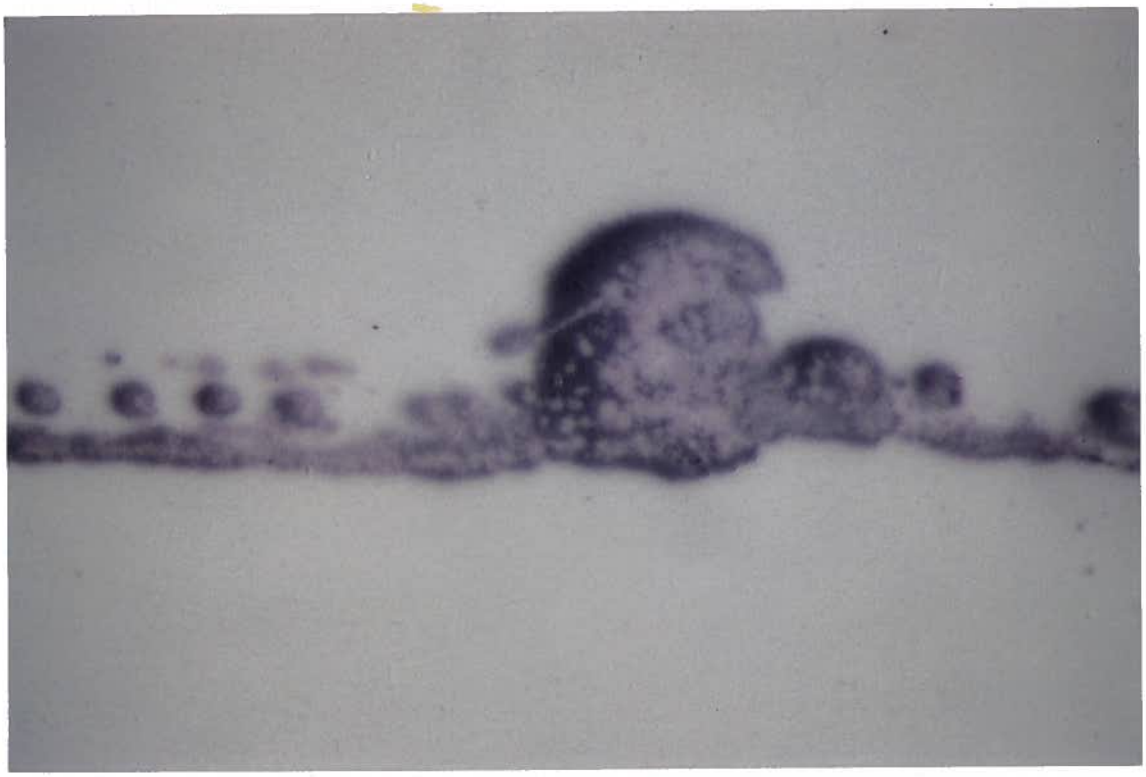


Fig. 11

Fig. 12. SBMV-C antigen detected in SBMV-C-infected cowpea primary leaf blade and vein tissue by tissue print immunoblotting. A. Cross section of leaf blade and lateral vein, (magnification, 100X). B. Cross section of leaf and lateral vein, (magnification, 200X). Dark purple areas indicate viral (SBMV-C) antigen is present.



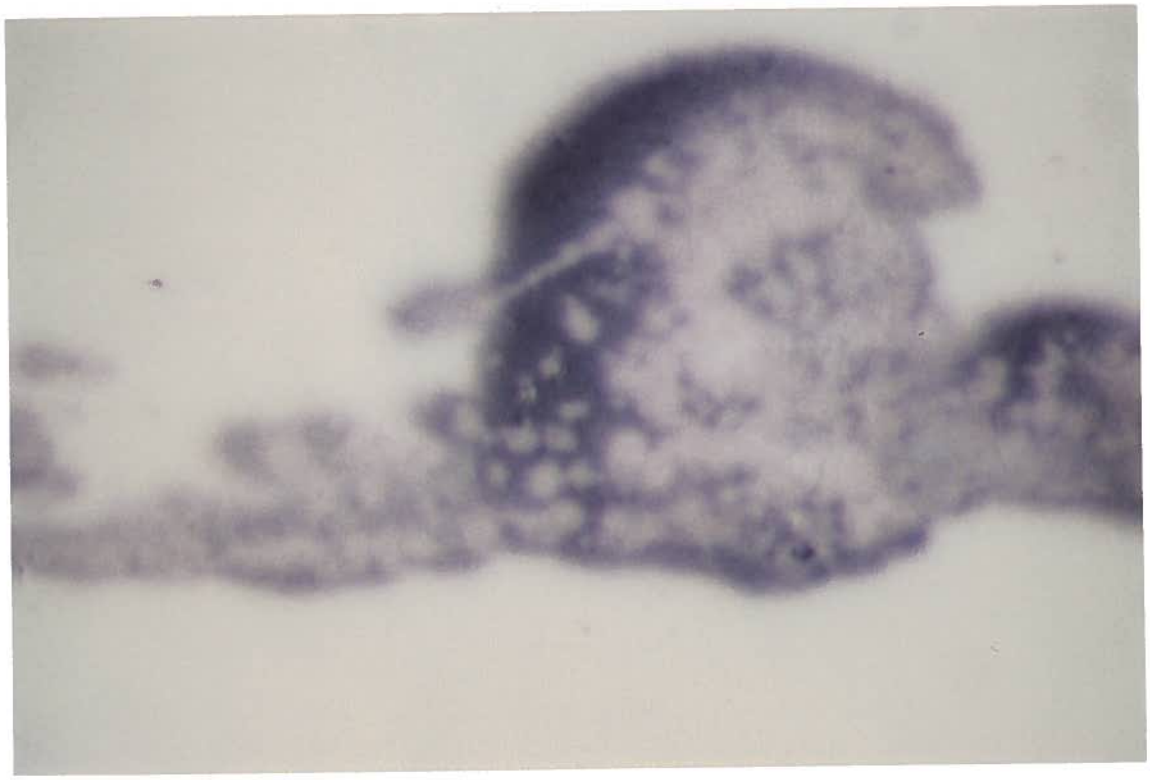


Fig. 12

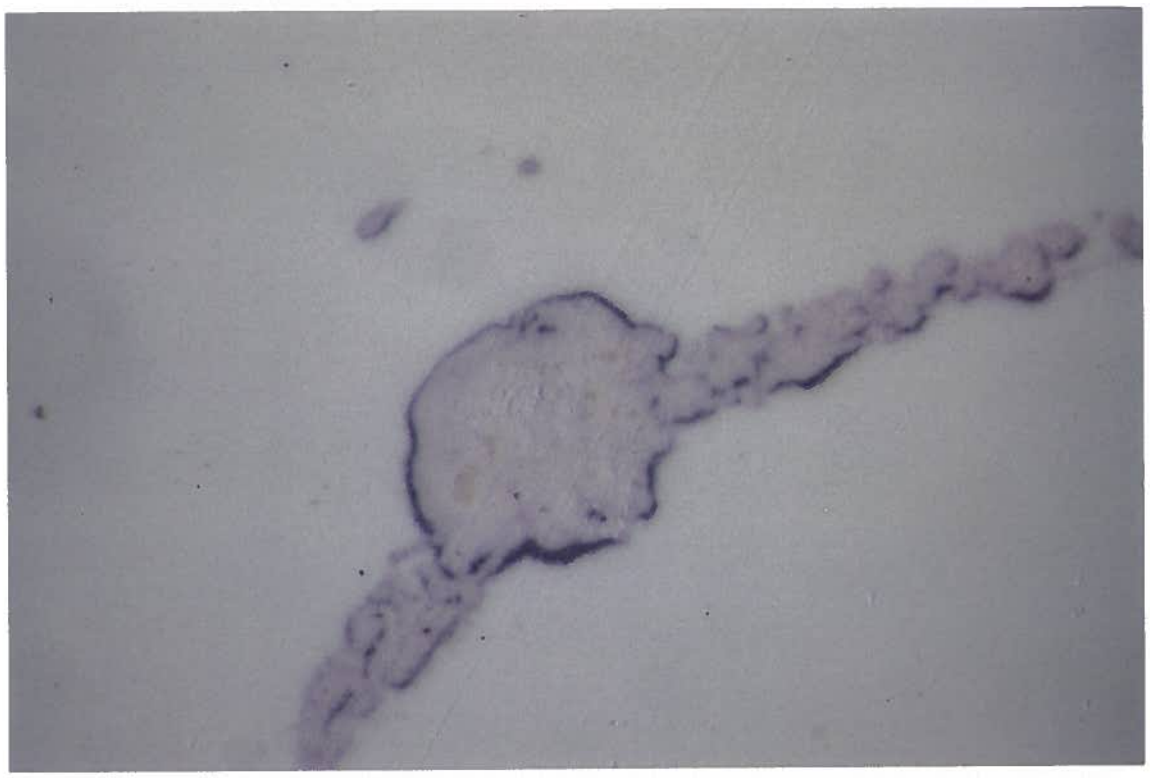
signal was detected in the vascular bundles of the vein (Fig. 13A, B). The light purple zones represent background but are clearly distinguishable from the positive dark purple areas, as can be seen by comparing prints from mixedly-infected primary bean leaves (Fig. 13A, B) to the healthy bean tissue prints (Fig. 14A, B).

4.3.4 SBMV-C antigen distribution in thick sections by cryosectioning and immunofluorescent labelling

Thick sections of bean tissue inoculated with SBMV-B + SHMV, SBMV-C + SHMV or mock-inoculated were fixed and infiltrated in sucrose. Samples mounted on pins and frozen in liquid N_2 were cut into 3 μm sections and treated as described in section 2.4.5.

In three replicated experiments, mesophyll cells and phloem companion cells in tissue infected with SBMV-B + SHMV fluoresced strongly (Fig. 15A, B). At antiserum dilutions of 1/100 some autofluorescence and background could be seen in healthy controls (Fig. 16A, B) while very little background was found at antiserum dilutions of 1/500 (Fig. 17A, B). No fluorescence was found in tissue infected with SBMV-C + SHMV. In all of three replications of this experiment, this tissue presented great difficulties in cutting, much more so than the SBMV-B + SHMV infected tissue. The reason for this is unknown.

Fig. 13. SBMV-C antigen detected in "Bountiful" bean primary leaf tissue doubly-inoculated with SBMV-C and SHMV by tissue print immunoblotting. Dark purple stain is present in epidermis. mesophyll and vascular parenchyma but not in conducting bundles. A. Cross section of leaf blade and lateral vein (magnification, 100X). B. Cross section of leaf blade and lateral vein (magnification, 200X).



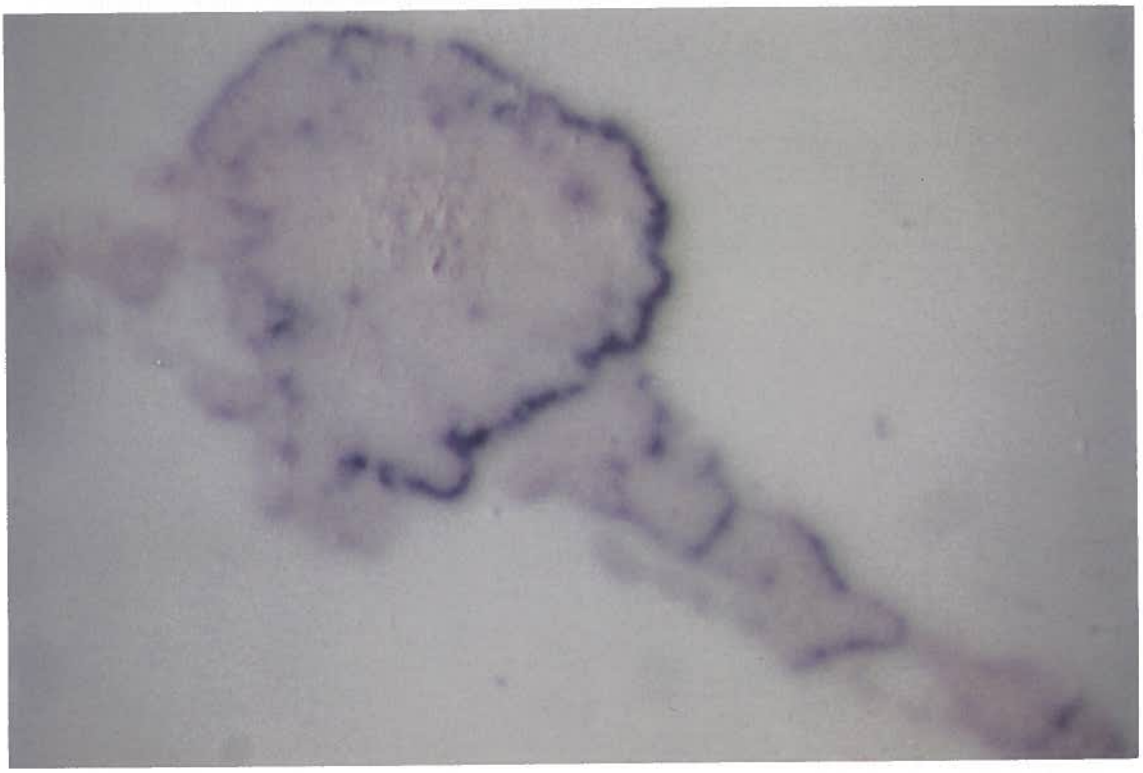
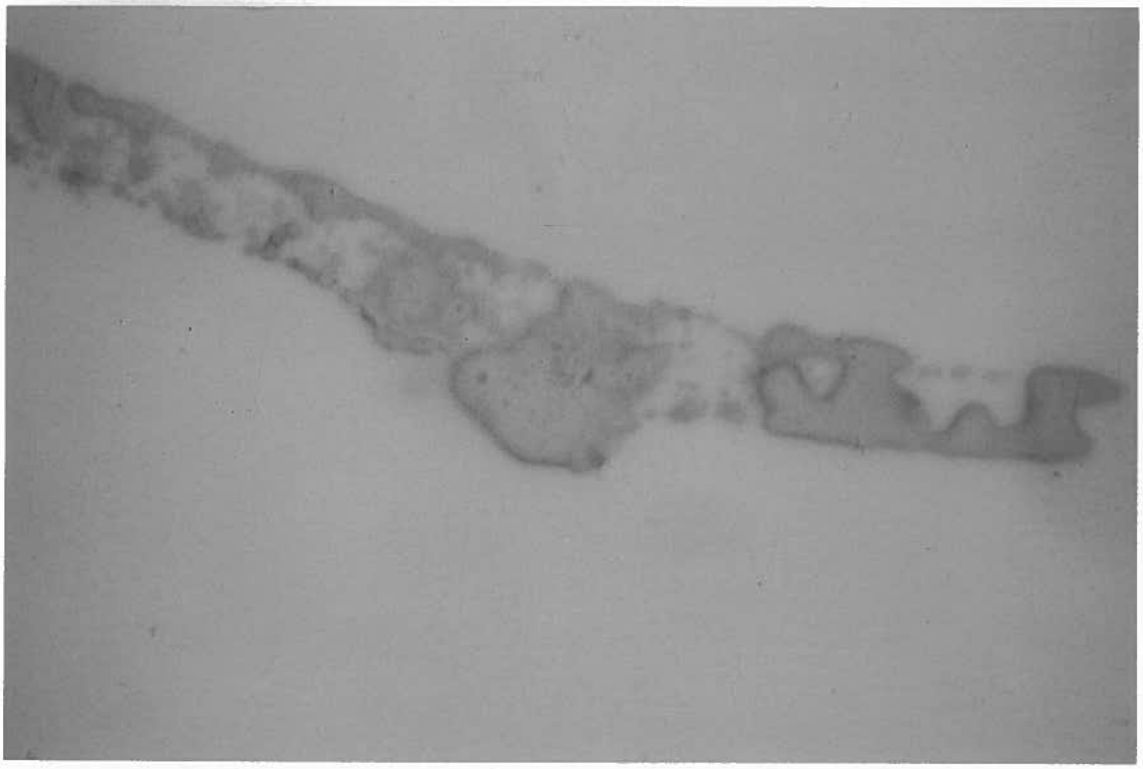
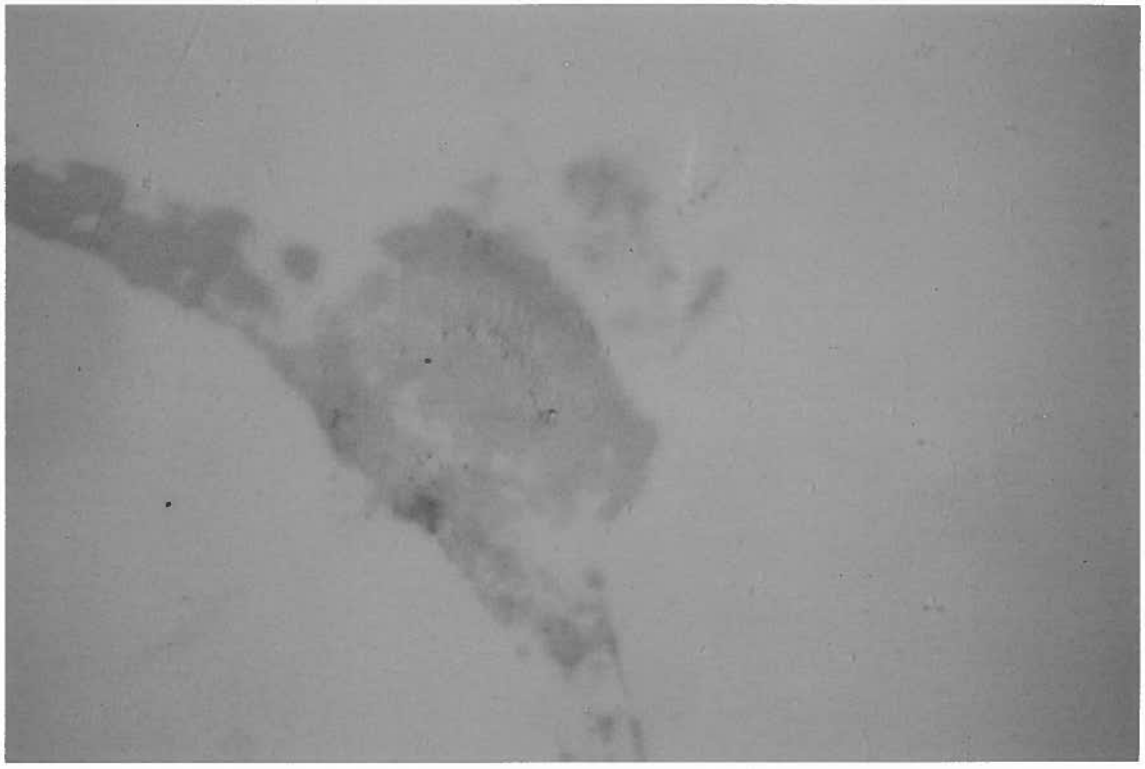


Fig. 13

Fig. 14. Tissue prints of healthy "Bountiful" primary leaf tissue showing a light purple background, clearly distinguishable from the positive dark purple coloring that results when the antigen is present. A. Cross section of leaf blade and lateral vein (magnification, 100X). B. Cross section of leaf blade and lateral vein (magnification, 200X).



A



B

Fig. 14

Fig. 15. Immunofluorescent labelling of a thick section of a primary "Bountiful" leaf doubly-inoculated with SBMV-B and SHMV. A) Mesophyll and vascular tissue cells under bright field microscope B) Same section under fluorescent microscope; mesophyll and companion cells fluoresce strongly when antiserum was diluted 1/500. (Magnification 430 X).

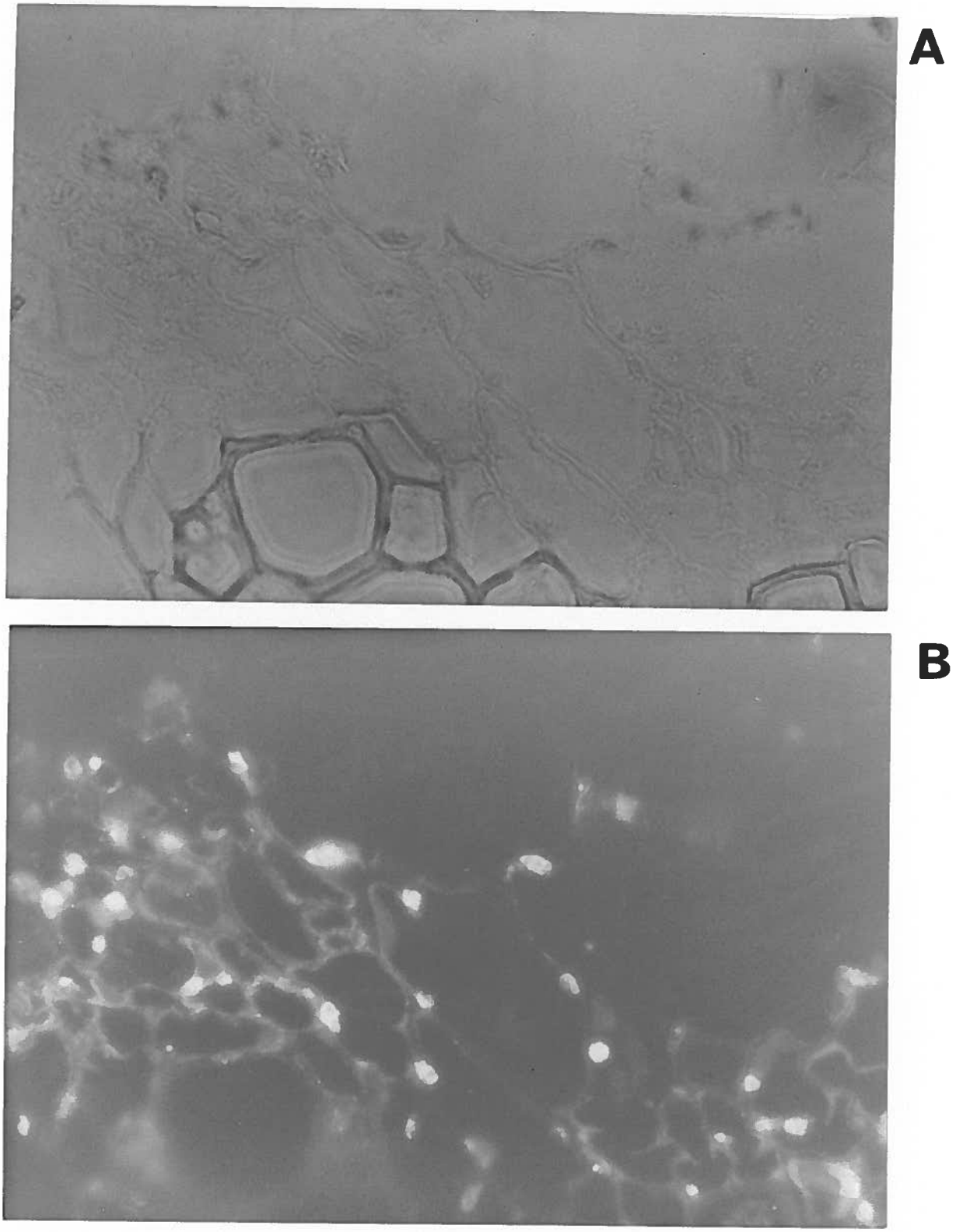


Fig. 15

Fig. 16. Immunofluorescent labelling of a thick section of "Bountiful" bean primary leaf mock-inoculated with phosphate buffer. A) Mesophyll and vascular tissue cells under bright field microscope B) Same section examined under fluorescent microscope; some background fluorescence can be seen when antiserum was used at 1/100 dilution. (Magnification 430 X).

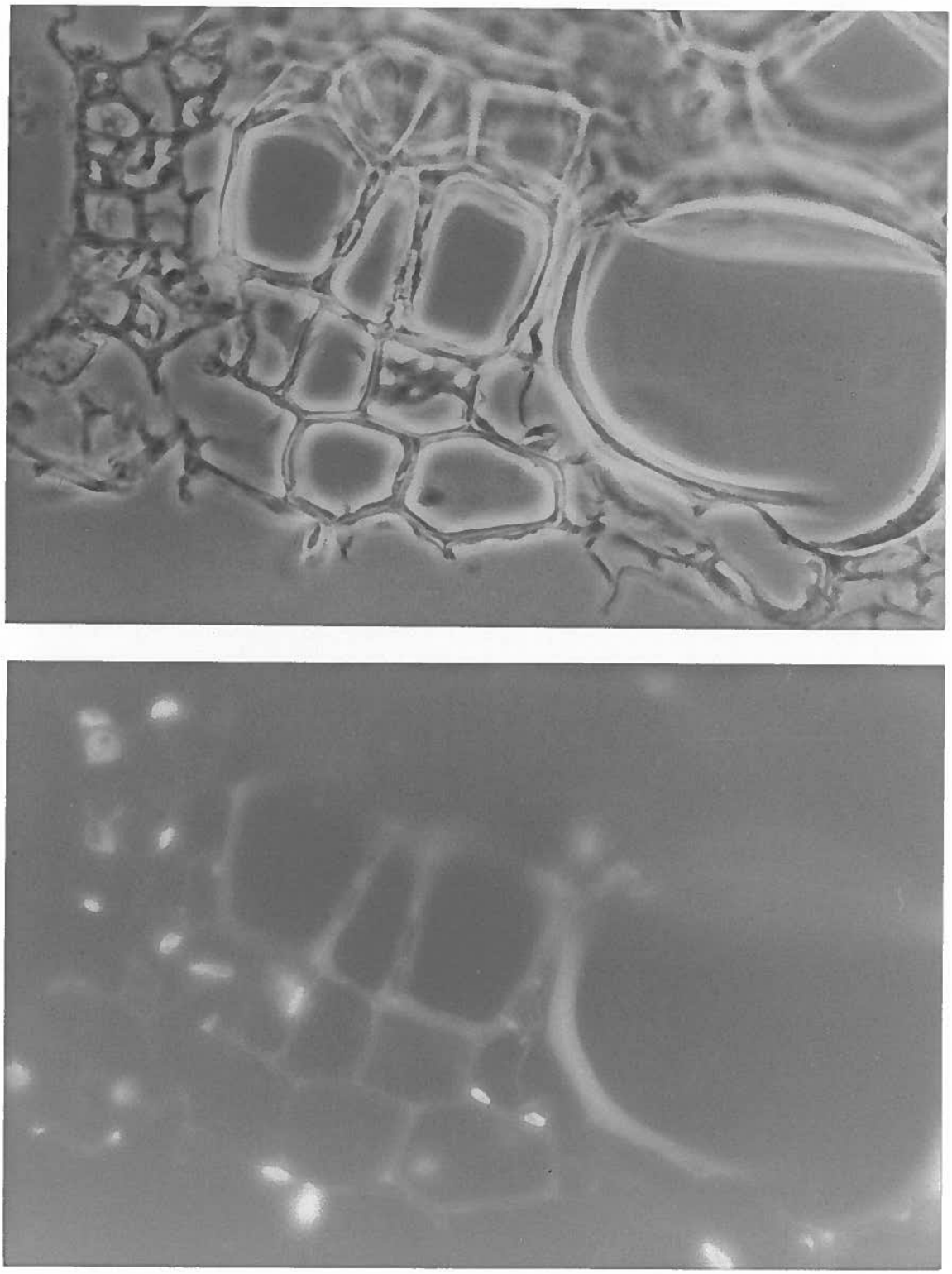
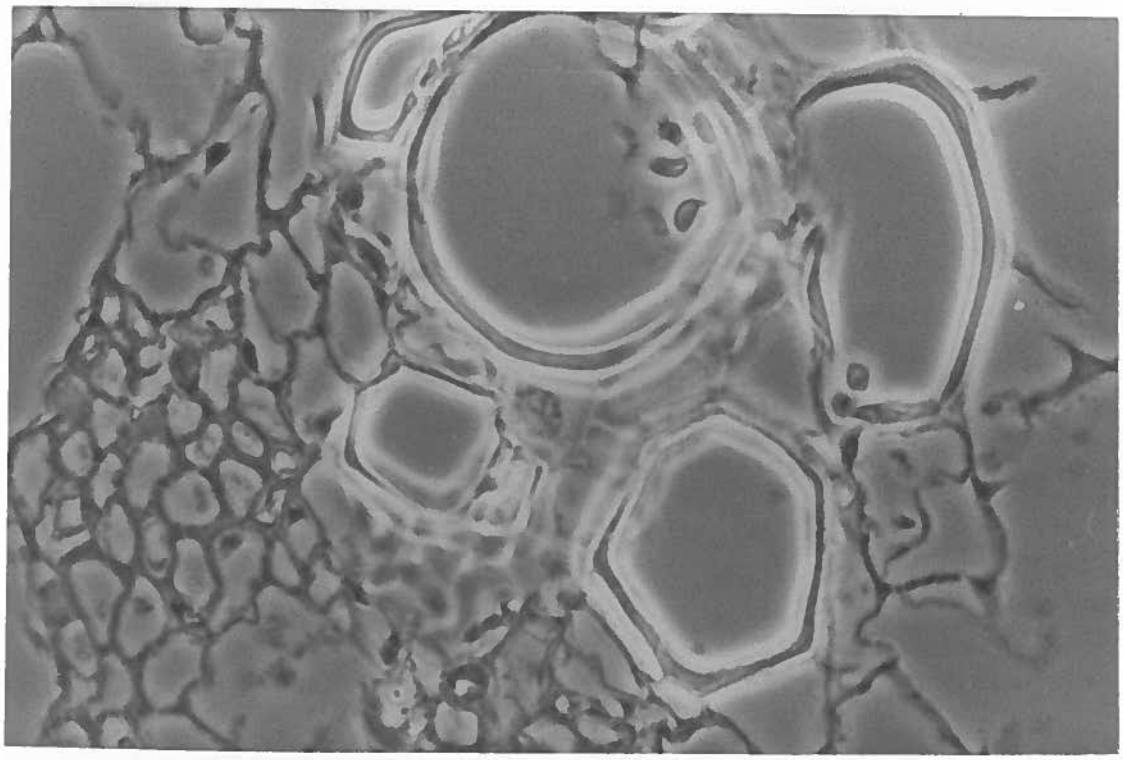
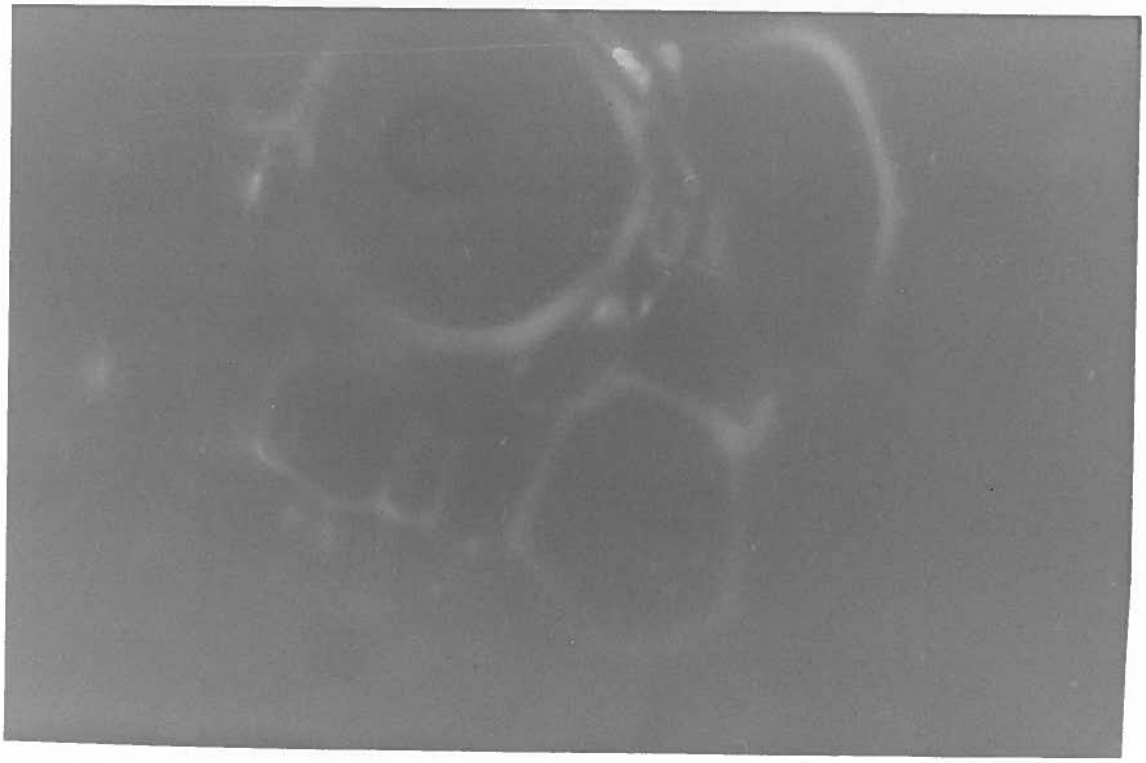


Fig. 16

Fig. 17. Immunofluorescent labelling of a thick section of "Bountiful" bean primary leaf mock-inoculated with phosphate buffer. A) Mesophyll and vascular tissue cells under bright field microscope B) Same section examined under fluorescent microscope; practically no background was present when antiserum was used at 1/500 dilution. (Magnification 430 X).



A



B

Fig. 17

4.3.5 Viral particle distribution in thin sections of leaf and petiole by standard osmium tetroxide fixation

Ultrathin sections of tissue embedded in LR White medium or Epon were observed with the electron microscope. Samples from three different treatments were compared; SBMV-B + SHMV- and SBMV-C + SHMV-infected Bountiful primary leaves and healthy Bountiful primary leaves. An average of 32 different grids for each treatment was examined.

Both viruses are clearly visible in mesophyll and phloem companion cells in sections of SBMV-B + SHMV-infected material (Fig.18A, B). SBMV-B was found in great amounts in vesicles in the cytoplasm as well as in the nucleus. In sections of SBMV-C + SHMV-infected tissue, only SHMV was clearly visible in mesophyll and phloem companion cells, but no vesicles with SBMV-C particles were found in the cytoplasm (Fig. 19). Some "virus-like" particles could be seen alongside the plasmalemma, but these were very much like ribosomes also found in healthy tissue (Fig. 20A, B).

In an attempt to distinguish virus particles from ribosomes, the tissue was treated with RNase. The RNase destroyed the ribosomes without harming the virus particles. In SBMV-B + SHMV-infected tissue, some

Fig. 18. Thin section of Epon-embedded "Bountiful" primary leaf tissue doubly inoculated with SHMV and SBMV-B. Both viruses were found in large amounts both in cytoplasm and in vesicles in mesophyll and companion cells. A) View of a cell with vesicles and cytoplasm containing both viruses; ve, vesicles; m, mitochondria; cw, cell wall; cl, chloroplast; v, virus. B) Close-up of two vesicles, one with spheres and another one with rods corresponding to SBMV-B and SHMV, respectively. (Bars represent 1 um).

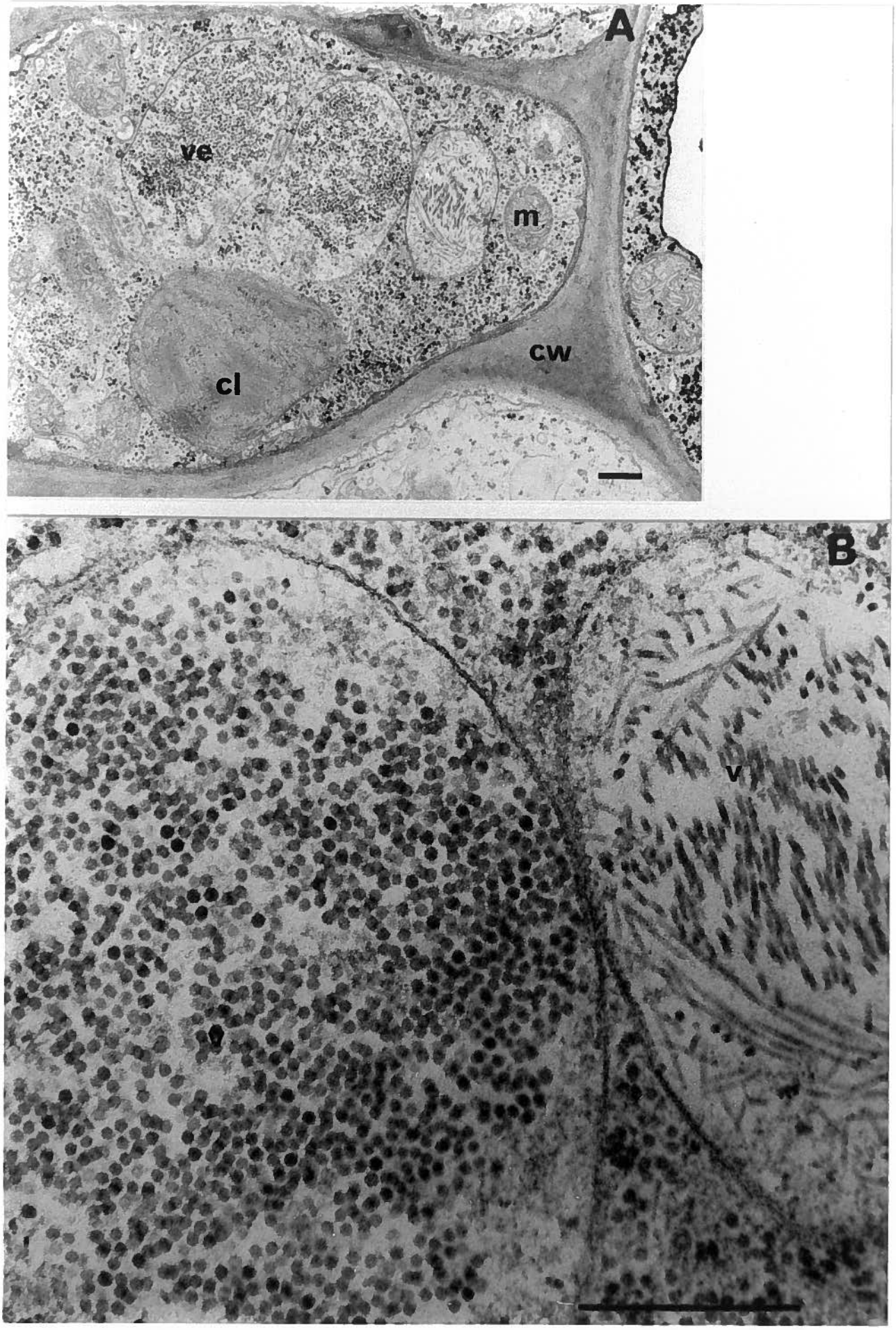


Fig. 18

Fig. 19. Thin section of Epon-embedded "Bountiful" primary leaf tissue doubly-inoculated with SHMV and SBMV-C. No spheres in vesicles or cytoplasm are visible, but large amounts of rods are present in some cells and are clearly visible when aligned in rows (v, virus). cc, companion cell; cl, chloroplast; v, virus; se, sieve element. (Bar represents 1 μm).

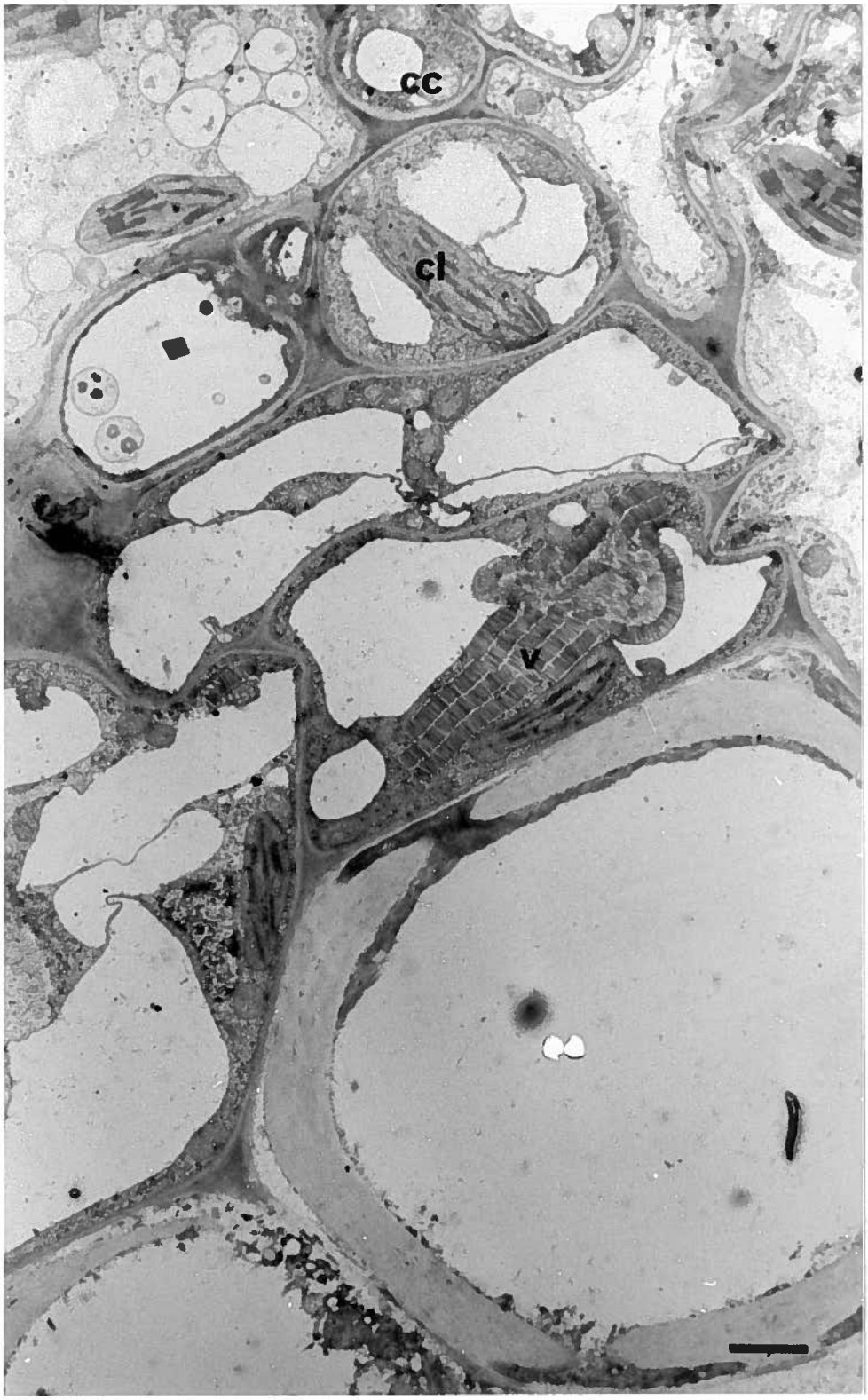


Fig. 19

Fig. 20. Thin sections of Epon-embedded "Bountiful" primary leaf tissue. A) Cell in leaf doubly inoculated with SHMV and SBMV-C; only rods are clearly visible, although ribosomes could be confused for viral spheres. B) Cell in leaf (mock-inoculated) showing ribosomes in the cytoplasm; r,ribosomes; cw, cell wall. (Bars represent 250 nm).

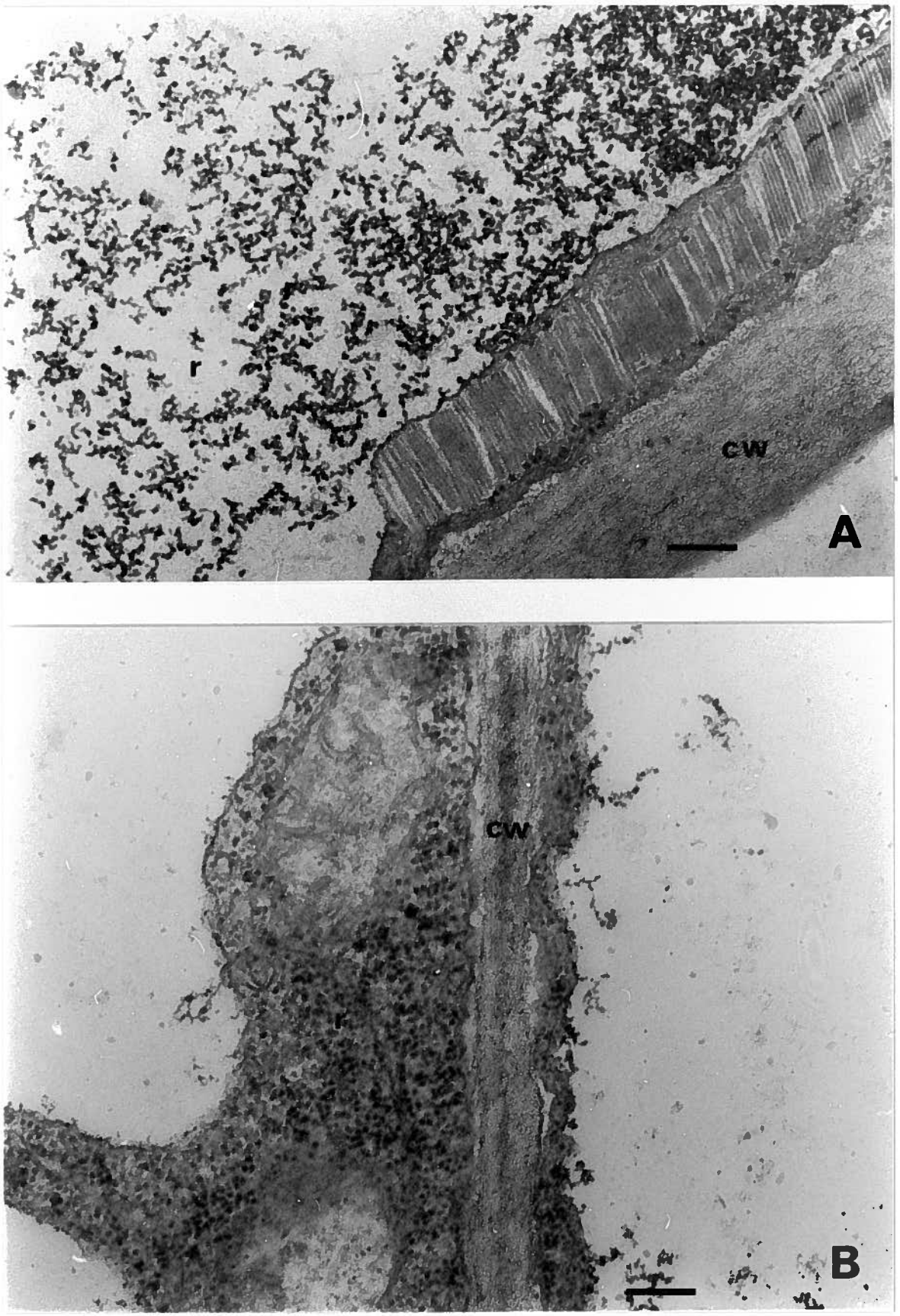


Fig. 20

deterioration of the tissue was observed after the treatment (general view of several cells in Fig. 21A, with details in B,C), but there were virtually no ribosomes present and both viruses were clearly visible (Fig. 21B, C). Tissue infected with SBMV-C + SHMV was very deteriorated; once again SHMV was present yet there was no evidence of SBMV-C particles in the cells (Fig.22A, B). Healthy controls also showed membrane disruption after treatment yet there were no ribosomes in the cells, proving that the enzyme treatment was effective (Fig. 23A, B).

4.3.6 Viral particle distribution in thin sections of leaves by immunogold labelling

Gold labelling was first tested on grids which had been floated on a homogenate of SBMV-C- or SBMV-B-infected tissue. Whole antisera as well as purified IgG were used; dilutions of 1/100 gave satisfactory results (Fig. 24 A, B).

Tissue from many different SBMV-C + SHMV-infected bean leaves was observed over a period of nine months. During these observations, no SBMV-C could be seen in any of the sections, although SBMV-B could be readily seen in SBMV-B + SHMV-infected bean and SBMV-C could be seen in SBMV-C-infected cowpea tissue.

Fig. 21. Thin sections of RNase-treated "Bountiful" primary leaf tissue doubly-inoculated with SHMV and SBMV-B. A) General view of a group of cells with large vacuoles. The granular matrix in the vacuoles (bottom left cell) as well as the granular material in the nucleus (top right cell) are representative of masses of virus particles shown at higher magnifications in B and C. B) Nucleus with SBMV-B and cytoplasm with SBMV-B and SHMV, C) Higher magnification (10 X that of B) of cytoplasm in B, both spheres and rods are present. (Bars represent 1 *u*m).

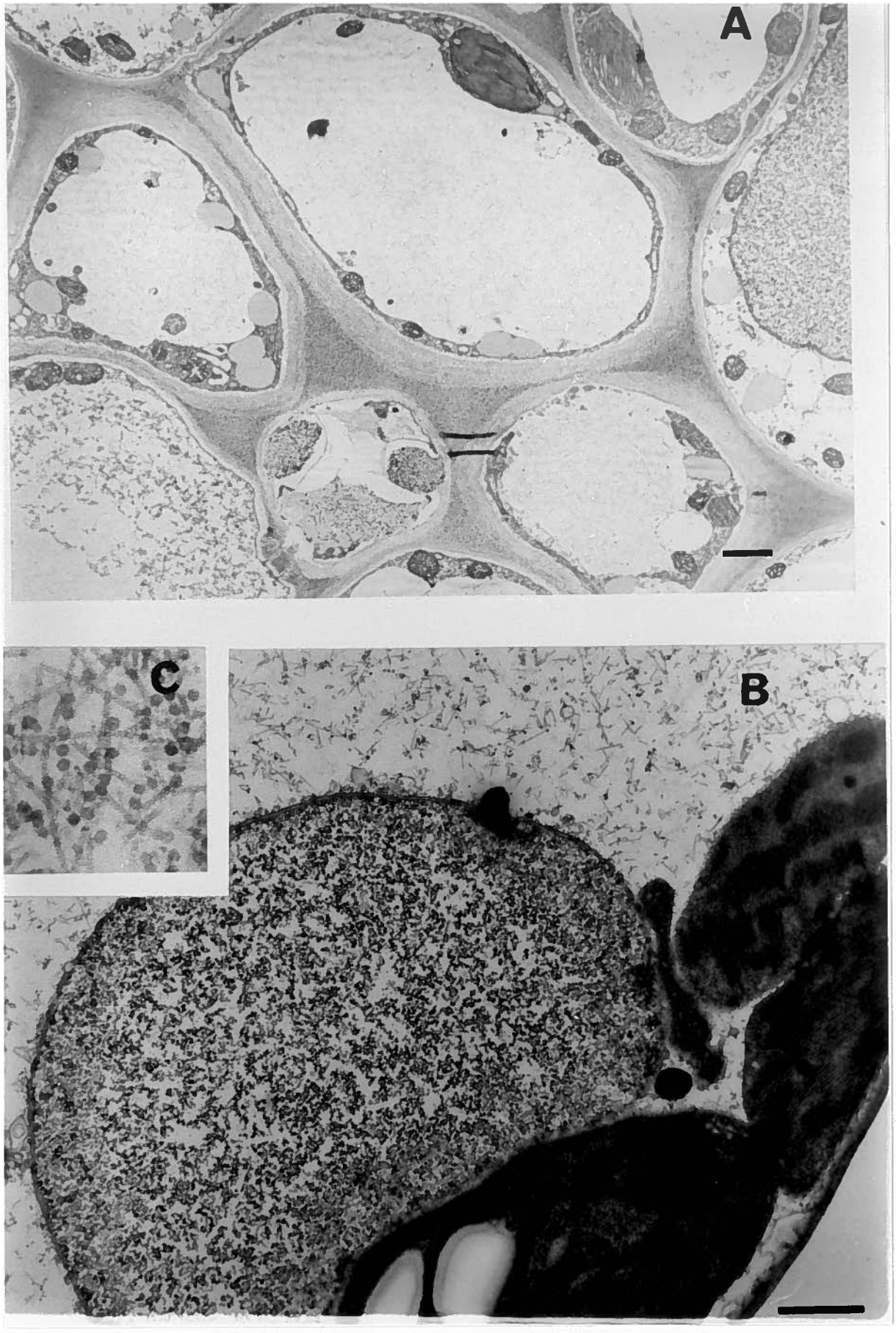


Fig. 21

Fig. 22. Thin section of RNase-treated "Bountiful" primary leaf tissue doubly-inoculated with SBMV-C and SHMV. A) Degraded ribosomes can be seen as clumps of amorphous material, vacuoles and cytoplasm contain large amounts of SHMV. v, virus; cc, companion cell; se, sieve element. B) Higher magnification of cytoplasm with virus and degraded ribosomes. (Bars represent 500 um).

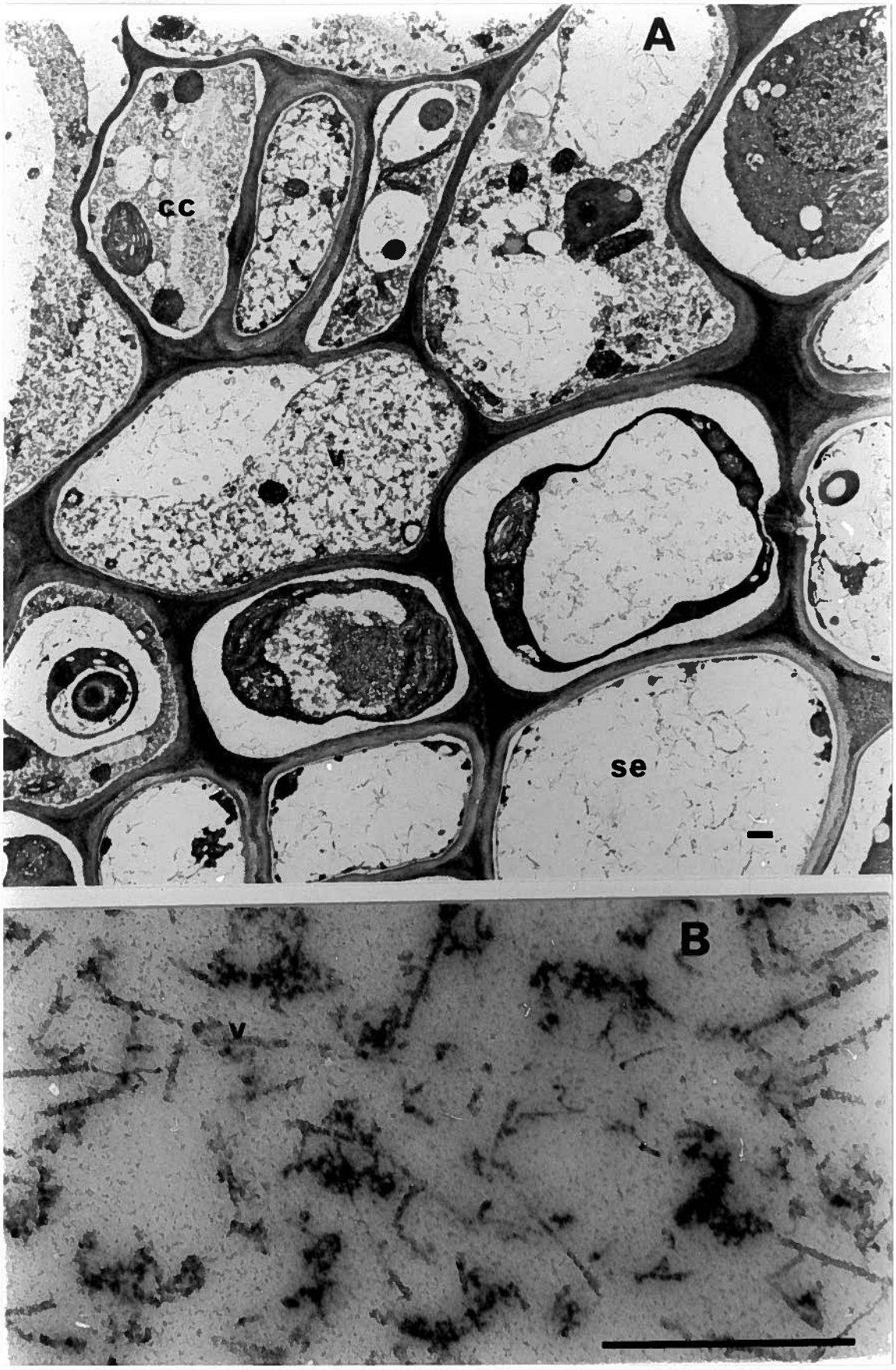


Fig. 22

Fig. 23. RNase-treated "Bountiful" primary leaf tissue mock-inoculated with phosphate buffer. A) Ribosomes in treated tissue can be seen as amorphous masses or in clumps, their distinct shape lost. B) Higher magnification of cells with degraded ribosomes. cl, chloroplast. (Bars represent 1 *u*m).

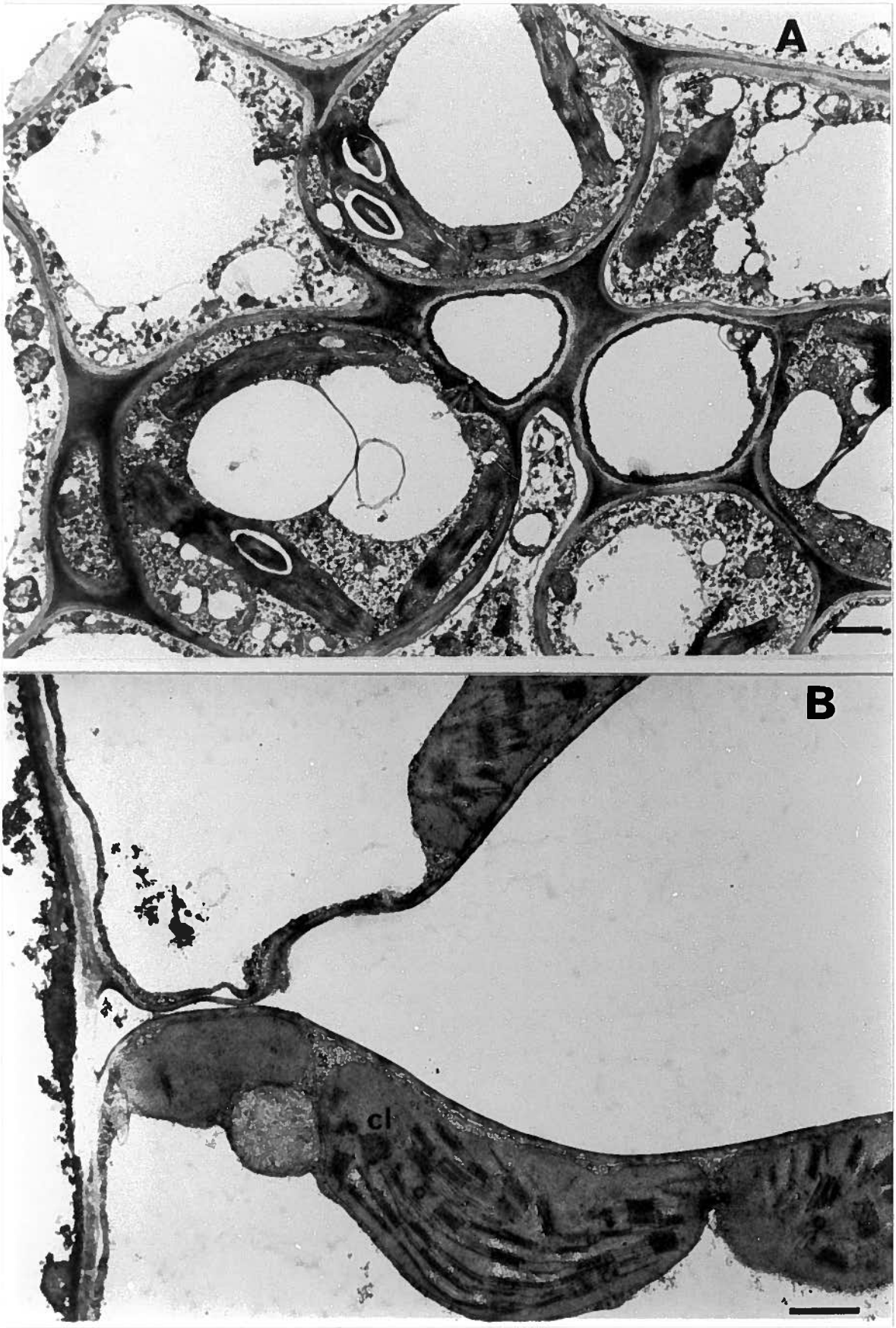


Fig. 23

Fig. 24. Specific gold-labelling of SBMV in leaf-dips of homogenates of infected tissue A) Homogenate of SBMV-B + SHMV-infected bean, spheres specifically are labelled. B) Homogenate of SBMV-C + SHMV-infected cowpea, spheres are specifically labelled. g, gold; v, virus. (Bars represent 100 nm).

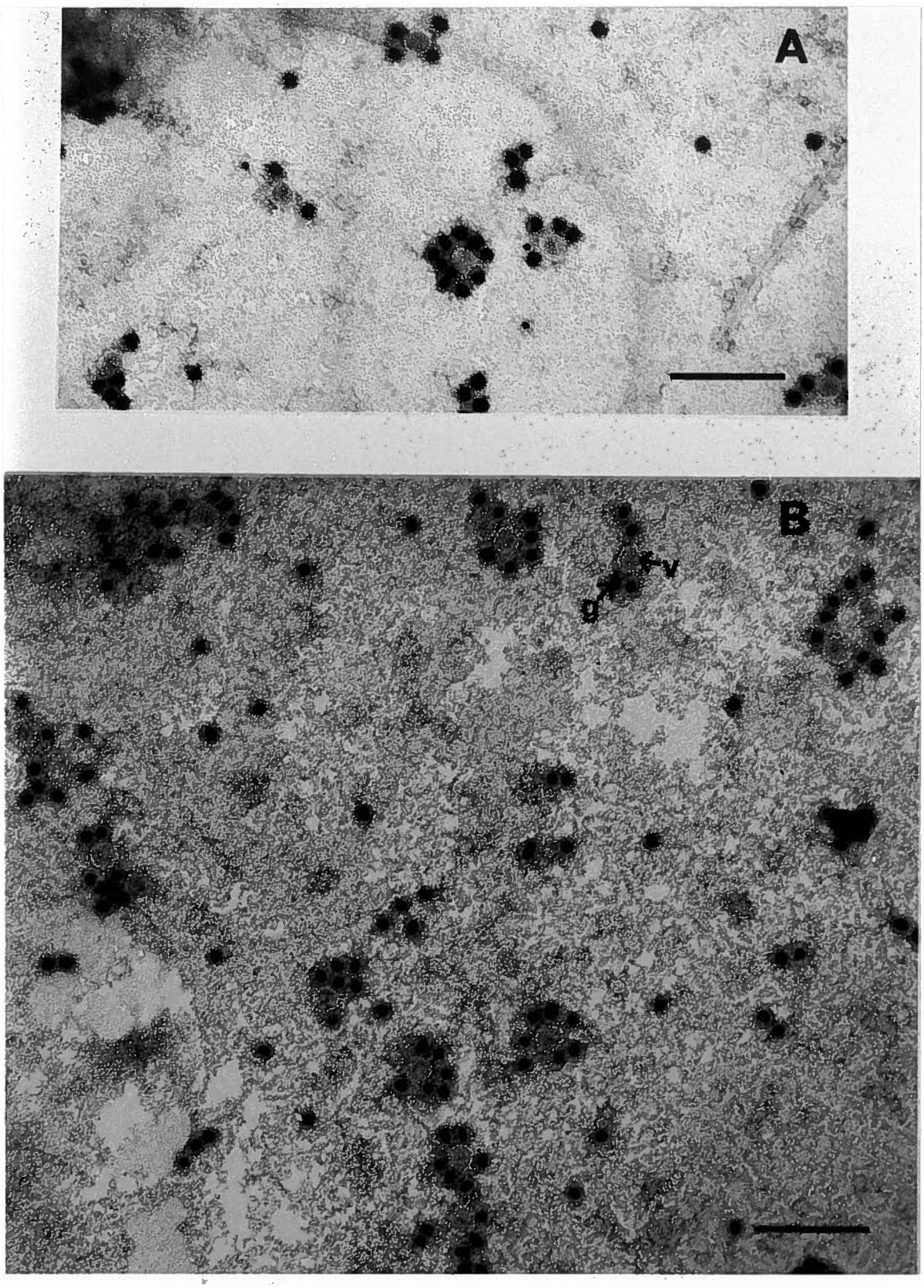


Fig. 24

Given these results, tissue was fixed for gold labelling after it had been blotted on nitrocellulose paper and tested for the presence of antigen, as described in section 4.2.5. Strips which showed positive signals on the filters were further treated and embedded in Epon and L.R. White resins. The fixation process involved the use of formaldehyde-glutaraldehyde and picric acid, and avoided the use of osmium tetroxide in order to maintain the antigenicity of the viral proteins.

After sectioning the blocks, several antibody and gold concentrations were assessed to determine optimal conditions for labelling. Whole antiserum and purified IgG were used at a 1/100 dilution and 15 nm and 10 nm gold particles were used at a dilution of 1/5 (v/v). Positive controls included bean tissue inoculated with SBMV-B + SHMV and cowpea tissue inoculated with SBMV-C. In both cases, large amounts of gold-labelled virus particles could be found in the mesophyll (Fig. 25A, B, C and Fig. 26A, B, C) and vascular parenchyma. No SBMV-C particles were found in bean tissue inoculated with SBMV-C + SHMV (Fig. 27A, B), but in some of the mesophyll cells, clumps of amorphous material appeared heavily labelled (Fig. 28 A, B, C and 29A, B). These clumps were seen in large vacuoles where SHMV particles were clearly present. These clumps were never observed in tissue infected with SBMV-B + SHMV, yet similar amorphous material has been observed in protoplasts inoculated with cowpea chlorotic mottle virus (van Lent, 1988).

Fig. 25. Gold-labelling of SBMV-B antigen in thin sections of "Bountiful" primary leaf tissue doubly-inoculated with SBMV-B and SHMV. A) Partial view of mesophyll cell with SBMV-B virus particles in nucleus and cytoplasm labelled with protein A-gold. Cl, chloroplast; N, nucleus; n, nucleolus. B) Higher magnification of virus particles in nucleus; g, gold particles; v, virus. C) Higher magnification of nucleus and nucleolus. (Bars represent 500 nm).

C
N
n
A
I
N
n
CL
B
V
Fg

Fig. 26. Gold-labelling of SBMV-C antigen in thin sections of cowpea primary leaf tissue doubly-inoculated with SBMV-C and SHMV. A) Partial view of mesophyll cell with SBMV-C virus particles in the cytoplasm. B) and C) Higher magnifications of the same area showing specific-labelling of the virus particles. v, virus; g, gold; cl, chloroplast. (Bars represent 500 nm).

C
CL
A
B
v
g

Fig. 27. Thin section of "Bountiful" primary leaf doubly-inoculated with SBMV-C and SHMV. A) No gold label was found in the nucleous, yet great amounts of SHMV could be seen in most cells. N, nucleous; cl, chloroplast. B) Some background labelling could be found in the cell wall, but no virus was labelled in the cytoplasm. cw, cell wall; c, cytoplasm; v, virus. (Bars represent 500 nm).

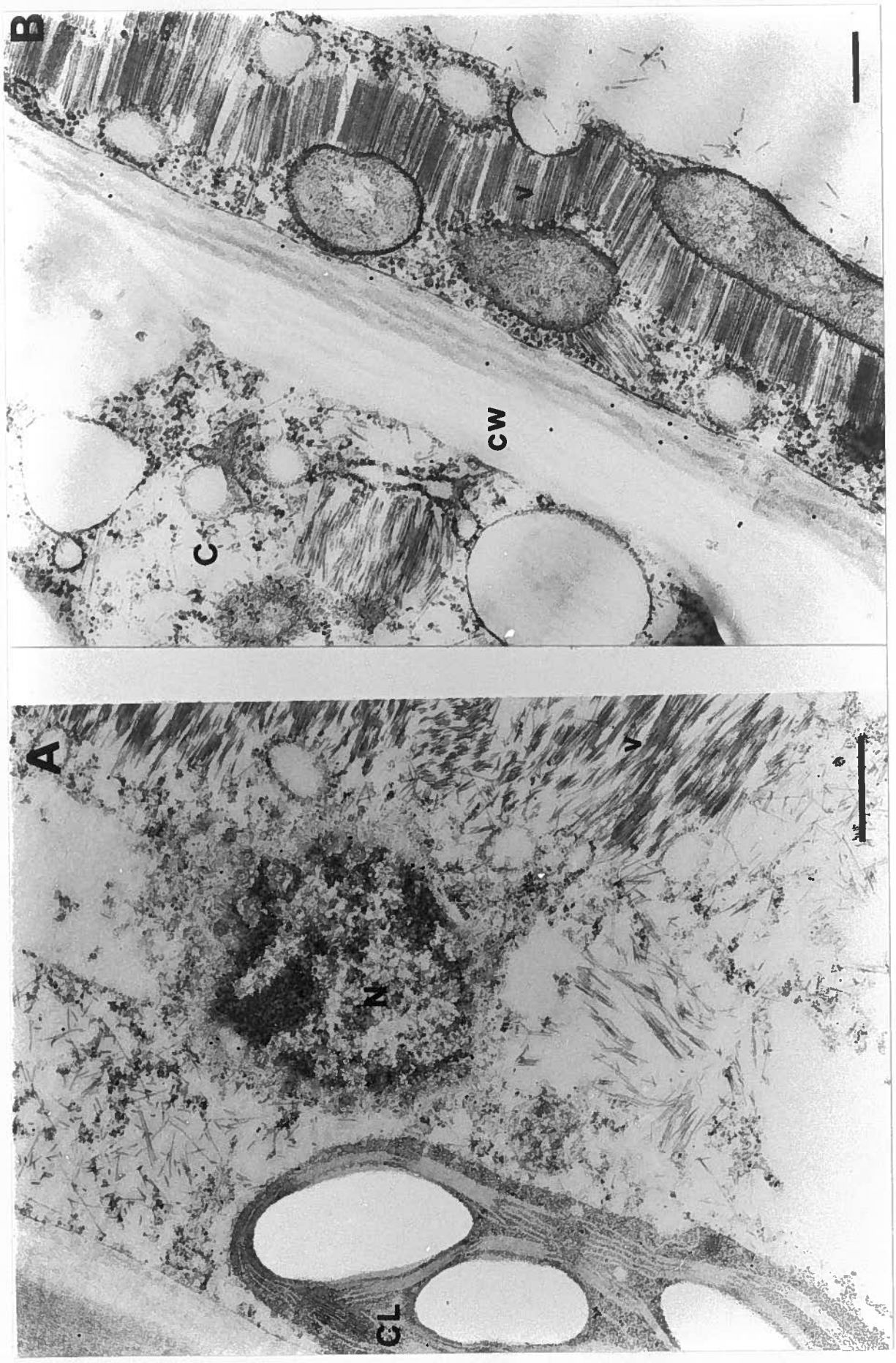

B
V
CW
C
A
V
N
CL

Fig. 28. Gold-labelling of thin section of "Bountiful" primary leaf doubly-inoculated with SBMV-C and SHMV. A, B and C are different magnifications of clumps of amorphous material found in vacuoles in some of the mesophyll cells. g, gold; v, virus (rod); p, protein. (Bars represent 500 nm).

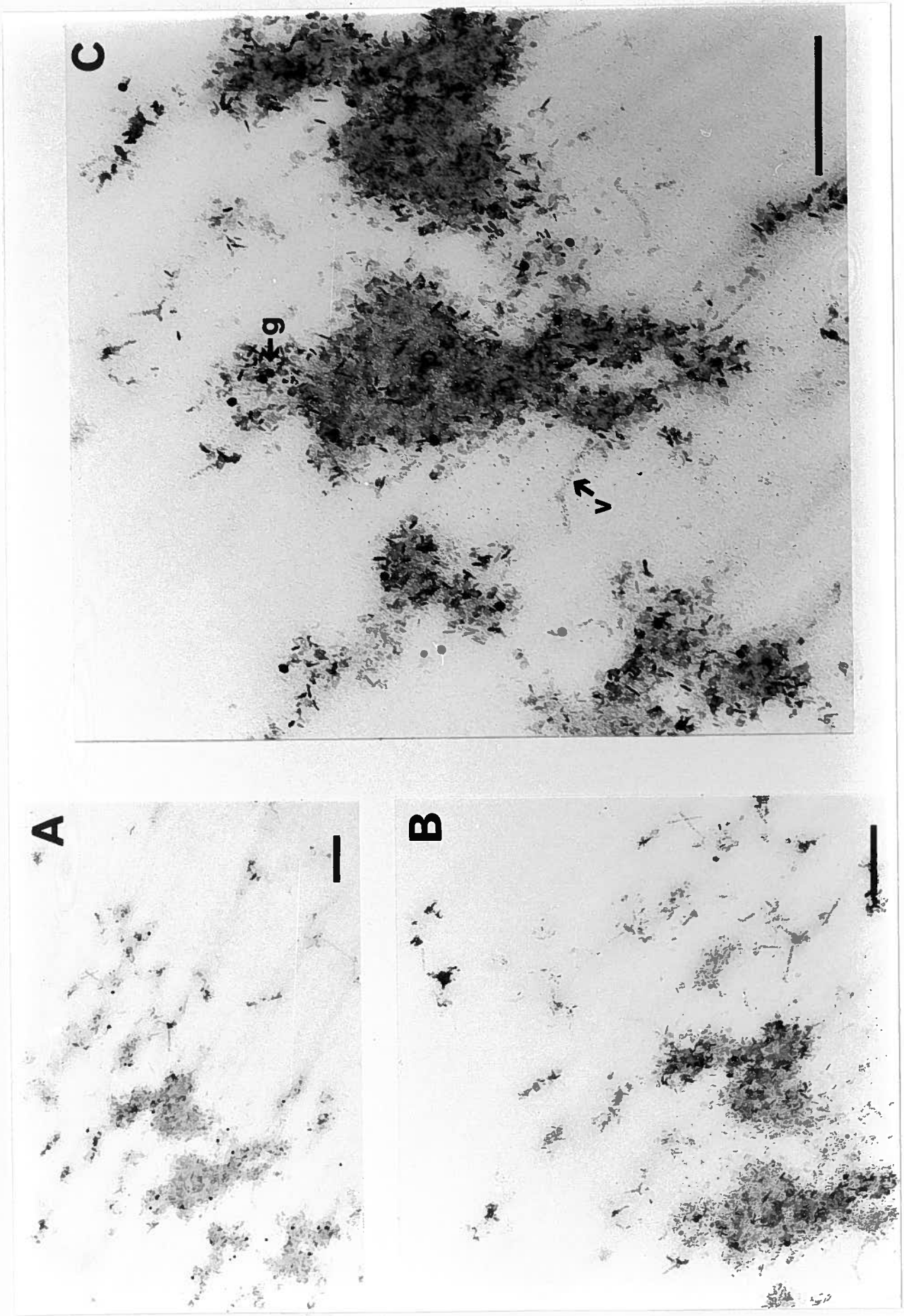
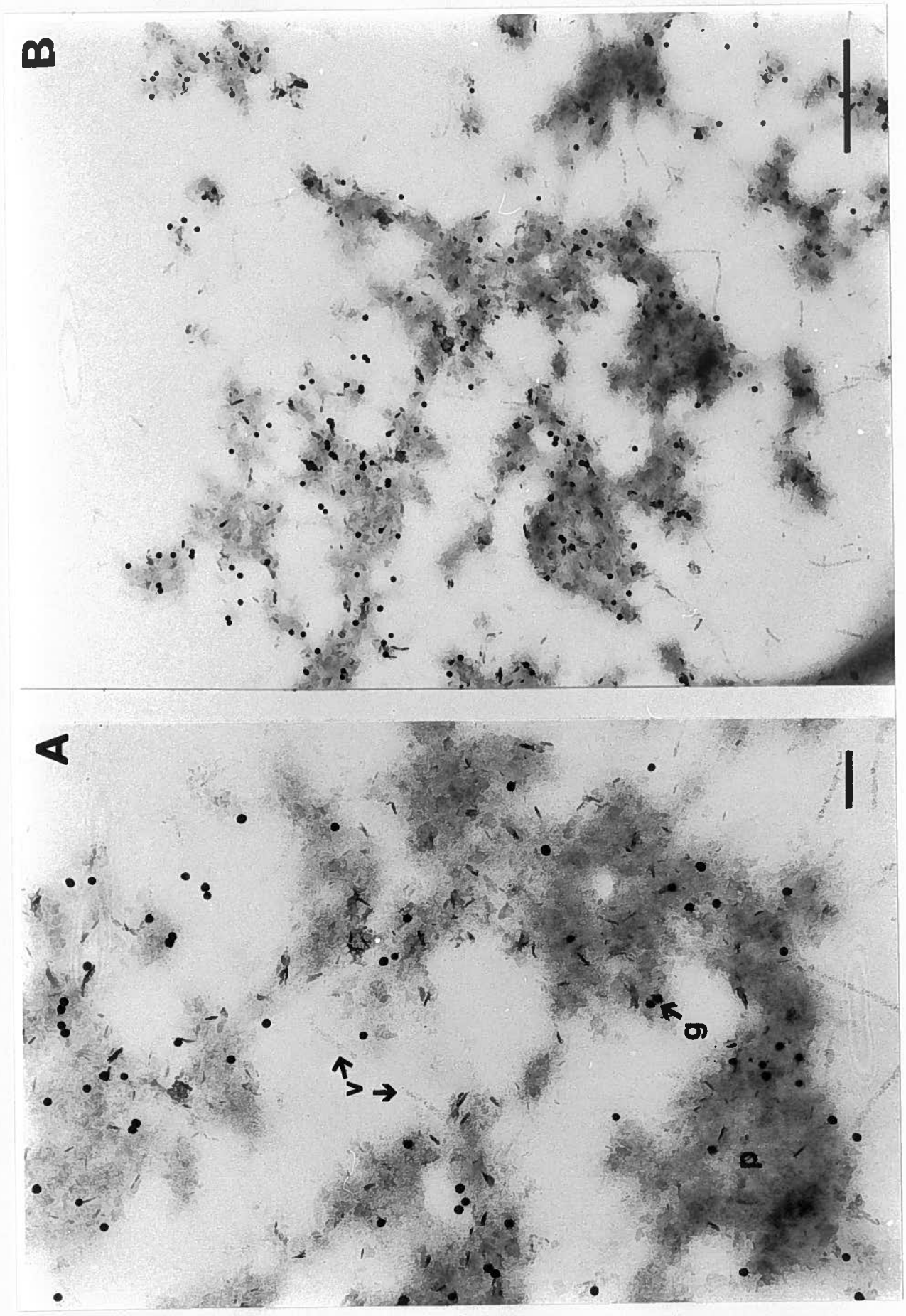

C
g
v
A
B

Fig. 29. Gold-labelling of thin sections of "Bountiful" primary leaves doubly-inoculated with SBMV-C and SHMV. A and B are different magnifications of clumps of heavily-labelled amorphous material found in vacuoles of mesophyll cells. v, virus (rods); g, gold; p, protein. (Bars represent 200 nm).


B
A

4.3.7 Electron microscopy observations after trapping of SBMV-C in mixed-infected leaves

Immunosorbent electron microscopy of extracts from primary bean leaves doubly inoculated with SBMV-C and SHMV resulted, in some cases, in grids covered with small particles of about 15-20 nm (Fig. 30A). These particles correspond to the T=1 structure which is assembled from 60 coat protein subunits instead of 180 subunits in typical virions. Homogenates from cowpea infected with SBMV-C and trapped with the same antibody resulted in grids covered with particles of about 28-30 nm (Fig. 30B), the size of typical virions (Hull,1987). No visible particles were seen in extracts of crude sap from healthy plants treated in the same manner (Fig. 30C).

4.3.8 Electrophoresis and Western blotting

Total soluble proteins from both infected and healthy bean and cowpea were electrophoresed on a denaturing SDS-polyacrylamide gel. When gels were transferred onto nylon membranes, incubated with either a polyclonal (not shown) or monoclonal SBMV-C antibody and then developed with NBT-BCIP substrate, no differences were found in the migration of SBMV-C coat protein (29 KDa) from cowpea or bean SBMV-C + SHMV-infected primary leaves (Fig. 31). The monoclonal antibody reacted with one other protein of

Fig. 30. SBMV-C-specific trapping of antigen from homogenates of infected tissue. A) Homogenate from "Bountiful" primary leaves doubly-inoculated with SBMV-C and SHMV. Trapped particles have a diameter of approximately 18 nm. B) Homogenate from cowpea primary leaves doubly-inoculated with SBMV-C and SHMV. Trapped particles have a normal diameter of approximately 30 nm. C) Homogenate from healthy plants; no particles can be seen. (Bars represent 100 nm).

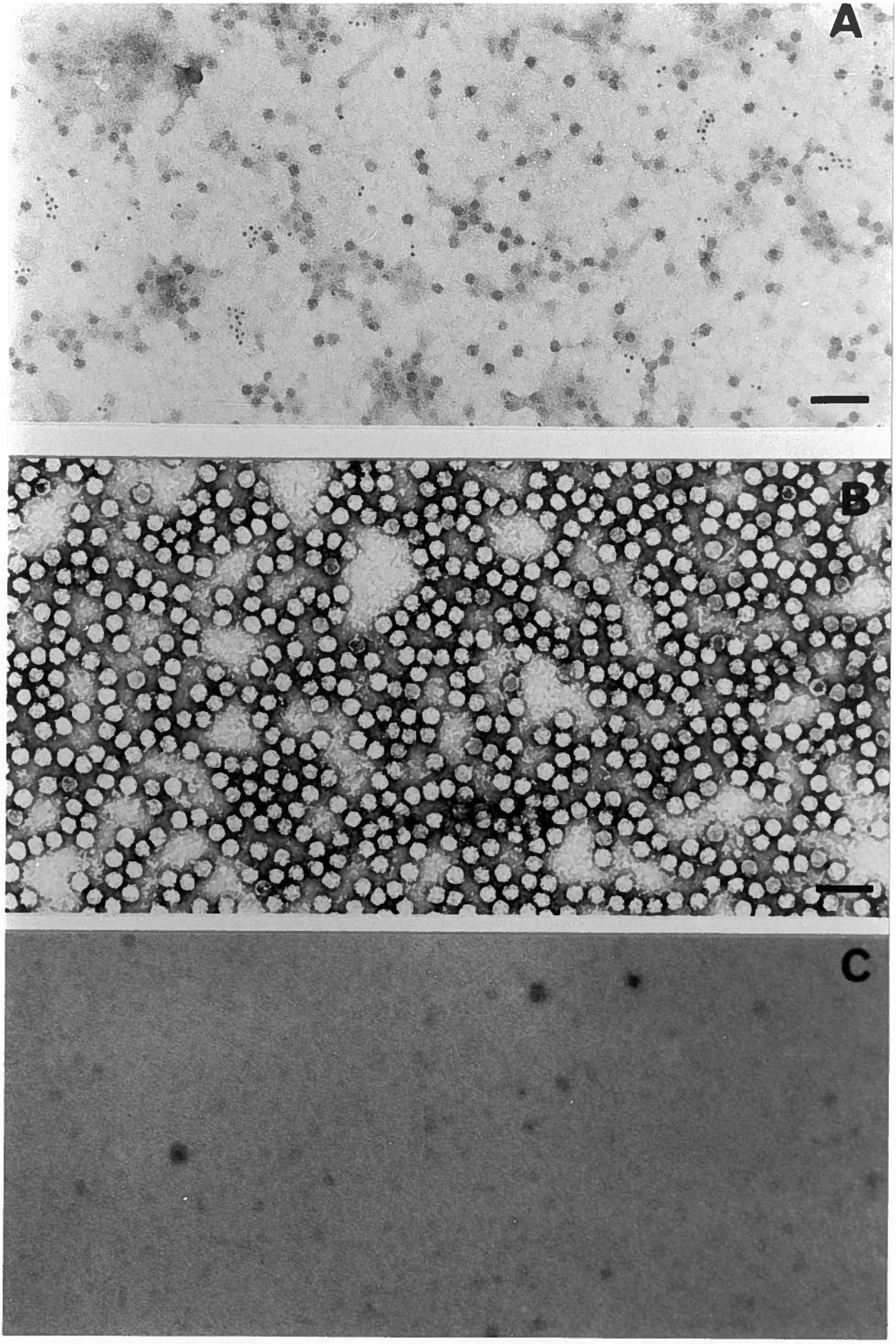


Fig. 30

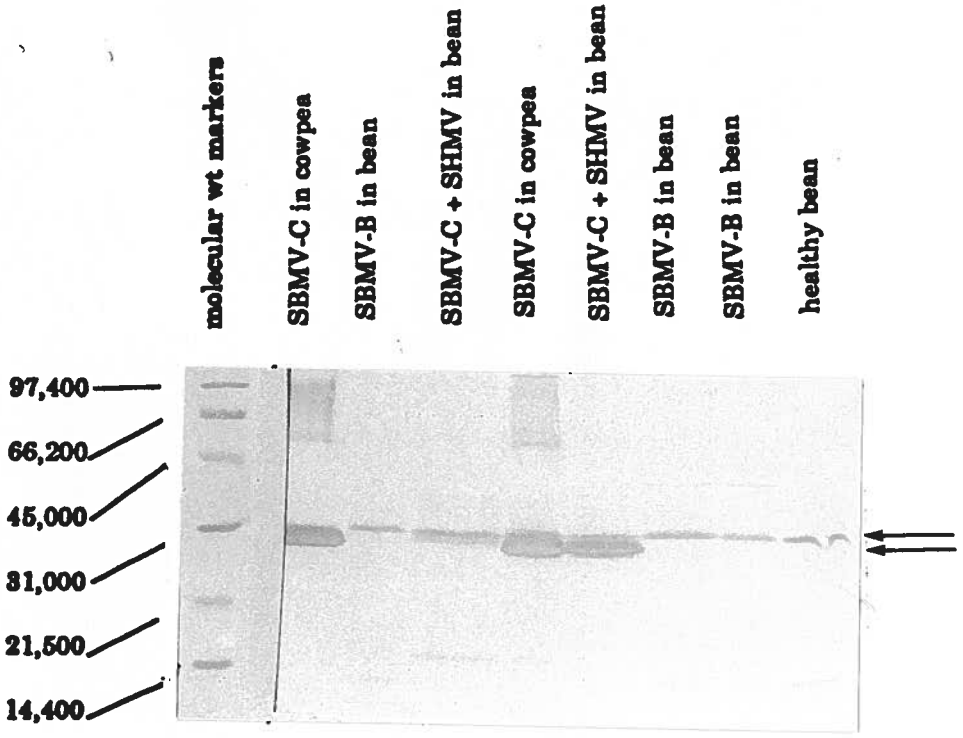


Fig. 31. Western blot analysis of SBMV-C coat protein migration from cowpea and bean primary inoculated leaves. Partially purified leaf proteins, extracted as described in Material and Methods, were electrophoresed through 12% (w/v) SDS-polyacrylamide gel, transferred to a nylon membrane, reacted with anti-SBMV-C specific monoclonal antibody and developed as described in Materials and Methods. Molecular weight markers were stained with Coomassie blue. A protein of approximately 32 KDa in both healthy and SBMV-B-infected bean reacted with monoclonal antibody (upper arrow). SBMV-C coat protein migrated to the same position as SBMV-C coat protein from cowpea (lower arrow), indicating that the size of the protein produced in SHMV + SBMV-C-infected primary bean leaves was the same as that produced in SBMV-C-infected cowpea primary leaves.

approximately 32 KDa, present in healthy bean (Fig. 31) and cowpea tissue (not shown). The unexpected reaction of the monoclonal antibody with a protein in healthy tissue could be due to the presence of a similar epitope, in an endogenous plant protein, to that against which the monoclonal was raised. The same antibody did not react against SBMV-B coat protein (Fig. 31). These results indicate that the SBMV-C coat protein produced in SBMV-C-infected bean was the same size as that produced by SBMV-C-infected cowpea.

Chapter 5

Discussion

The results of these experiments provide an example of a system in which one virus (SHMV) enables a second virus (SBMV-C) to move from cell to cell within the inoculated leaf of a plant that is not normally a host of SBMV-C. However, SHMV does not enable SBMV-C to move systemically through the plant.

The cowpea strain of SBMV replicated only subliminally in bean, having no effect on the appearance or development of inoculated plants. Conversely, when SBMV-C and SHMV were inoculated either simultaneously or sequentially (at intervals not exceeding 24 h), SBMV-C replicated and produced large amounts of infectious material and, in bean, cultivar "Pinto", caused characteristic necrotic pin-point local lesions. It is interesting to note that the bean strain of SBMV, in single inoculations, produced very similar local lesions in "Pinto" bean. This phenomenon seems to indicate that once SBMV-C concentration exceeds a certain threshold, it then triggers the defence mechanism in bean in a dose-dependent fashion, in a manner similar to that for SBMV-B. The host-virus reaction appeared to be somewhat specific, since SHMV alone failed to induce these symptoms in spite of the fact that it reached high titres in the leaf. The specificity of this particular host-virus reaction was confirmed by the fact that, upon mixed inoculation with SBMV-C and

SHMV, the other three bean cultivars ("Top Crop", "Tendergreen" and "Bountiful"), developed the same symptoms as those induced by single inoculation with SHMV.

The observed increase of infectious SBMV-C in mixed infections with SHMV could be due to either complementation of some replication function, which would augment the susceptibility of infected cells, or to complementation of a movement factor/function which would allow the dependent virus to move into neighbouring cells and replicate. The first hypothesis was tested by examining the behaviour of SBMV-C in bean protoplasts. Results showed that SBMV-C replicated and produced infective particles in bean protoplasts to the same degree as SBMV-B. This suggests that the limited recovery of SBMV-C from inoculated bean leaves was not due to the inability of the cells to support viral replication. Further experiments showed that SHMV had no effect on the accumulation of SBMV-C in bean protoplasts. Thus, SHMV infection most likely augmented SBMV-C concentration in whole leaves by complementation of a movement factor. This could be a plant and/or viral factor, since spread of a virus within a plant depends on both the genome of the plant (Zaitlin and Hull, 1987) and the translation products of the viral genome (Atabekov and Dorokhov, 1984). One possible factor is the "transport protein" which is important in determining the host range of plant viruses (Taliansky et al, 1982b).

Mixed infection of bean with SBMV-C and SHMV produced high levels of SBMV-C antigen and infectious material only in inoculated primary leaves. Thus,

in the SBMV-C/SHMV system, the helper effect provided by SHMV was limited to short distance (cell-to-cell) transport. This phenomenon was not temperature-dependent, since the same results were obtained with inoculated plants grown at either 32 C or 25-28 C (Fuentes and Hamilton, 1988, 1991). Malyshenko et al (1989) obtained similar results in complementation experiments between different viruses within the tobamovirus group and between unrelated viruses from different taxonomic groups. The complementation of transport function was limited to spread of the dependent virus only in the inoculated primary leaves.

Comparison of the accumulation of SBMV-C and SBMV-B viral antigen in different parts of inoculated plants showed that, in mixed infections with SHMV, SBMV-C moved at the same rate as SBMV-B between epidermal and parenchyma cells of the leaf blade (Fig.8). However, the comparatively low quantities of SBMV-C in the veinal system of inoculated leaves and its absence in petioles, stems, roots and trifoliate leaves suggested that the virus was not being transferred from parenchyma into the conducting system. These results were further confirmed when virus antigens were detected in thick leaf sections after transfer onto nitrocellulose paper. When tissue from cowpea inoculated with SBMV-C was compared to bean tissue inoculated with a mixture of SBMV-C and SHMV, it was evident that, in the cowpea tissue, viral antigen could be found in the mesophyll and the conducting bundle, whereas in the bean tissue SBMV-C could only be found in epidermal and mesophyll cells of the leaf (Figs.11 to 14). The absence of infectious material in tissues other

than those in the inoculated leaves was also confirmed by bioassay and Southern-blot hybridization analysis (Fig.9).

The fact that SHMV, in both single and mixed infection, moved systemically in the host, but did not complement the long distance movement of SBMV-C, implies that a specific virus-plant interaction regulates viral movement between parenchyma cells and vascular tissue. Recent reviews suggest that viral movement is probably regulated at several levels by different host and viral genes (Atabekov and Taliansky, 1990; Hull, 1989). It is generally accepted that viruses move through plasmodesmata (for reviews see Esau, 1968; Meshi and Okada, 1987; Zaitlin and Hull, 1987; Hull, 1989; Robards and Lucas, 1990) and plasmodesmatal connections function differently in different symplastic domains (symplasm meaning a community of living plant cell protoplasts interconnected by plasmodesmata) (Erwee and Goodwin, 1985). Thus, it is likely that different plant and/or viral genes regulate cell-to-cell and long distance viral movement (eg. between parenchyma and vascular tissues).

One mechanism to explain cell-to-cell movement, proposed by Citovsky et al (1990) and Citovsky and Zambryski (1991), is based on their finding that the TMV 30 KDa movement protein is both an RNA- and single-stranded DNA-binding protein. They proposed that cell-to-cell transport of plant viruses occurs by the formation of an unfolded nucleic acid-movement protein complex which is targeted

to the plasmodesmata. The movement protein then interacts with the plasmodesmata, changing their size-exclusion limits, thus allowing for translocation of the viral nucleic acid.

Since the binding of RNA by the 30 KDa protein is non-specific (Citovsky et al, 1990), an analogous transport protein of SHMV could bind SBMV-C single-stranded RNA and transport it from cell to cell. In fact, Malyshenko et al (1988) showed that SHMV facilitated cell-to-cell transport of the B component RNA of red clover mottle comovirus (RCMV), which does not encode either the transport 58 KDa/48 KDa protein or the coat protein, in cowpea. This provides evidence that a virus may act as a helper for a viral RNA component which does not encode its own transport or coat protein. On the other hand, a more recent study by Taliansky et al (1992) suggested that the presence of the full length TMV genome or a certain TMV-encoded product(s) other than the 30 KDa protein is required for complementation of the RCMV-B RNA. Thus, the interaction of the movement protein with the viral RNA and with the plasmodesmata are not enough to promote viral movement.

Another factor that the 30 KDa protein-RNA binding model does not account for is the relative host specificity of movement complementation. When cowpea plants were simultaneously inoculated with SBMV-B and SHMV, complementation of SBMV-B movement by SHMV did not occur. Thus, SHMV will

act as a helper to SBMV-C in bean but will have no effect in the reciprocal situation in cowpea; SHMV infects both hosts systemically. Citovsky and Zambrysky (1991) explain the relative specificity of viral RNA translocation as a result of compartmentalization of viral replication which would increase the probability of the 30 KDa protein associating with viral nucleic acid rather than with host RNA. The same could be true for the interaction of the 30 KDa protein of a helper virus with the RNA of a dependent virus. The probability of the 30 KDa protein of a helper virus associating with the RNA of a dependent virus would be greater if both viruses replicated in the same cell compartment. For example, compartmentalization of replication and translation of SBMV-C and SHMV may differ in bean and cowpea, with both viruses replicating in the same cell compartment in bean. This model would by no means explain all helper-dependent virus phenomena, since certain virus groups such as comoviruses have different strategies for cell-to-cell movement (Wellink and van Kammen, 1989), and the nature of the infectious agent can be different for different viruses (Maule, 1991).

Repeated observations of thin sections, treated and stained as described in Materials and Methods, failed to reveal the presence of intact SBMV-C virions in leaf tissue doubly infected with SHMV and SBMV-C (Fig. 19, 20 and 22). Results of immunosorbent electron microscopy of leaf dips showed that although whole SBMV-C particles were present, they were fewer in number than those from SBMV-B-infected bean and occasionally the antibody-coated grids were covered with particles of a

diameter corresponding to a T=1 configuration instead of the normal T=3 pattern (Fig. 30). Furthermore, sections from SBMV-C + SHMV-infected leaves labelled with gold revealed the presence of heavily labelled clumps or strands of amorphous material, but no whole particles (Fig. 28 and 29). The presence of this amorphous material, which appears to be viral, is correlated with the high levels of antigen detected by ELISA and suggests that SBMV-C virions are either unstable or fail to assemble efficiently in bean.

Studies on the movement of free TMV RNA mutants or mutants with deletions or insertions in the coat protein have shown that their cell-to-cell movement is just as efficient as wild type TMV, yet long distance spread occurs inefficiently and sporadically compared to that of the wild type (Siegel et al., 1962; Dawson et al, 1988). Saito et al (1990), showed that a mutant with modifications in the origin of assembly displayed reduced capacity for both long distance movement and assembly in tobacco. On the other hand, a hybrid virus containing an exact exchange of the coat protein open reading frame of odontoglossum ringspot virus (ORSV) for that of TMV was able to replicate and efficiently move from cell to cell in tobacco leaves. However, although virions accumulated to a high level, the chimeric virus was unable to move systemically (Dawson and Hilf, 1992). Thus, it appears that, in some cases, a compatible capsid protein may be one of the factors required for virion entry into the conductive tissue.

In the case of SBMV-C, several factors could be responsible for the lack of systemic movement of the virus in bean. One of these factors may be the absence of the compatible capsid protein. Due to the absence of proper recognition sites in the capsid protein of SBMV-C, it would be unable to interact with the host factor that would allow modification of the cell and subsequent movement of the virus into the vascular system. Since the bean strain of SBMV does move systemically in bean, one way of investigating the role of the coat protein would be to replace the gene coding for the SBMV-C coat protein with that from SBMV-B and then observe the behaviour of this hybrid in bean.

A second cause of the lack of SBMV-C long-distance mobility in bean could be the fact that SBMV-C virions seem to be unstable and occur in low concentration in mixedly infected plants. Although gel electrophoresis of coat proteins of SBMV-C from bean plants and SBMV-C from cowpea plants showed no difference in their migration (Fig. 31), and hence their molecular weight, results of the electron microscopy showed that whole virions were either in very low concentration, in an unstable configuration or completely absent. SBMV-C particles are stabilized by three types of bond: 1) divalent cations; 2) pH-dependent contacts; and 3) salt linkages between protein and RNA (Hull, 1977). It is possible that in bean, one or several of these SBMV-C structural contacts is altered, resulting in unstable particles. SBMV could belong to the group of viruses that requires assembly of virus particles or formation of a specific ribonucleoprotein-complex for efficient long

distance movement, as occurs with TMV (Saito et al., 1990,) brome mosaic virus (Sacher and Ahlquist, 1989) and beet necrotic yellow vein virus (Quillet et el., 1989).

A third cause for the lack of systemic movement of SBMV-C in bean, which would not exclude either one of the mechanisms proposed above, could be related to differences in the routes by which viruses move systemically. Gergerich and Scott (1988) examined the movement of purified virus particles in the xylem and subsequent initiation of primary infection in non-wounded cells using a modification of the steamed-stem techniques described by Caldwell (1930) and Schneider and Worley (1959). Beetle-transmissible viruses, such as SBMV-B, were translocated through the steam-killed stem sections and initiated infection above the steam-killed area in bean. Viruses not transmitted by beetles, such as SHMV, did not move through the steam-killed sections and were not able to infect non-wounded cells above the steam-killed sections. These results and those from previous work (Bennett, 1940; Schneider and Worley, 1959; Roberts, 1970), would indicate that SBMV is translocated mainly in the xylem while SHMV moves through the phloem. Thus, although SBMV-C is able to move from cell to cell with the aid of SHMV, once it reaches the cells neighbouring the conducting tissue, other requirements for its translocation are not met.

The above observations would imply: 1) that the nature of the infectious SBMV-C agent that moves from cell-to-cell in SBMV-C/SHMV infected bean is

probably different from that in long-distance movement; and 2) that these types of movement are distinct processes controlled by different factors. Long distance movement may involve one or more interactions of host factors with viral products, different from, or in addition to, those involved in cell-to-cell movement. In the first place, loading of the mobile form of the virus from the mesophyll into the lumen of the sieve element may occur through plasmodesmata of the phloem parenchyma (in the symplasm), or it could involve an apoplasmic step. In either case, it would seem that the cellular control process which regulates the properties of plasmodesmata in phloem parenchyma is different from that of plasmodesmata in the mesophyll (Robards and Lucas, 1990). Thus, virus products other than, or in addition to, the movement protein, may be required to allow viral translocation into the conducting elements (Hull, 1989). It can be speculated that in addition to modifications of the plasmodesmata in the parenchyma cells surrounding the phloem, other virus products, such as the coat protein, interact with plant factors in order to overcome, or be protected against, the plant's natural defences present in the vascular system. These plant factors could be components in the cell wall which bind to virus particles or mobile forms and allow them to be released into the conducting stream. The availability of these complementary plant factors could be the result of a long process of co-evolution between a specific plant species and virus, and would determine whether a particular plant is a host for a virus.

In a preliminary study, BYMV, a potyvirus, and BRMV, a

comovirus, were found to act as helpers for SBMV-C replication in inoculated primary leaves of "Bountiful" bean. These results, together with the results from other virus-host combinations for which complementation of the movement function has been demonstrated (Taliansky et al., 1982 a,b; Carr & Kim, 1983; Barker, 1987,1989; Malyshenko et al., 1987, 1988, 1989), have been interpreted to suggest that transport complementation is relatively non-specific (Taliansky et al. 1982 b, Atabekov and Taliansky, 1990). However, there are many examples for which complementation between viruses is not known to occur (Barker, 1989, Malyshenko et al, 1989). Three viruses that infect bean systemically, alfalfa mosaic virus, tomato ringspot virus and bean golden mosaic virus, which were also tested as part of this study, failed to facilitate SBMV-C movement in bean. Currently there is no simple explanation for this phenomenon. Viruses have been grouped according to amino acid sequence similarities of their putative transport proteins, yet no correlation was found among sequence similarities in transport proteins of viruses and their ability to complement each other (Melcher, 1990).

This study provides a unique example in which the "helper effect" of one virus is limited to cell-to-cell movement of the "dependent" virus in the inoculated leaf. Thus, it allows for a clear separation between the processes of short and long-distance movement. This division, which has been widely used before to describe viral movement, supports the hypothesis that short and long-distance movement of viruses require different mechanisms involving different viral and plant factors. One

of these factors in the SBMV-C/SHMV complementation system seems to be proper assembly of the SBMV-C coat protein, which is apparently not required for cell-to-cell movement complementation but presumably necessary for complementation of long-distance movement.

Systemic invasion of plants by viruses is usually accompanied by disease and consequent production losses (Matthews, 1991). Long-distance movement is of prime importance since it is the main route for the establishment of a systemic infection and is probably related to the efficiency of vertical spread of viruses through seed transmission (Maule, 1991). A system such as the one described in this work could be used to identify the plant factors involved in blocking long-distance movement and those viral factors necessary for long distance movement. A consequence of the use of a model system such as this might be the development of disease-resistant crops.

Understanding the nature of the interactions between viruses and plant factors which affect the movement process is required in order to categorize and predict the behaviour of viruses in mixed infections. In this study, the interaction of SHMV and SBMV-C in bean has been characterized to a large extent, thus allowing comparison with other mixed virus infection combinations.

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