PATHOLOGY AND DISTRIBUTION IN THE HOST OF PEA SEED-BORNE MOSAIC VIRUS

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Thesis submitted to the University of Adelaide in fulfillment of the requirement for the degree Doctor of Philisophy

December, 1992

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Summary

Five isolates of pea seed-borne mosaic virus (PSbMV; isolates US, Q, S4, S6 and T) were compared by host range and symptomatology on 16 *Pisum sativum* cultivars and lines, 21 lines of *Lathyrus* and *Lens spp.* and several indicator species. All selections of *Pisum sativum* , except cv. Greenfeast, were susceptible to all isolates, but Greenfeast was susceptible to the isolate US . All isolates except isolate T infected the *Lathyrus* and *Lens spp.* through mechanical and aphid transmissions. *Chenopodium amaranticolor* and *Vicia faba* reacted similarly to all isolates while *Phaseolus vulgaris* cv. Hawkesbury Wonder was infected by none. The North American isolate (US) was distinguished from the Australian isolates S4, S6, Q, and T by infecting *Nicotiana clevelandii* and Greenfeast pea.

Four of the PSbMV isolates were tentatively classified using pea differentials as follows: isolates US and Q were placed in pathotype P1 and isolates S4 and S6 in pathotype P4. Using the grouping system, isolates US and Q were placed in group III and isolates S4 and S6 in group V.

The infectivity assay and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) showed that PSbMV was present in 5 areas of South Australia, but at a low incidence (2-3%).

In all cases the highest rate of seed transmission occurred in the largest seed (83-92%) and the lowest was in the smallest seed (29-40%). Infected seed in the largest size classes was lighter in weight than the corresponding uninfected seed. Infected seed in all classes had a significantly lower germination rate than uninfected seed although the greatest reduction in germinability was in the smallest seed. In each size class uninfected seed was heavier than infected seed and germinated better.

Two-dimensional immunodiffusion tests showed that precipitin lines between all the isolates and either the US and S6 antisera were confluent with no evidence of spurs. A rapid and sensitive indirect dot-immunobinding assay (DIBA) on nitrocellulose membrane for PSbMV was developed. Non-specific binding of conjugate to the healthy antigen was partially removed by using mannose and glucose in all buffers, and completely eliminated by using either healthy plant sap, healthy seed extract or a combination of both (1:1) as the

blocking agent. The limit of detection of antigen was about 32 ng per sample. Both of the antisera detected antigen in sap extracted from peas infected with the 5 standard PSbMV isolates, as well as an additional isolate from Denmark and all isolates were detected at similar antiserum dilution endpoints.

Isolates US, Q, S4 and S6 were used in a study of the survival and partitioning of PSbMV under conditions of continuous seed transmission in the commercial pea cultivar Dundale. Under the conditions of these experiments, seed transmission was at rates exceeding 90% for all virus isolates.

Assays suitable for detecting virus in small tissue samples were used, and included DIBA with antisera to both PSbMV and cytoplasmic inclusion body (CIB) protein, and dot hybridization assay (DHA) with cDNA transcribed from virus RNA.

Virus was detectable by serology and symptoms in inoculated plants, and in all vegetative tissue of second generation (G2) plants raised from seed of the inoculated plants. However, in the third (G3), fourth (G4) and fifth (G5) sequential generations raised from seed, all plants were symptomless. Neither virus nor CIB were detectable in leaf, stem or roots by serology, but were readily detectable in some floral parts, and in immature and mature green seeds. Mature seed contained virus and CIB antigen in the testa, cotyledon and embryo. Inoculum prepared from whole seeds was infectious. The testa was shown not to be involved in transmission between generations, thus implicating the embryo alone in vertical transmission. Although virus antigen could not be detected in the emerging cotyledons of germinating seed and any true leaves by serology, the leaves contained PSbMV RNA detectable by DHA. This inability to detect PSbMV in the vegetative tissue of plants is defined as an eclipse phase.

These results show that PSbMV infection can be transferred through the vegetative phase at a subliminal level, and reaches relatively high concentrations in floral parts and seeds. Thus PSbMV may be maintained at a high level of infection in seed in the absence of any apparent symptoms in the plant, and without a requirement for horizontal transmission between plants by vectors. Such a mechanism may explain the high levels of infection commonly reported in pea breeding lines.

A study to determine whether symptomless plants exhibited cross protection showed that there was an uneven distribution of antigen after plants from G5 were challenged with the homologous isolate US. Plants in the eclipse phase in G5 thus exhibited an apparent resistance to infection by systemic movement. These results support the conclusion that peas in the eclipse phase are subliminally infected.

STATEMENT

This thesis contains no material which has been previously presented for any other degree or diploma in any university and to the best of my knowledge and belief, does not contain material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Julio S. Ligat

Acknowledgement

I thank Dr. John W. Randles, my principal supervisor, for his invaluable supervision and encouragement during the course of this study. I am grateful to the late Dr. R. I. B. Francki, my other supervisor, for his helpful advice and supervision when Dr. Randles was on sabbatical leave.

I thank Professor H. R. Wallace, the then Head of the Department of Plant Pathology, for his permission to allow me to study in his department and his help at hard times. I also thank Professor A. Kerr, the then Head of the Department of Crop Protection, for his helpful advice. I also thank Professor O. Schmidt, the Head of the Department of Crop Protection, for his consideration and help.

My special appreciation is expressed to Mr. D. Cartwright for his help during the field survey and for PSbMV inocula; to Dr. R. Dietzgen for his help on DIBA; Dr. M. Ali for pea seeds; Dr. R. Hampton, Dr. J. Thomas, Dr. J. Fletcher, Dr. M. Albrechtsen, Mr. D. Graddon, Mr. D. Munro for isolates of virus and antisera, and Dr. G. Johnstone for an aphid culture.

I thank Mr. R. Miles for his excellent assistance in electron microscopy. Thanks are due to Messrs D. Miller and C. Grivell for their help with techniques; D. Talfourd for supply and care of plants; T. Feckner for organizing the material; Mr. A. Dunbar and Ms J. Groom for their assistance in photography. I also thank those people whose names are not mentioned in the then Department of Plant Pathology and the now Department of Crop Protection, for their kind help.

I thank my parents, brothers and sisters for their understanding and spiritual support. I also thank my Filipino friends and the Christian Family Group members at St. Paul's Retreat and Modbury Heights Christian Centre for their prayers and spiritual support.

I acknowledge the scholarship from the Australian International Development Assistance Bureau. Without this financial support, this thesis would not have been possible.

Chapter 1 General Introduction

1.1 History and economic importance

Pea seed-borne mosaic virus (PSbMV, Hampton and Mink 1975) has various synonyms:pea leaf rolling virus (Musil 1966); false pea leaf roll virus (Thottappilly and Schmutter 1968); pea fizzle top virus (Hampton 1969); a seed-borne virus of pea (Mink *et al.* 1969); a new seed-borne virus of pea (Stevenson and Hagedorn 1969); pea leaf roll mosaic virus (Bos 1970); and pea leaf-rolling mosaic virus (Musil 1970). A typical, apparently distinct member of the potyvirus group. The virus has filamentous particles c. 770 x 12 nm, and contains RNA. Transmission is by mechanical inoculation of sap, in the non-persistent manner by aphids, and through seed. It is disseminated primarily through infected pea seed.

The virus was reported initially from Czechoslovakia in 1966 (Musil), then from Japan (Inouye, 1967), West Germany (Thottappilly and Schumutter, 1968), USA (Mink *et al.*, 1969; Hampton, 1969; Stevenson and Hagedorn, 1969), Netherlands (Bos, 1970), Canada (Zimmer and Ali-Khan, 1976), Yugoslavia (Milicic and Grbrelja, 1977), German Democratic Republic (Karl and Schmidt, 1978), Poland (Kowalska, 1979), Switzerland (Pelet, 1980), New Zealand (Fry and Young, 1980), UK (Matthews *et al.*, 1981), and India (Thakur *et al.*, 1984). Lindsten *et al.* (1976) reported its possible occurrence in Sweden. In Australia, Munro (1978) detected PSbMV in seeds imported from Sweden. In 1987, Chang *et al.* (1989) isolated the virus from diseased peas in Ear-Lin, Taiwan. The virus is distributed primarily in infected pea seed and probably occurs worldwide, as several pathotypes, in pea germplasm (Alconero and Hoch, 1989).

PSbMV is a pathogen of high economic potential importance because of its high rate of transmission through seeds (Khetarpal and Maury, 1987). Commercial seed lots containing up to 90% infected pea seed have been reported from the USA (Mink *et al.*, 1969; Knesek and Mink, 1970) and Switzerland (Pelet, 1980). The virus has been detected in pea germplasm collections in Canada (Ali-Khan and Zimmer, 1979), USA (Hampton and Braverman, 1979), New Zealand (Fry and Young, 1980), and UK (Matthews *et al.*, 1981).

1.1.1 Incidence of PSbMV

In 1968, a previously unreported, seedborne, mosaic-type virus disease was discovered in Washington-grown, midseason, perfection-type peas. The virus was detected by indexing 53 fresh plant samples having virus-like symptoms and in 4 of 113 random pea seed lots from Washington and Idaho (Knesek and Mink, 1970). The virus could not be detected in random lots of fresh processing peas grown in 1969 from seed produced in the Pacific Northwest.

Hampton (1972) reported that samples of 570 Plant Introduction (PI) seedlot accessions of *Lens culinaris* were planted in greenhouses and the resultant seedlings were tested for the presence of seedborne virus by local lesion assays on *Chenopodium amaranticolor* leaves . A seedborne virus, designated the lentil strain of PSbMV-L was detected in 38 of the 570 accessions. The incidence of PSbMV-L in 5 selected accessions ranged from 5 to 10%. PSbMV-L was transmitted by *Acyrthosiphon pisum* at frequencies ranging from 55 to 88%.

Of 1,835 lines of *Pisum sativum* tested for the presence of PSbMV, 420 (23%) were found to be infected New York, USA (Hampton and Braverman, 1979). The intensity of symptoms induced in pea seedlings varied greatly among infected lines and 37 lines were infected and symptomless. The incidence of PSbMV was much higher in post-1970 than in pre-1970 PI lines, the increase being attributable principally to lines introduced from northern India. Seedlots collected in India and Peru were confirmed as sources of introduced PSbMV inoculum, whereas 22 seedlots of pre-1962 introductions from 11 countries contained no PSbMV. Twenty-three PI lines principally from northern India contained new sources of PSbMV-immune germplasm.

PSbMV was detected in pea seed imported from Sweden to Tasmania by electron microscopy and aphid transmission (*Myzus persicae*) to *Vicia faba* and *Pisum sativum* cv. Perfection 447 (Munro, 1987). In 1987, a virus was isolated from disease peas in Ear-Lin, Taiwan (Chang *et al.*, 1989). The virus was shown to be transmitted by manual inoculation and by *Myzus persicae* in a non-persistent manner.

Alconero and Hoch (1989) obtained 189 isolates of PSbMV from seedlings of 435 pea (*Pisum sativum)* germplasm introductions originally acquired from India, Turkey, Latin America and Europe. Fifty-eight percent of the isolates were identified as belonging to pathotype P-1, 22% to pathotype P-4, and 7% to the mild form of the lentil pathotype L-1.

1.1.2 Crop losses due to PSbMV infection

PSbMV is a continuing threat to the pea seed and processing industry (Kraft and Hampton, 1980). When the plants were inoculated at three or eight leaf stages, the growth stage at inoculation did not significantly affect yields of seed or levels of virus transmission through seed (Chiko and Zimmer, 1978) . Average yields of plots inoculated at all 3 stages were reduced 8% in cv. Trapper and 70% in cv. Century. In both cultivars, yield reduction was attributable mainly to reduction in seed weight rather than total number of seeds. Transmission of PSbMV through seed from inoculated plots averaged 5-8 and 0.5%, respectively. In both cultivars, cracked seed coats were most prevalent in seeds from plots inoculated at the 2 youngest stages of growth.

Field responses of 6 processing pea cultivars to PSbMV were tested in an isolated location near Prosser, WA, USA in 1977 and 1978 (Kraft and Hampton, 1980). Test plots were mechanically inoculated either 2 or 3 wk after emergence. Disease incidence, determined visually, was higher in all plots in 1977 than in 1978 and was generally correlated with rate of seed transmission and loss in green pea and seed yields. The later maturing, more determinate cultivars (Mars, Conway, and Corfu) were more severely affected than earlier maturing, indeterminate cultivars (Small Sieve Alaska and A-45) in both years. Losses in green pea and seed yields in 1977 were greater in plots of all cultivars inoculated 2 weeks after emergence than in those inoculated 3 weeks after emergence.

In the UK, 10% PSbMV infection was detected in seedlings of pea cv. 'petit'pois' . In glasshouse tests, plants infected through the seed yielded 63% fewer pods and 84% less seed, and plants inoculated before flowering produced 8% fewer pods and 36% less seed, than uninfected plants (Anon, 1985).

Khetarpal *et al.* (1988) showed that early infection caused a delay of 4 to 5 weeks in maturity of three commercial pea varieties. Seeds harvested at maturity from the infected plants were smaller in diameter: a reduction in 1000-grain weight of 16% was recorded for cvs. Belinda and Finale in 1986, and of 28% for cv. Amino in 1984. In the latter case, the marked reduction in grain weight was found to be partially compensated for by an increase in the number of pods produced by the plants. In addition, in all 3 cultivars, small seeds were found to transmit PSbMV at a rate markedly higher than those of average and large seeds.

1.2 Biological Properties of PSbMV

PSbMV was first reported in peas in the USA in 1968 (Stevenson and Hagedorn, 1969). .In Wisconsin, field symptoms included stunting, rosetting, tendril cupping, leaf cupping, and a severe reduction in yield. Pea leaf roll mosaic disease was first observed by Hubbeling and Huyberts in the pea varieties 'Wyola', Olympic, and a Dutch breeding line in field trials in the Netherlands (Bos, 1970). The symptoms were narrowing and downward rolling of leaves, sometimes accompanied by a faint mosaic or mottling. The virus isolate (designated E210) may have been related to 2 viruses described independently and incompletely under the names pea leaf roll virus in Czechoslovakia (Musil, 1966) and pea seed-borne mosaic virus in Japan (Inouye, 1967).

Pea fizzletop disease was first described in 1968 (Hampton and Bagget, 1970). Filamentous virus particles are associated with this disease; it is seedborne, and clearly distinguishable from other seedborne virus diseases of peas by symptomatology; it was transmissible by *Acyrthosiphon pisum.* Pea seed-borne mosaic virus (PSbMV), sometimes called pea fizzletop virus(PFV), was transmitted by *Myzus persicae*, *Acyrthosiphon pisum*, and *Macrosiphum euphorbiae* when apterae or alatae of these aphid species were allowed single access probes of 10-90 sec. (Gonzales and Hagedorn, 1970). Hampton (1972) found out that PFV isolates produced uniform symptom profiles on selected hosts, and exhibited uniform properties. Most seedlings raised from PFV-infected seed exhibited discernible and varying degrees of downward leaf roll, vein protrusion, and veinbanding when 1 to 3 weeks old, and were visually identified with high accuracy.

Forty-seven plant species representing 12 families were susceptible to PSbMV by either mechanical or aphid inoculation (Aapola *et al.*, 1974). The susceptible species were classified as either: (i) highly susceptible species in which most individual plants could be readily infected by either rub-inoculation or by aphids; (ii) marginally susceptible species in which only one or 2 plants were infected regardless of the inoculation method; and, (iii) species whose apparent susceptibility or immunity was dependent upon the inoculation technique.

1.3 Pathotypes of PSbMV

1.3.1 Variation in pathogenicity

The F_1, F_2, F_3 and backcross data from pea crosses between 2 mosaic-susceptible varieties and 2 immune varieties indicated that immunity is controlled by a single recessive gene, with the proposed designation *mo* (Yen and Fry,1956) . The susceptible heterozygote differs from the immune homozygote (*mo mo*) by: (i) timing of the appearance of the mosaic symptom after inoculation; (ii) the type of symptom, 3 being described. The latter, however, appears to depend on the variety, or the parentage in the case of crosses, and the strain or isolate of the virus.

Stevenson and Hagedorn (1971) reported that the disease reaction of 143 processing pea cultivars and 528 plant introductions in the field and greenhouse were determined to find resistance to the PSbMV. Pea seedlings were artificially inoculated and disease development noted. Symptoms developed on most pea lines, but all seedlings of Plant Introduction (PI) 193586 and the green-axiled plants of PI 193835 remained symptomless under repeated tests.

Greenhouse tests of 1326 PI pea lines identified resistance to pea fizzletop virus (PFV) in 3, possibly 4, lines (Baggett and Hampton,1972). PI 269774 and PI 193586 were uniformly resistant, although the former was variable in seed type. PI 269818 appeared to be either a resistant line containing a few susceptible variants, or it was susceptible but difficult to infect. An additional line, PI 175877, was symptomless in repeated trials, but absence of PFV was not determined by assay.

The F_1 progenies were all susceptible when *Pisum sativum* P.I.'s 193586 and 193835, resistant to PSbMV, were crossed with 8 susceptible commercial cultivars (Hagedorn and Gritton, 1973). Backcross and progeny-tested F_2 segregation ratios fitted the hypothesis that resistance to the virus is conditioned by a single recessive gene. Populations evaluated in F_2 were somewhat variable, but in general, they support this hypothesis. Both PI's appeared to possess the same genetic factor for resistance. Hagedorn and Gritton (1973) proposed that the recessive factor for resistance to PSbMV be designated *sbm*.

A pea latent strain (PLS) of PSbMV caused transient vein clearing but no leaf rolling or stunting (Milicic and Plavsic,1978) . It rarely formed x-bodies but produced typical pinwheels, and differed from the typical virus by shorter stability *in vitro*.

Immunity to the Oregon (Standard) and Yugoslavian isolates of PSbMV was conferred by *Pisum* gene *sbm* in 3 of 17 differential accessions of pea (Goodell and Hampton, 1983). Immunity to the lentil and New Zealand isolates was conferred by gene *mo* which also confers resistance to bean yellow mosaic virus but not to the Standard isolate of PSbMV.

Three isolates of PSbMV, P-1 and P-4 from pea (*Pisum sativum*) and L-1 from lentil (*Lens culinaris*) germ plasm accessions, were distinguished by their capacity to infect pea genotypes (Alconero *et al.*, 1986). Resistance to the L-1 isolate was associated with bean yellow mosaic virus resistance and also with a delayed reaction to isolate P-4. All isolate were infective to 26 genetic lines of chickpea (*Cicer arietinum*), a new host, but not to 12 accessions of pigeon pea (*Cajanus cajan*).

Resistance to a newly recognized pathotype of PSbMV, PSbMV-P4, was found in PI 347492, an accesion of *Pisum sativum* from India (Provvidenti and Alconero, 1988) . In cross and back-cross populations between PI 347492 and the susceptible cultivars Bonneville, Ranger, and PI 269816, resistance was determined to be monogenic and recessive. The symbol *sbm*-4 is proposed for the gene conferring resistance to this pathotype of PSbMV. Thirty-nine accessions of *Pisum sativum* (Hampton, 1980) were listed as resistant to PSbMV-4 (Provvidenti and Alconero, 1988). Twenty eight of these lines (72%) were from India, of which 25 derived from Uttar Pradesh. These and a few other lines were tested with 5 isolates of PSbMV [(Standard (ST), Pea 1 (P1), Lentil L (L), Lentil 1 (L1), and Pea 4 (P4)]. All lines reacted identically to the ST and P1 isolates, which incited similar foliar symptoms in susceptible genotypes. Hence, these 2 isolates should be considered as members of the same pathotype, PSbMV-ST. Resistance is conferred by the gene *sbm*-1, which is located on chromosome 6. Secondly, all lines responded identically to the L and L1 isolates. However, in susceptible genotypes, L caused a mild mottle and a slight downward leaf cupping, whereas L1 incited a prominent chlorotic mottle, upward leaf curling, flower abortion, and severe stunting. Consequently, these isolates should be regarded as 2 different strains of the same pathotype, PSbMV-L. The genes *sbm*-2 and *sbm*-3, independently, confer resistance to both strains of this pathotype, and may be duplicate entities. The gene *sbm*-2 is located on chromosome 2. Thirdly, resistance to P4 was found to be associated with that to PSbMV-ST and PSbMV-L or with 1 of these 2 pathotypes. In susceptible plants, PSbMV-P4 caused a

moderate to prominent mottle with a partial recovery on subsequent growth. Resistance was conferred by *sbm*-4

A virulent pathotype, PSbMV-Pi, capable of partially overcoming the recessive resistance gene *sbm* 1, has been recently identified in an Indian pea line (Khetarpal *et al.*, 1990) . It did not produce visible symptoms on *sbm* 1 lines, on which it had reduced multiplication and could not be detected by ELISA 7 weeks after inoculation. However, it multiplied normally on the susceptible cultivars and could be differentiated from other strains on a set of strain differentials. In addition, another strain, of the virulent pathotype PSbMV-Pv, that appeared to multiply slightly better than PSbMV-Pi on *sbm* 1 lines, was recovered from the local strain of PSbMV.

A seed lot of pea cv. Belinda was shown to transmit a latent strain of PSbMV with high frequency. It was detectable in ELISA only 5 weeks after plant growth (Khetarpal and Maury, 1990). This strain did not induce apparent symptoms on plants, and was not detectable in plants older than 9 weeks.

1.3.2 Relationships among pathotypes of PSbMV

A seed-borne virus disease of peas described in Washington, Oregon, and Wisconsin was found to be related serologically to a PSbMV isolate described in Japan (Mink *et al.*, 1974). The discrepancies among particle length measurements reported for the US and Japanese isolates appear to be due to methods which were used to prepare the US isolates for electron microscopy.

Seven isolates of PSbMV were compared on selected *Pisum sativum* L. differentials and by microprecipitin and SDS-gel serology and particle length (Hampton *et al.*, 1981) . All isolates were characterized by modal particle-length of 750 nm and were closely related serologically, but some were readily distinguished on *P. sativum* differentials. Isolate distinctions were of the magnitude typical for virus strains.

1.4 Seed transmission

Stevenson and Hagedorn (1973) reported that there was a correlation between seed transmission and seeds of small size, abnormal shape, or seeds with growth cracking of the seed coats . The virus was isolated from the embryo and cotyledons of mature seed growth cracks within the seed coat, and from the embryo, endosperm, and testa of immature

developing seeds. .All parts of inflorescences from infected plants including carpels, filaments, petals, pollen, and sepals, contained virus. They showed that PSbMV was transmitted to seed both by the female parent and by pollen. In contrast, Wang and Maule (1992) found that pollen transmission of PSbMV did not occur.

PSbMV was seed-transmitted in *Lens culinaris* at frequencies of 32 to 44% (Hampton and Muehlbauer,1977). Plants mechanically inoculated 16 days after emergence yielded seed which germinated at only 47% the rate of normal seeds; and 44% of the germinating seeds produced seedlings containing PSbMV. Virus was transmitted through 32% of the germinating seeds from lentil plants that had been aphid-inoculated with PSbMV 5 weeks after emergence.

The sensitivity of direct-seed assay was tested by triturating 100 presoaked seeds from an infected seed lot individually in a mortar and inoculating each to a half leaf of *Chenopodium amaranticolor* (Mink and Parsons, 1978). Another 100 seeds from the same lot were germinated and raised for 28 days in a growth chamber and observed for symptoms. Fourteen and 20% infection were detected, respectively. In a second test, 100 presoaked seeds were individually indexed on both *C. amaranticolor* and the pea cultivar 447. Virus was detected in 16 seeds by using *C. amaranticolor* and in 15 seeds by using pea cultivar 447.

The incidence of PSbMV was determined by germinating seeds, observing seedling symptoms, and confirmatory assays on *C. amaranticolor*, or by assaying individual presoaked seeds on *C. amaranticolor* (Naim and Hampton, 1979) . Seeds were infected at 1.7 to 3 times the average rate for the seed lot. Although the highest observed infection rate per class, 80% and 90%, respectively, was associated with seeds exhibiting growth-cracked coats, most classes of seeds with this coat deformity were no more frequently infected than the seed lot average. Seeds with green seed coats were more frequently infected than the seed lot average, and there was a slightly higher than average incidence of PSbMV in the smallest and next-to-largest seed sizes.

More than 100 pea cultivars as well as 6 broad bean, 5 lentil and 4 vetch cultivars were observed to be susceptible to infection with pea leaf rolling mosaic virus (PSbMV). Only 3 PI pea lines of American origin were not susceptible to all. The virus was transmitted by seeds of all susceptible cultivars of pea, lentil and broad bean. In vetch, however, seed transmission was found in 1 cultivar ('Lyra') only. The seed transmission percentage of the virus (between

<1% and >50%) was influenced by the cultivar involved as well as by the time of plant infection and by environmental conditions, and above all by the temperature during flowering and seed formation (Musil, 1980)

PSbMV-L was seed-transmitted at rates of 5 and 0.5%, respectively, in *Lens culinaris* Tekoa, and *P. sativum* Tempter (Goodell and Hampton,1984). The virus was detected in seeds of the USDA lentil plant introduction (germplasm) accessions and in seed-increase plots at levels as high as 16.7%.

The assays for PSbMV-infected seed in the experimental lot greatly overestimated the known percentage of seed transmission, due to the extraction of non-seed transmissible virus from the seed coats (Maury *et al.*,1987). The removal of the seed coat was imperative and before grouping of the embryos, the maximum size of a group of embryos could be determined on the basis of the variation in PSbMV titer among about 20 infected embryos of a given cultivar.

Seed transmission of PSbMV in 20 cultivars ranged from 0% in cvs. Maro, Princess and Progreta to 74% in cv. Vedette (Wang *et al.*, 1992). There was no obvious relation between virus content and the efficiency of seed transmission in different cultivars. Wide variation in percentage seed transmission between individuals of single cultivars was observed; for example, for seven cvs. tested ST was 2% to 49% and all showed some plants escaping seed transmission altogether.

Cross-pollination experiments showed that pollen transmission of PSbMV did not occur and virus was not detected in pollen grains by ELISA or electron microscopy (Wang and Maule, 1992). Comparative studies between 2 pea cultivars with either high or nil incidence of seed transmission showed that PSbMV infected the floral tissues (sepals, petals, anther and carpel) of both cultivars, but was not detected in ovules prior to fertilisation. Virus was detected equally well in seed coats of the progeny in both cultivars. Analysis of virus incidence and concentration in pea seed of different developmental stages demonstrated that, in the cultivar with a high incidence of seed transmission, PSbMV directly invaded immature embryos, multiplied in the embryonic tissues and persisted during seed maturation. In contrast, the cultivar without seed transmission did not show invasion of immature embryos by the virus; there was no evidence for virus multiplication or persistence during embryo development and

seed maturation. Hence, seed transmission of PSbMV resulted from direct invasion of immature pea embryos by the virus and the block to seed transmission in a non-permissive cultivar probably occurred at this step.

1.5 Purification of PSbMV

The infectivity of PSbMV in leaf extracts was lost following by clarification with the organic solvents, carbon tetrachloride-ether, and butanol (Inouye, 1967). Stevenson and Hagedorn (1973 c) used a high molarity buffer, chloroform and low speed centrifugation for initial clarification of pea extracts prior to polyethylene glycol (M.W. 6000) precipitation and two cycles of differential ultracentrifugation for the concentration of particles.

Several procedures for purification of PSbMV have been compared and it was found out that the best results were obtained when infected roots were extracted in 10 mM sodium diethyldithiocarbamate (NaDIECA) + 10 mM cysteine and when an infected leaf was extracted in NaDIECA + cysteine containing 10 mM EDTA both followed by clarification in 0.5 volume of chloroform (Knesek *et al.*, 1974) . The use of sucrose density gradients has led to the successful purification of several potyviruses in adequate yields, with a high degree of purity and without significant aggregation or change in particle size distribution (Moghal and Francki, 1976).

Substantial yields of PSbMV were obtained with the modification of the method of Huttinga (1973): leaves were blended in 100 mM tris-HCL pH 9 containing 0.2% 2-mercaptoethanol (3.6 volumes), carbon tetrachloride (one volume) and chloroform (one volume); virus was sedimented at 26500 *g* for 1.5 h and 25000 rpm for 5 h with 0.1% Ijepon T-73 through a sucrose cushion (Hamilton and Nichols, 1978)

The purification of PSbMV by centrifugation in isopycnic density gradients of Nycodenz gave no significant losses of particles, they appeared intact, and the degree of purity of partially purified preparations was increased considerably (Gugerli, 1984). Alternatively, 10 PSbMV isolates have been purified successfully using caesium sulphate gradient (Wang *et al.*, 1992).

1.6 Molecular properties of PSbMV

Preparations usually contain one sedimenting component with a sedimentation coefficient ($s^{o}20,w$) of 154 S and buoyant density in CsCl of 1.329 g/cm^3. A 260/280 is 1.14-1.18. Absorbance at 260 nm (1 mg/ml, 1 cm light path) is 2.5 (Huttinga, 1975). Particles are slightly

flexuous filaments c. 770 x 12 nm. Some isolates appear highly susceptible to breakage unless fixed with 3.5% glutaraldehyde (Hampton *et al.*, 1974). Nucleic acid is ssRNA, representing 5.3+1% of the particle weight. The molar percentage of nucleotides is G22.8; A44.0; C17.6; U15.6 (Knesek *et al.*, 1974). Protein is about 94% of the particle weight. Subunit M.Wt is 34,000 (Huttinga, 1975). Relative amino acid molar ratio is Ala 3.3, Arg 2.5, Asx 4.7, Glx 4.9, Gly 2.7, His 1.0, Iso 1.8, Leu 2.2, Lys 1.5, Met 1.9, Phe 1.1, Pro 1.4, Ser 2.2, Thr 2.0, Tyr 1.2, Val 2.4, Cys and Try not determined (Knesek *et al.*, 1974).

1.7 Diagnosis of PSbMV

1.7.1 Detection of PSbMV by electron microscopy

Electron microscopic examination of partially purified or dip preparations from fizzletop-affected peas revealed an abundance of flexuous, anisometric particles with a normal length of 517 m*u*. (Hampton, 1969) Particle lengths of PSbMV were significantly shorter in leaf-dip and in partially purified preparations fixed with formalin, than those derived from either preparation which had been fixed with glutaraldehyde. Inherent structural properties, possibly unique strains of this virus, and unfavorable preparatory procedures caused particle breakage and anomalous modal particle-lengths. Preparations obtained by a sequence of differential rate-zonal density gradient-and sucrose polyethylene glycol ultracentrifugation were highly infectious and showed virus particle-length modes of 750 nm to 770 nm when fixed with glutaraldehyde (Hampton *et al.*, 1974) .

1.7.2 Serological studies

Two antisera, one prepared from PSbMV particles and the other from D-protein from PSbMV particles were prepared by Stevenson and Hagedorn (1973). These antisera, when used in the microprecipitin test, could be used to identify PSbMV in partially purified virus extracts. PSbMV was detected in clarified sap from naturally infected pea plants using the antiserum to intact PSbMV in the microprecipitin procedure. Double-diffusion tests in agar with either antiserum were unsuccessful.

Immunodiffusion in gels containing 0.5% sodium dodecyl sulfate, enzyme-linked immunosorbent assay (ELISA), and serologically specific electron microscopy (SSEM) were evaluated for the detection of PSbMV in pea tissues (Hamilton and Nichols, 1978) . The virus was readily detected by immunodiffusion of leaf homogenates from single infected plants but

not in homogenates from composite samples containing less than 25% PSbMV-infected leaves. In contrast, detection of PSbMV in composite samples of leaves containing 5-10% PSbMV-infected leaves was obtained using ELISA or SSEM. Each of these latter methods also detected the virus in homogenates of bulk samples of seed from a seedlot containing 25% infected seed; levels of 1% seed infection were detected consistently by SSEM.

The modification of an immunodiffusion -in-gel method for detection of PSbMV has improved the visual acuity of the test (Zimmer, 1979). Precipitin band formation was clearer and separation of the band from a nonspecific precipitate ring around the antiserum well was more distinct on Bacto agar than on special Noble agar. A reduction in the concentration of sodium dodecyl sulfate (SDS) concentration from 1.0-1.5% also gave more distinct separation. Crystal formation, a problem at times around some antigen wells, was aggravated by cool temperatures (18^{o}C) and by low concentrations of SDS (0.25%) and NaN_3 (0.5%).

A dot immunobinding assay for the rapid detection of plant viruses in infected tissues has been developed with the advantages of avoiding non-specific reactions and minimizing the amount of antibodies used (Hibi and Saito, 1985) . With this method, less than 1 ng of tobacco mosaic virus could be detected in several milligrams of infected tobacco leaves. This simple, rapid and sensitive assay promises to be a useful and practicable diagnostic technique for plant virus diseases. A single antibody dot immunoassay (SADI) was developed for use as a rapid, simple and sensitive technique for the direct assay of virus bound to nitrocellulose membranes (Graddon and Randles, 1986). SADI has been tested for the detection of subterranean clover mottle virus (SCMoV) in small amounts of infected tissue and purified virus preparations, and the technique was found to be 12 times more sensitive than ELISA in terms of total antigen detected. DIBA, an indirect dot immunoassay, was about twice as sensitive as SADI, but the latter was more specific for the detection of SCMoV and is a simpler, more rapid assay, requiring less than 3 h to complete. DIBA was adapted for detection of citrus tristeza virus (CTV) and compared with DAS-ELISA and DAS-indirect ELISA (Rocha-Pena *et al.*, 1991) . DIBA was easy to perform and as sensitive as either ELISA procedure for CTV diagnosis. The entire test could be performed in 2-3 h using polyclonal antibodies, with minimal laboratory equipment. Three different polyclonal antibodies gave a strong positive reaction with 12 selected CTV isolates; however, each serum had to be cross-absorbed with sap from healthy

plants before use. The broad spectrum 3DFI monoclonal antibody reacted with most of the CTV isolates. The MCA-13 strain-specific monoclonal antibody was specific for most severe CTV isolates. As blocking agents, 3% bovine serum albumin (BSA), 3% gelatin, 0.5% non-fat dry milk or 5% Triton X-100 gave an adequate white background on the nitrocellulose membranes and permitted discrimination between infected and healthy samples. However, 3% gelatin gave the best contrast between green for the healthy samples, and purple color for infected samples.

Clover yellow vein virus (CYVV) and homologous antisera were used to test the effects of time and temperature on enzyme-linked immunosorbent assay (ELISA) in polystyrene substrate plates (McLaughlin *et al.*, 1981). Replicated lattice square and Youden square experimental designs were used to measure and account for variation in absorption values associated with sample position within polystyrene plates. Adsorption of coating antibody to polystyrene was relatively rapid, reaching optimum assay efficiency in 1 h at 5°C when applied at 2.5 *ug*/ml. Binding of antigen and enzyme-linked antibody (conjugate) in their respective steps during ELISA was also rapid. Incubation of antigen and conjugate for 2 h each was adequate to enable detection of 20 ng CYVV in a 100 *ul* sample, but longer incubation of either reactant improved results. At this virus concentration, reduction of antigen incubation time to one-half could be compensated for by doubling the conjugate incubation time and vice versa. Incubation of conjugates at 5°C rather than 30°C increased final ELISA readings (A400 nm) more than two-fold.

The ELISA procedure easily detected tobacco ringspot virus (TRSV) or soybean mosaic virus (SMV) in individual soybean seed, to 1/2500 and 1/160 (w/v) dilutions, respectively, of seed extracts made in a phosphate-buffered saline containing polyvinyl pyrrolidone (MW 40,000) (Lister, 1978). Sensitivity for detection of the viruses in leaf extracts relative to seed extracts was similar to TRSV and about 4 times greater for SMV. The results indicated that both viruses would also be detectable in extracts from seed batches containing low proportions of infected seed: leass than 1% for TRSV and about 2-4% for SMV. Germination of the test seeds significantly improved the sensitivity of such tests for SMV. The ELISA tests may be widely applicable for testing seed for viruses, especially with large-seeded species such as

legumes and cereals. Testing seedlings instead of seed could further broaden applicability to other systems.

1.8 Cytology of PSbMV infection

Ultrathin sections and negatively stained dip-preparations of pea and broad bean leaves infected with PSbMV (Inouye, 1967) have been examined under the electron microscope (Inouye, 1971). Characteristic inclusions, such as pinwheels, rings, circles, tubes or bundles were observed in cytoplasm, especially in the swollen parts of cytoplasm, of PSbMV-infected plant cells. The profiles of sections of these cytoplasmic inclusions appeared very similar to those of watermelon mosaic virus (Purcifull and Edwardson, 1967) and several other viruses of the PVY group. Small numbers of virus particles were distributed throughout the cytoplasm. Negatively stained dip-preparations of PSbMV-infected plants showed that the oblong or scroll shaped fragments of inclusions were composed of plates or sheets on which parallel linear striations were seen. These linear striations were about 5 μm apart. Fine structure of cytoplasmic inclusions observed here was very similar to that of many other viruses of the potyvirus group.

Eleven isolates of PSbMV from US seedlot were compared by thin-section electron microscopy to appraise the cytopathological diversity among isolates (Hampton *et al.*, 1973). Nine isolates uniformly induced formation of typical pinwheel inclusions, 2 of these also inducing tonoplast aggregates (TA) in a few cells. One isolate predominantly produced TA, accompanied in a few cells by pinwheel inclusions. The remaining isolate induced formation of dense bodies and laminated aggregates like those reported for bean yellow mosaic virus (Kamei *et al.*,1969). Other cytological abnormalities induced by one or more PSbMV isolates included extensive masses of convoluted endoplasmic reticulum, aggregates of viruslike particles, and crystalline inclusions consisting of ultrastructural hexagons. Cytological symptoms induced by the 11 isolates in species from which they originated fell within the range previously reported for this virus (Hampton, 1972). Root parenchyma cells from PSbMV-infected plants contained few inclusions, but instead contained extensive masses of viruslike particles that occupied most of the cytoplasmic volume in some cells. Samples of purified virus prepared separately from infected root and leaf tissues were morphologically indistinguishable.

The *in situ* relationships between the cylindrical inclusion (CI) bodies of PSbMV and structures of the host cell, and the function of the CI protein were examined in virus-infected cells (Calder and Ingerfeld, 1990). Of the 3 morphological types of CI bodies, only the pinwheels were associated with cellular components. The bundles are associated with both the plasmodesmata and the membrane of the rough endoplasmic reticulum (rER). The rER associated with CIs produces large numbers of smooth-surfaced vesicles. Some of the vesicles, particularly those associated with the arms of the pinwheels, had electron-dense contents. The cytoplasmic associations suggest that there are two functions for the CI protein. The first is the induction of vesicles analogous to the 2BC protein of picornaviruses. The 2BC protein of poliovirus (a picornavirus) is known to associate with rER and this responds by producing large numbers of vesicles. The vesicular membranes provide a matrix to which the replication complex of poliovirus is bound. The second function is in the cell-to-cell spread of the virus.

An immunogold labelling procedure was developed to detect PSbMV virion and pinwheel inclusion body proteins in leaf and seed tissues of pea and to study the cytology of PSbMV infection in this host (Wang *et al.*, 1991). Ultrathin sections were treated with immunoglobulin prepared against either PSbMV particles or PSbMV pinwheel inclusion bodies. The sections were then probed with gold-labelled goat anti-rabbit immunoglobulin. With immunoglobulin against PSbMV particles, virus particles, but not pinwheel and crystalline inclusions, were specifically labelled. With immunoglobulin against pinwheel inclusion bodies, pinwheel inclusion bodies (bundles and scrolls) were specifically labelled, but neither PSbMV particles nor its crystalline inclusion bodies were labelled, showing that the crystalline inclusion body protein was unrelated to either virus coat protein or the pinwheel inclusion protein. When cotyledon and plumule sections cut from fixed seed tissues were treated with the 2 types of immunoglobulin, only PSbMV particles were labelled, indicating that in seed tissues, the pinwheel inclusion was either absent or structurally different from that in leaf tissues.

1.9 Control Measures

During the 1974 growing season, several USDA-ARS pea breeding lines at Prosser and Pullman, Washington were found to be infected with PSbMV (Hampton *et al.*, 1976). Infection of the USDA lines was traced to Brawley, California where they had been grown near

Canadian pea breeding lines for seed increase during the winter of 1973-74. Canadian lines planted at Brawley again in November 1974, although previously assumed to be disease free, were found to be heavily infected with PSbMV. It was concluded that these lines had been the inoculum source from which USDA lines became infected. Seed of all contaminated USDA lines was destroyed, and prompt eradicative measures have been undertaken for all Canadian pea seed programs.

PSbMV was first identified in Canadian field pea (*Pisum sativum* L.) breeding lines in 1974 (Ali-Khan and Zimmer, 1979). Since then, an extensive program has been underway to eradicate this virus from the breeding lines. At the Morden Research Station, nearly 2000 breeding lines were evaluated. The virus was assayed by infectivity tests using the local lesion host *Chenopodium amaranticolor*, and by a gel immunodiffusion test. PSbMV was detected in 136 lines. The level of infection within lines varied from 1 to 3%. Due to the restricted extent of the virus in the breeding lines, it was possible to continue the breeding program without a serious loss in germplasm.

In New Zealand, PSbMV infected lines that were sown were traced and the resulting crops ground for stock feed (Fry and Young , 1980). Seed indexing is being carried out by the Ministry's seed testing station, Palmerston North. Seedlines that are to be used for further seed production, that is parent seedlines, are submitted for indexing by seed companies. Participating firms have agreed to destroy or grind for stock feed any seedlines in which virus is detected. Seed that is produced from indexed parent lines will be able to carry the certification that it was grown from seed in which no PSbMV had been found.

Significant reduction of genetic diversity was observed in seed coat colour and electrophoretic isozyme genotypes when infected plants were eliminated from genetically heterogeneous pea populations (Alconero *et al.*, 1985). Some accesions where genetic homogeneity was expected were apparently mixtures. Detection of latent PSbMV infections by ELISA was more effective on single-plant samples and older plants.

1.10 Cross protection studies

Cross protection is one of several approaches by which virus diseases in plants can be controlled (Ponz and Bruening, 1986). For example, a mild variant of zucchini yellow mosaic virus (ZYMV-WK) that had been selected from a severe strain of ZYMV in France was used

for cross protection tests in cucurbit crops in Taiwan (Wang *et al.*, 1991). Under greenhouse conditions, ZYMV-WK provided protection in cucumber, melon, and zucchini squash against four severe strains originally from Connecticut, Florida, France, and Taiwan. Cross protection was more effective against the French strain, from which the mild strain was derived. Two field trials with zucchini squash under moderate and high disease pressures showed that ZYMV-WK provided excellent cross protection against the Taiwan strain of ZYMV.

Rezende *et al.* (1992) reported that the expression of cross protection between two strains of tobacco mosaic virus (TMV-C and PMV-P) differed in *Arabidopsis thaliana* cv. Columbia and *Nicotiana tabacum* cvs. Samsun and Xanthi. Protection in *A. thaliana* cv. Columbia was expressed as a prevention of systemic movement of the challenge strain, regardless of the protecting strain of TMV. Protection in *N. tabacum* cvs. Samsun and Xanthi was expressed as an inhibition of an early event in the infection process.

The expression of virus capsid proteins in genetically engineered plants has been shown to delay disease development and/or diminish invasion by viruses representing a range of groups (Beachy *et al.*, 1990). Tobacco plants expressing the coat protein of a lilac isolate of arabis mosaic virus poorly supported the replication of this virus and did not display any of the signs of systemic invasion produced in their untransformed counterparts or in transgenic plants expressing a different gene (β-glucuronidase). These effects were manifest whether the inoculum was virions or RNA (Bertioli *et al.*, 1992). Transgenic tobacco plants expressing the CP of tobacco rattle virus (TRV) strain TCM were found to be resistant to infection with the homologous virus but not to infection with the PLB strain of TRV. The amino acid sequence identity between the CP of TRV strains TCM and PLB is 39% and the two CP genes do not cross-hybridize. On the other hand, there is extensive cross hybridization between the CP genes of TRV-TCM and a Dutch isolate of pea early browning virus (PEBV). The transgenic plants accumulating TRV-TCM CP showed a considerable resistance to infection with PEBV (Van Dun and Bol, 1988). In the protoplasts from TCM-CP plants, the synthesis of RNA-1b and 16K protein was blocked when inoculation was performed with TRV-TCM virions but not when the inoculum consisted of TRV-TCM RNA. This suggests that an interaction between CP and plus-strand viral RNA in the parental virus particles results in the synthesis of minus-strand RNAs that do not serve as templates for the synthesis of RNA-1b. It could be that the

endogenous CP prevents a complete uncoating of the incoming virus particles. CP molecules that remain bound to the parental RNA could introduce mistakes in the transcription of minus-strand RNA that affect the subgenomic promoter for RNA-1b synthesis (Angenent *et al.*, 1990). Transformation with the coat protein gene of potato leaf roll virus has induced a measure of resistance to virus multiplication in two potato cultivars. Among a number of conventionally-bred clones of potato, some were resistant to virus multiplication and were found to accumulate little virus (Barker *et al.*, 1992).

A normal host-pathogen relationship can be disrupted, leading to resistance, if the host organism expresses a pathogen-derived gene product which interferes with the pathogen's normal replicative cycle (Sanford and Johnston, 1985). Lindbo and Dougherty (1992) have generated transgenic tobacco plants which express untranslatable sense or antisense forms of the tobacco etch virus potyvirus (TEV) coat protein (CP) gene sequence. One of seven transgenic plant lines expressing a CP gene antisense transcript showed an attenuation of symptoms when inoculated with TEV. Three of ten transgenic plant lines expressing untranslatable sense transcripts did not develop symptoms when inoculated with TEV. These lines were resistant to either aphid or mechanically transmitted TEV. In contrast to CP-mediated resistance reported for other viruses, resistance was (1) mediated by an RNA molecule; (2) TEV-specific (i.e., "broad-spectrum resistance" was not observed); (3) independent of inoculum levels; (4) not dependent on plant size and; (5) due to decreased levels of virus replication. Protoplasts experiments were used to demonstrate that resistant plant lines did not support the production of virus protein and progeny virus at wild-type levels.

1.11 Nucleotide sequence of the coat protein gene and RNA genome of PSbMV

The nucleotide sequence of a 1355 bp cDNA representing the 3'-terminal sequences of PSbMV was determined (Timmerman *et al.*, 1990) . This sequence contained a single long open reading frame (ORF) of 1189 bp ending with a single TAA termination codon. Downstream from the ORF was an untranslatable region of 189 bp followed by 8 bp of polyadenylate. The probable location of the PSbMV coat protein codons within the long ORF was determined by comparing the inferred amino acid sequence with other potyviral coat protein sequences and by examining the sequence for a potyviral polyprotein cleavage cassette

sequence. Direct chemical sequencing of the PSbMV coat protein revealed it to be blocked at its amino terminus. A partial amino acid sequence representing the N-terminus of the protease-resistant core of the coat protein was determined, however. Alignment of the PSbMV coat protein sequence and the sequences of 7 other potyviral coat proteins revealed significant homology, ranging from 53.7% for potato virus Y strain D to 43.2% for tobacco vein mottling virus. Mere recently, the complete nucleotide sequence of the RNA genome of PSbMV has been determined from cloned cDNA and by direct sequencing of viral RNA (Johansen *et al.*, 1991). The PSbMV genomic sequence was determined to be 9924 nucleotides in length excluding the poly (A) tract. The RNA contained an open reading frame (ORF) of 9618 nucleotides with the potential to encode a polyprotein with a calculated M_r of 364000 (364K). The ORF was flanked by a 5' untranslated leader sequence of 143 nucleotides and a 3' untranslated region of 163 nucleotides. A comparison of the PSbMV polyprotein with the polyproteins of the potyviruses tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), plum pox virus (PPV) and potato virus Y (PVY) showed that PSbMV had a similar genome organization. The polyproteins had a high level of amino acid identity except in the N-terminal region, which varied in both sequence and length. Putative proteolytic cleavage sites were identified in the polyprotein of PSbMV by comparison with those identified for other potyviruses. The cleavage site between the 6K protein and the 49K proteinase is thought to occur at the C-terminal side of glutamine as in other potyviruses. In addition to the 5 proteolytic cleavage sites for the 49K proteinase identified previously, a 6th putative cleavage site was identified internally in the 49K proteinase of PSbMV, as well as in the 49K proteinases of TEV, TVMV, PPV, PVY and soybean mosaic virus.

1.12 Nucleic acid hybridization studies

The properties of a virus are determined by the sequence of the four nucleotide residues in the nucleic acid of its genome. The ultimate comparison of viruses must therefore be done at the level of their nucleic acids; their complete sequence, gene content, and gene arrangement (Gould and Symons, 1983). It is the expression of the coat protein genes that has allowed the extensive comparison of viruses by serological techniques (Clark, 1981; Van Regenmortel, 1978 and 1982), whereas, it is the complex and very poorly characterized interaction of the viral nucleic acids and their gene products with host plant that leads to the phenotypic

expression of disease symptoms. The technique of hybridization analysis using complementary DNA (cDNA) is a powerful method for (i) estimating the extent of sequence homology between viral nucleic acids, (ii) probing the finer aspects of the organization of the viral genome, (iii) detecting and quantifying the presence of minor RNA species, and (iv) the rapid diagnosis of viral and viroid infections (Gould and Symons, 1983). Huang and Pagano (1977) revealed that the prime application of hybridization techniques is for the detection of viral genomes that cannot be disclosed by tests for infectivity. *In vitro* systems, for synthesis of viral DNA or RNA require identification and quantitation of the products by specific hybridization. Nucleic acid hybridization provides the only accessible method for the identification of messenger RNA. It provides the ultimate method for the determination of homology between viruses both in kind and in degree. It is possible to determine the percentage of the genome or transcripts of the genome present in cells or tissue.

Hybridization signals may be given not only by the homologous virus, but also by heterologous viruses belonging to the same or different taxonomic groups (Koenig *et al.*, 1988). Quantitative dot-blot hybridization tests with extracted viral RNAs proved to be very sensitive in differentiating closely related viruses which were barely distinguishable in serological tests.

A randomly ^{32}P-labeled DNA probe was prepared by reverse-transcribing tobacco mosaic virus (TMV) RNA to produce single-stranded complementary DNA (cDNA) (Sela *et al.*, 1984). A dot hybridization technique using the (^{32}P) cDNA probe was adopted for detecting TMV-RNA in crude leaf sap and from protoplasts derived from cultured cells. Tissue homogenates or lysed protoplasts were briefly denatured and applied direcctly to a nitrocellulose membrane without further clarification. As little as 2.5 pg of purified TMV-RNA could be detected.The method was about twice as sensitive and detected the appearance of new TMV-RNA earlier in infection than the ELISA method which only detected new capsid protein.

Scope of this thesis

The theme of this thesis is the pathology and distribution in the host of PSbMV. The biological and serological properties of several Australian isolates and known overseas isolates of PSbMV were compared. An indirect dot-immunobinding assay (DIBA) on nitrocellulose

membrane was developed, which is a simple and rapid test for the diagnosis of these isolates. DIBA and dot hybridization assay (DHA) with cDNA to PSbMV RNA were used to further investigate the transfer of PSbMV between generations by seed. This thesis reports that the virus becomes subliminal in the vegetative stage of the plant, but that it reaches a relatively high concentration in the seed. The implications of these findings for virus epidemiology and control are discussed.

Part of the work reported in this thesis has been published (see Appendix III).

Chapter 2 General Materials and Methods

2.1 Materials

2.1.1 Virus isolates

The isolates used, their geographic origins, and source are shown in Table 2.1. The isolates were maintained both in plants and seed of *P. sativum* cvs Dundale and Early Dun. These cultivars were used for all further studies with pea. Storage in desiccated leaf was found to be unreliable. Local lesion tests were made on *Chenopodium amaranticolor* Coste and Reyn. grown at 25°C under continuous light at 2 000 lx.

Table 2.1. Pea seed-borne mosaic virus isolates, sources

Isolate	Geographic Origin	Source
US	USA	R. O. Hampton[a]
Q	Queensland	J. Thomas[b]
T	Tasmania[c]	D. Munro[d]
S4	South Australia	D. Cartwright[e]
S6	South Australia	D. Cartwright
D	Denmark	M. Albrechtsen[f]
NZ	New Zealand	J. Fletcher[g]

[a]Oregon State University, Corvallis; [b]Department of Primary Industries, Queensland; [c]University of Tasmania germplasm collection; [d]Department of Agriculture, Tasmania; [e]Department of Agriculture, South Australia; [f]Plantevaerns Centret, Denmark; [g]Department of Scientific and Industrial Research, New Zealand.

2.1.2 Plants

All plants unless otherwise specified were grown either in a glasshouse at 20-25°C or in a growth room (25°C) under continuous light at 2,000 lx. The seeds and cultivars used are listed in Table 3.1.

2.1.3 Bio-chemicals

The main biochemicals used in this study are listed in Appendix I-1.

2.1.4 Miscellaneous chemicals

The main chemicals used in this study are listed in Appendix I-2. Other chemicals not listed were all analytical grade.

2.1.5 Water

Autoclaved double distilled water (DDW) was used in all experiments.

2.2 Methods

2.2.1 Comparative host range

Sixteen pea cultivars and lines, 21 other legumes and two indicator species (Table 3.1) were inoculated with the five isolates either mechanically or with aphids. Test plants were back-indexed to pea and *C. amaranticolor*. Both *Myzus persicae* Sulz. and *Acyrthosiphon pisum* Harris were used for aphid inoculations. A group of five aphids was used for each plant, and 10 plants of each host selection were used for each aphid species. Acquisition feeds were 30-45 seconds and inoculation feeds were 2 hours.

2.2.2 Classification of PSbMV isolates with pea differentials

Six pea differentials: Plant Introductions (PI) 272171, 269774, 193836, 347484, 347422 and 347329 (originating from the USDA germplasm collection and kindly provided by Dr. J. Fletcher, DSIR, New Zealand) were used to classify the PSbMV isolates. Four groups of 10 seeds from each line were sown. At the 3 leaf stage, each group was mechanically inoculated with isolates US, Q, S4 or S6. All plants were tested individually by DIBA at two weeks after inoculation.

2.2.3 Seed transmission

2.2.3.1 Detection of PSbMV in *Pisum sativum* cv. Dun and Dundale

Seed samples of pea cultivar Dundale from Willunga and Dun from Willunga, McLaren Vale, Maitland, Keith and Maslins Beach were supplied by the South Australian Department of Agriculture, Northfield. From each seed source, 300 seeds were divided into 3 lots of 100. Seeds from the first lot were triturated individually with 50 mM sodium borate buffer, pH 8.1 (w/v) and mechanically inoculated to individual *Chenopodium amaranticolor* plants. The second lot of seeds was also triturated as above and tested for PSbMV by DAS-ELISA. The third lot were sown, and at the three leaf stage, leaf samples were triturated as above and were also tested by DAS-ELISA. The seedlings from this lot

were assessed visually for symptoms of PSbMV.

2.2.3.2 Relationship between seed size and rate of seed transmission

To study the relationship between seed size and rate of seed transmission of PSbMV, seedlings of cv Dundale were inoculated with all virus isolates and all infected plants grown to maturity. Harvested seed was classed according to size with a sieve, average seed weights were obtained, and samples comprising either 50 seeds or 2-week old seedlings were tested individually for virus by DAS-ELISA (Clark and Adams, 1977).

2.2.4 Virus purification

The US, Q, S4 and S6 isolates were propagated in pea , and purified using a modification (see Table 4.6) of the Triton method of Moghal and Francki (1976). Leaf tissue was blended in one volume (w/v) of 500 mM sodium-borate buffer, pH 8.1, containing 0.15% thioglycollic acid and 0.5 volume of chloroform. The resulting emulsion was centrifuged at 10,000 g for 15 min. The supernatant was recovered, Triton-X 100 was added to 5%, and it was stirred for 30 min. at 4°C. Virus was sedimented by centrifugation at 311,000 g for 1 h in the 70 Ti rotor. After resuspension in 50 mM sodium-borate buffer, pH 8.1 containing 5 mM EDTA for 12 h at 4°C, and clarification at 10,000 g for 10 min., virus was sedimented through a 20% (w/v) sucrose cushion at 372,000 g for 20 min in the TLA 100.3 rotor of a Beckman TL 100 centrifuge. The pellet was resuspended in 50 mM sodium-borate buffer, pH 8.1 for 2 h, clarified at 10, 000 g for 10 min., and fractionated by centrifugation through a 10-40% (w/v) linear sucrose density gradient at 165,000 g for 1.5 h in the SW 41 rotor. Virus was recovered from appropriate fractions by high speed centrifugation and the resuspended pellet was layered over a 30-60% gradient of Nycodenz (Nyegaard; Gugerli, 1984) dissolved in 50 mM sodium-borate buffer pH 8.1, and subjected to isopycnic density gradient centrifugation for 4 h at 264,000 g in the TLA 100.3 rotor. The single light scattering band three-fourths of the way down the gradient was drawn out with a sterile syringe and sedimented through a 30% (w/v) sucrose cushion to remove the Nycodenz. The resuspended pellet was mixed with 50% glycerol before storage at -20°C.

The purified preparations were negatively stained with either neutral 2% phosphotungstic acid or 2% uranyl acetate and examined under the electron microscope to assess the purity, state of fragmentation and aggregation of virus particles.

2.2.5 Serology

2.2.5.1 Immunization of rabbits for antiserum production

Two rabbits were immunized with the isolates US and S6 and single rabbits were immunized for isolates Q and S4. In one rabbit of each pair, the first injection of 200 µg of virus in Freund's complete adjuvant (FCA) was given subcutaneously, the second and third [200 µg in Freund's incomplete adjuvant (FICA)] were given 7 and 14 days later. A fourth subcutaneous injection of 500 µg of virus in FICA was given at 42 days. In the second rabbit, the first 3 injections were given intravenously, the fourth was intramuscular with FICA.

2.2.5.2 Two-dimensional immunodiffusion test

Two-dimensional immunodiffusion tests (Van Regenmortel, 1982) were done in 0.75% agarose in 0.02 M phosphate buffer, pH 7.6 containing 0.02% (w/v) sodium azide. All virus antigen preparations were at 1 mg/ml and were sonicated for 15 min. at 0°C in a Labsonic 1510 (B. Braun) unit immediately before use.

2.2.5.3 DAS-ELISA

2.2.5.3.1 Purification of γ-globulin

One ml of PSbMV antiserum was diluted with 9 ml of sterile DDW, mixed with 10 ml of saturated ammonium sulphate solution and left at room temperature for 30 min. The precipitate was collected by centrifugation at 3,000 *g* for 10 minutes and dissolved in 2 ml of 1/2-strength phosphate buffered saline (PBS) (Clark and Adams, 1977). The dissolved precipitate was dialyzed 3 times against 500 ml 1/2-strength PBS at 4°C. The γ–globulins were further purified by DE 22 cellulose column chromatography and the effluents were collected in silicone coated tubes. The purified γ-globulin fractions were adjusted to approximately 1 mg/ml (OD 280 = 1.4) and stored in silicone-treated tubes at -15°C.

2.2.5.3.2 Conjugation of enzyme with γ-globulin

Approximately 3 mg of alkaline phosphatase was dissolved in 1 ml of purified γ-globulin and dialyzed 3 times against 500 ml PBS at 4°C. Glutaraldehyde solution was added to 0.06% final concentration and left for 4 h at room temperature. Glutaraldehyde was removed by dialyzing 3 times against 500 ml PBS. Bovine serum albumin (BSA) was added to about 5 mg/ml and the preparation was stored at 4°C.

2.2.5.3.3 Procedure for carrying out microplate DAS-ELISA technique

To each well of the microtitre plate 200 µl (2 µg/ml) of purified γ–globulin in sodium carbonate buffer, pH 9 was added and incubated for 3 h at 25°C. Plates were washed 3 times each for 3 minutes by flooding wells with PBS-Tween wash buffer. Aliquots of 200 µl test sample in PBS (w/v) were placed in duplicate wells and incubated for 12 h at 4°C. Plates were washed again as above. Aliquots of 200 µl enzyme-labelled globulin was added to each well and incubated for 4 h at 25°C . Plates were washed as above. Aliquots of 200 µl freshly prepared enzyme substrate were added to each well and incubated for 30 minutes to 1 h at 25°C. The results were assessed by visual observation or measurement of absorbance at 405 nm in a Bio-Rad Model 2550 EIA Reader.

2.2.5.4 Dot-immunobinding assay (DIBA)

The DIBA of Hibi and Saito (1985) was modified (see 5.4) for testing virus, tissue and seed samples on nitrocellulose membranes. One microlitre of each antigen sample was spotted onto individual squares of the sheet (Schleicher and Schuell, BA85, 0.45 µm pore diameter) and allowed to air dry. The test sheet was incubated with blocking phosphate buffered saline (PBS) buffer containing either healthy leaf sap (1/10), healthy seed extract (1/10) or both (1:1) for 30 min. at 25°C. The first antibody was polyclonal antiserum diluted 1/5000 and the second was goat anti-rabbit IgG-alkaline phosphatase conjugate(Sigma Chemical Company) diluted 1/5000 and incubated for 1 hour at 25°C. Alkaline phosphatase was detected with substrate containing 75 mg/ml nitro blue tetrazolium, 2 mg/ml phenazine methosulphate and 40 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Company) at 37°C for 2-5 min. The reaction was stopped by adding 10 mM Tris-HCL, pH 7.5 containing 5 mM EDTA. The time required to complete the assay was 5 hours.

Problems of membrane background were overcome by diluting the antiserum and increasing the time of washing. Non-specific binding was removed by adding glucose and mannose each at 500 mM to all buffers and by using healthy leaf or dry seed extracted by crushing in nine volumes of PBS, and used as the blocking agent.

Cytoplasmic inclusion protein (CIP) antiserum (kindly supplied by Dr. M. Albrechtsen, Plantevaerns Centret, Lottenborgget, Lyngby, Denmark) was also used to identify PSbMV infection, using the same assay procedure as above.

2.2.5.5 Electron microscopy

Copper grids of 400 mesh coated with formvar and a carbon film were briefly discharged with a ST 4 M Spark tester before use. A drop of about 20 µl of appropriately diluted virus preparation was pipetted on to a grid for 1 min and extra liquid removed with a piece of filter paper. The grid was then stained with either 2% phosphotungstic acid (PTA), pH 4.5 (Hatta and Francki, 1984) or 2% uranyl acetate, pH 6.5 for 1 min and extra liquid removed as above, then placed on a Whatman filter paper to dry. The leaf dip method (Noordam, 1983) was used to examine PSbMV particles in an infected plant. This was done by cutting the leaf and touching the cut edge to a grid with a drop of stain on it for about 30 seconds. The viruses were viewed in a JOEL 100 CX electron microscope.

2.2.5.6 Immunosorbent electron microscopy (ISEM)

Leaf and seed extracts were tested using a modification of the method of Roberts & Harrison (1979). Carbon-coated grids were floated film side down on 5 µl drops of antiserum diluted 1:100,000 in phosphate buffered saline for 1 h at 25°C. The grids were then drained briefly by touching to a filter paper. Grids coated with antiserum were then floated on 20 µl drops of plant extract placed on aluminum foil in petri dishes containing moist filter paper, for 12 h at 4°C. Grids with attached virus were washed once with double distilled water, then negatively stained with either neutral 2% phosphotungstic acid or 2% uranyl acetate and examined under the electron microscope.

2.2.6 Spectrophotometry

Ultraviolet absorption spectra were determined using a Beckman DU-8B spectrophotometer with a 1 cm-path quartz cuvette. Concentrations were determined using the extinction coefficient $E^{0.1\%}_{260} = 3$ (Brunt, 1970).

2.2.7 Nucleic acid studies

2.2.7.1 Precautions against RNase activity

Whenever possible all equipment, glassware, buffers and chemicals were autoclaved to inactivate RNase. Otherwise items were washed with alcoholic KOH (100 g KOH, 100 ml DDW and 900 ml ethanol) and rinsed thoroughly with DDW.

2.2.7.2 Preparation of total plant nucleic acids

Seed and leaf samples from healthy or infected pea were triturated with a pestle and mortar in 2 volumes (w/v) each of 50 mM Tris-HCl pH 7.5, 10 g/litre sodium dodecyl sulphate (SDS) and aqueous phenol (900 g/litre containing 1 g/litre 8-hydroxyquinoline). The mixture was shaken for 45 min., centrifuged at 10,000*g* for 10 min. and the aqueous supernatant fraction was extracted once with 0.5 volumes each of phenol and chloroform. Nucleic acids were precipitated with 3 volumes of ethanol in the presence of 200 mM sodium acetate.

2.2.7.3 Preparation of virus RNA

PSbMV-RNA was prepared by incubating virus in 50 mM Tris-HCl pH 7.5, 10 g/litre SDS, containing 200 mM sodium acetate and protease type VI (Sigma, 2 mg/ml) for at least 12 h at 25°C or 3 h at 37°C. Mixtures were extracted once with 0.5 volumes of phenol-cresol (9:1 ratio) and RNA was precipitated with ethanol.

2.2.7.4 Synthesis of cDNA

Virus RNA (approximately 1 µg) in water was incubated in the presence of hexa-deoxynucleotide primers (62.5 µg/ml; Pharmacia), 10 mM $MgCl_2$, 50 mM Tris-HCl pH 7.4, 50 µM-*d*ATP, -*d*GTP, and -*d*TTP, 30 µCi[^{32}P]*d*CTP(3000 Ci/mmol, Amersham), 500 µM 2-mercaptoethanol, and MMuLV reverse transcriptase(Bethesda Research Laboratories, 400 units). The volume of the reaction mixture was 31 µl, with incubation for 5 h at 37°C. The transcribed probe was separated on a G-50 Sephadex column, boiled in TE buffer, and added to hybridization buffer to give between 0.5 x 10^6 and 1.0 x 10^6 cpm/ml (Randles & Rohde, 1990).

2.2.7.4.1 Measurement of radioactivity

^{32}P was measured directly in a Beckman LS 5000 TD Liquid Scintillation System. The relative radioactivity was expressed as counts per minute (CPM).

2.2.7.5 Hybridization assays

Nucleic acid extracts dissolved in water were applied as 1 µl dots to nylon membrane (Zeta-probe, Biorad). The nylon was baked at 80°C for 1.5 h, prehybridized at 42°C for 16 h in 20x SSC (3 M NaCl, 0.3 M sodium citrate) containing 5 mM-EDTA, 2 g/litre SDS, 50 mM-Na phosphate pH 6.5, 0.2 g/litre each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolidone (PVP, Mr 40,000), 50% deionized formamide and boiled herring sperm DNA (0.25 mg/ml). Hybridization buffer was as above but contained 2 g/litre each of BSA, Ficoll 400, and PVP 40,000, 50% dextran sulphate, 1 mg/ml boiled carrier DNA and boiled cDNA. Incubation was at 42°C for 16 h. Membranes were washed at 65°C in 1x SSC with 1 g/litre SDS for 1 h, then in 0.1x SSC with 1 g/litre SDS for 1 h before autoradiography at -70°C using an intensifying screen.

2.2.8 Studies on virus distribution in pea

Groups of twenty *P. sativum* cv. Dundale seedlings at the 3 leaf stage were each mechanically inoculated with isolates US, Q, S4 or S6. Two weeks after inoculation, plants were tested for PSbMV by DIBA using homologous polyclonal antisera. For each virus isolate twenty seeds were collected at random from infected mature plants and sown to produce the second vegetative generation (G2). The first true leaves were assayed immediately after expansion by DIBA for PSbMV and CIB. As the plants grew, the leaves, stipule and peduncle were sampled as soon as they became available and tested as above. At the reproductive stage, the flowers were excised and dissected and the floral parts (see Table 6.1 and Figure 6.1) were individually tested by DIBA. Some pods with green seeds were also dissected and the pod wall, midrib plus funicle, testa, cotyledon and embryo separated and tested. The remaining pods were left to mature.

At the completion of the second generation, 40 seeds were again taken for each of the 4 isolates and divided into 2 groups of 20 seeds. For one group the seeds were left intact, whereas in the other the testas were removed. Seeds were sown to give the third generation (G3) and leaves 1-9 of each plant were tested. After pod set, pods with green seeds were again dissected and tested.

Twenty mature dry seeds were collected from the plants raised from intact seed and these were sown to provide the fourth generation (G4). All leaves of fully grown plants, and green

and dry mature seeds were then tested by both DIBA and dot hybridization assay using ^{32}P-cDNA specific for PSbMV RNA. This was repeated for the fifth generation (G5).

2.2.9 Cross Protection Studies

Groups of ten seedlings of Dundale with or without suspected subliminal infection with PSbMV were mechanically inoculated at the three-leaf stage with the isolate US . Two weeks after inoculation, plants were tested for PSbMV by DIBA and DAS-ELISA. All the different leaf stages from 1-22 (see Figure 6.1) were sampled and tested. At the reproductive stage, the whole flower and whole mature green seed were also sampled and tested.

Chapter 3 Biological Properties of PSbMV

Introduction

PSbMV causes various degrees of stunting, downward rolling of leaflets, and a transient clearing and swelling of leaf veins of most cultivars of *Pisum sativum* L. Infected plants may produce distorted flowers, which often give rise to small distorted pods. Ovule development is affected in pods and may be uneven, with only one or two seeds produced. Seed coats may split as the seeds mature (Hampton and Mink, 1975).

PSbMV is economically important both because of its high rate of seed transmission and because the virus can reduce pod yield by 63% and seed yield by 84% (Anon, 1985). The virus is spread in the field by 21 species of aphids (Khetarpal & Maury, 1987), and secondary spread is thought to account for high levels of infection in crops and seed (Hampton & Mink, 1975).

The symptoms induced by PSbMV vary according to virus isolate and host genotype. Differential genotypes of *Pisum* have been used for standardizing isolates and strains of PSbMV (Hampton,1980). Symptoms induced in these differentials by PSbMV range from very rapid development of whole-plant necrosis at one extreme to very slight leaf rolling and/or vein clearing at the other. The pattern of resistance to these differentials, and the symptoms produced, have allowed isolates to be classed into either one of 3 pathotypes (P1, P4 and L1; Alconero *et al.*, 1986) or one of 5 groups (I-V; Hampton *et al.*, 1981).

This chapter describes the biological properties of several Australian isolates and compares them with a type isolate of PSbMV from the USA.

Results and discussion

3.1 Origin of isolates S4 and S6

Biological indexing (Mink and Parsons, 1978) detected a virus resembling PSbMV in 2 of 12 certified lines from the Seed Services Section of the South Australian Department of Agriculture at Northfield (Ligat *et al.*, 1991). The identity of the virus was confirmed as PSbMV by indirect ELISA at Washington State University, USA (S. Jones,

personal communication). They were designated as isolates S4 and S6. Their identity was confirmed by host range, pathogenicity to pea differentials and by serological studies with antisera to the isolates Q and US as PSbMV.

3.2 Comparative host ranges

Isolates US, Q, T, S4 and S6 produced local necrotic lesions on *Chenopodium amaranticolor* (Table 3.1). Only the US isolate infected *Nicotiana clevelandii* while no isolate infected *Phaseolus vulgaris* cv. Hawkesbury Wonder. All isolates infected *Vicia faba* inducing the same symptoms. The isolates caused distinctive symptoms on selected pea cultivars. For example on cv. Dundale; isolate US showed a severe mosaic, isolate S4 showed a chlorotic mottle, isolate S6 showed a severe leaf roll, isolate Q showed a vein-banding, and isolate T showed a mild mosaic and leaf roll (Figure 3.1). Its ability to infect Greenfeast pea distinguished the US isolate from the others. Isolate T was distinguished from the others by its inability to infect *Lathyrus* and *Lens spp.*

The results of aphid transmission generally paralleled those of mechanical inoculation, except that isolate T was not transmitted to Maitland pea by either aphid species.

3.3 Classification of the PSbMV isolates with pea differentials

With the limited number of pea differentials, the PSbMV isolates did not fall clearly into groups or pathotypes (Table 3.2). However, it can be seen that all 4 isolates were distinguished with this group of pea differentials. A tentative classification of the isolates can be made as follows. Isolate US is in group III, because it infects PI 272171. However, it also infects PI 347422 which is resistant to all strains. Isolate US is in pathotype P1, because it infects PI 347329. Isolate Q is in group III, because it infects PI 272171. However, it also infects PI 347422 which is resistant to all strains. Isolate Q is in pathotype P1, because it infects both PI 347484 and PI 347329. Isolates S4 and S6 are in group V and pathotype P4, because they infect PI 269774. However, isolate S6 infects PI 347422 which is resistant to all strains.

Table 3.1. Comparison of host range, symptoms and transmissibility by aphids of 5 isolates of PSbMV

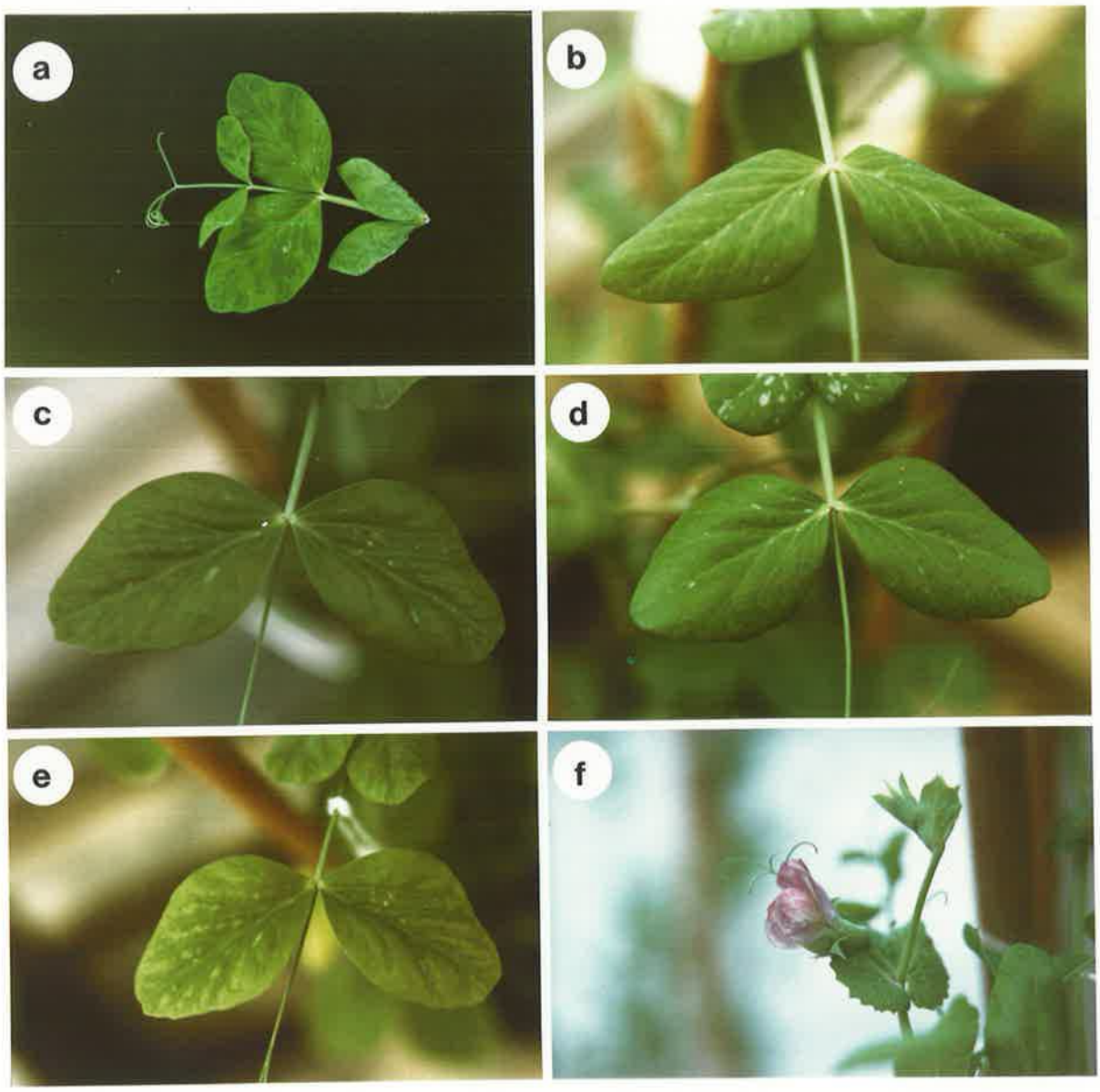
Host	SYMPTOMS[a]					APHID TRANSMISSIBILITY[b]									
	US	Q	T	S4	S6	US		Q		T		S4		S6	
						M[c]	A[d]	M	A	M	A	M	A	M	A
Chenopodium amaranticolor Coste and Reyn	NLL	NLL	nll	NLL	NLL	0	0	0	0	0	0	0	0	0	0
Nicotiana clevelandii A. Gray	m,vc	-	-	-	-	0	0	0	0	0	0	0	0	0	0
Phaseolus vulgaris L.	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0
Vicia faba L.	m,lr	m,lr	m,lr	m,lr	m,lr	4	5	4	4	3	4	3	3	3	3
Pisum sativum L.															
cv. Alma	M,lr	VB,lr	m,lr	CM,lr	m,LR	4	5	5	5	2	2	4	5	3	4
Blue Boiler	m,lr	lr	lr	lr	lr	2	2	2	2	1	1	2	2	2	2
Buckley	M,lr	VB,lr	m,lr	m,lr	m,LR	4	4	4	4	3	3	3	3	3	3
Collegian	M,lr	VB,lr	m,lr	m,lr	m,LR	4	5	4	5	2	3	4	4	3	4
Derrimut	M,lr	m,lr	m,lr	m,lr	m,LR	4	4	4	4	1	1	3	4	3	3
Dundale	M,lr	VB,lr	m,lr	CM,lr	m,LR	4	4	4	4	3	3	3	4	4	4
Early Dun	M,lr	VB,lr	m,lr	M,lr	m,LR	4	5	4	5	3	3	4	4	4	5
Green Feast	m,lr	0	0	0	0	2	3	0	0	0	0	0	0	0	0
Maitland	m,lr	m,lr	m,lr	m,lr	m,lr	3	4	2	2	0	0	2	2	2	2
Pennant	M,lr	VB,lr	m,lr	m,lr	m,LR	4	5	4	5	3	3	4	5	4	4
Prussian Blue	m, lr	lr	lr	lr	lr	3	3	2	2	1	1	2	2	2	2
Whero	m, lr	M, lr	m, lr	Cm, lr	m, LR	4	4	4	3	3	3	4	4	5	4
Wirrega	M,lr	VB,lr	m,lr	CM,lr	m,LR	4	4	4	4	2	2	4	4	4	4
line no. P225-2	M,lr	VB,lr	m,lr	CM,lr	m,LR	5	5	4	4	2	2	4	4	4	3
SA 432	m,lr	vb,lr	m,lr	m,lr	m,lr	3	3	3	3	2	2	3	3	3	3
SA 433	m,lr	vb,lr	m,lr	m,lr	m,lr	4	4	3	3	2	2	2	3	3	3
Lathyrus cicera L.															
line no. SA 17011	m,lr	vb,lr	-	m,lr	m,lr	3	3	3	3	0	0	2	2	2	2
SA 22117	m,lr	vb,lr	-	m,lr	m,lr	2	3	2	2	0	0	2	2	2	2
SA 22149	m,lr	vb,lr	-	m,lr	m,lr	3	3	2	3	0	0	2	3	2	2
SA 22083	M,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	2	2	2	2
SA 22118	M,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	2	2	2	2

Lathyrus ochrus L.																
line no.	SA 22075	m,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	2	2	2	2
	SA 22073	M,lr	vb,lr	-	m,lr	m,lr	3	4	2	2	0	0	2	2	2	2
	SA 22074	m,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	1	2	1	2
	SA 22077	m,lr	vb,lr	-	m,lr	m,lr	3	3	1	2	0	0	1	1	1	1
	SA 22076	M,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	1	2	1	2
Lathyrus sativus L.																
line no.	SA 19667	m,lr	vb,lr	-	m,lr	m,lr	2	3	2	2	0	0	1	1	1	1
	SA 22078	m,lr	m,lr	-	m,lr	m,lr	3	3	2	2	0	0	1	1	1	2
	SA 22081	M,lr	m,lr	-	m,lr	m,lr	2	2	1	2	0	0	1	1	1	1
	SA 17024	m,lr	vb,lr	-	m,lr	m,lr	2	3	2	2	0	0	1	2	1	2
	SA 16214	m,lr	vb,lr	-	m,lr	m,lr	3	3	2	2	0	0	2	2	1	2
Lens culinaris L.																
cv. Laird		m,lr	vb,lr	-	m,lr	m,lr	3	2	1	1	0	0	1	1	1	1
Kye		m,lr	vb,lr	-	m,lr	m,lr	2	2	1	1	0	0	1	1	1	1
line no.	1LL 20	m,lr	vb,lr	-	m,lr	m,lr	2	3	2	2	0	0	1	2	1	1
	1LL 5731	m,lr	lr	-	m,lr	m,lr	3	3	1	2	0	0	1	2	1	1
	1LL 5716	m,lr	vb,lr	-	m,lr	m,lr	2	3	1	2	0	0	1	2	2	2
	1LL 4401	m,lr	vb,lr	-	m,lr	m,lr	3	3	1	2	0	0	1	2	1	2

(a) Mechanical or aphid inoculation, (b) number of plants infected from 10 test plants each inoculated with 5 aptera, (c) *Myzus persicae*, (d) *Acyrthosiphon pisum*.

Key to abbreviations: M, severe mosaic; m, mild mosaic; LR, severe leaf roll; lr, mild leaf roll; VB, severe vein-banding; vb, mild vein-banding; vc, mild vein-clearing; CM, severe chlorotic leaf mottle; NLL, severe necrotic local lesion; nll, mild necrotic local lesion.

Fig. 3.1 Distinguishing symptoms of five PSbMV isolates in *Pisum sativum* cv. Dundale: a) isolate S4, chlorotic mottle; b) isolate S6, severe leaf roll; c) isolate Q, vein-banding; d) isolate T, mild mosaic and leaf roll; e) isolate US, severe mosaic; and, f) isolate US, colour break.


a
b
c
d
e
f

3.4 Seed Transmission

3.4.1 Detection of PSbMV in *Pisum sativum* cv. Dun and Dundale

To determine whether PSbMV had become established in commercial crops in South Australia, the South Australian Department of Agriculture at Northfield conducted a limited survey of PSbMV infection in crops of field pea (*Pisum sativum* L.) in 1984-1985 by collecting samples from two commercial seed receival points (Ligat *et al.*, 1991). The

Table 3.2. Classification of the PSbMV isolates using pea differentials

	Isolates				Susceptibility	
Pea Differentials	**US**	**Q**	**S4**	**S6**	**Group**	**Pathotype**
PI 272171	+	+	+	+	III	
PI 269774	-	-	+	+	V	P4
PI 193836	-	-	-	-		L1 and P4
PI 347484	-	+	-	-		P1 and P4
PI 347422	+	+	-	+		Resistant to all strains
PI 347329	+	+	+	+		P1

collection comprised 83 samples representing crops from York Peninsula, the Adelaide region, the mid-north and the southeast of South Australia. Twelve certified pea lines from the Seed Services Section, South Australian Department of Agriculture, were also sampled. Six samples representing 5 areas of South Australia were supplied by the department for testing for PSbMV (see 2.2.3.1).

As shown in Table 3.3, there was a low percentage of PSbMV infection in the seed samples collected from the five rural areas of South Australia. Seedlings from pea cv. Dun and Dundale had mild symptoms of mosaic and leaf roll. Mild chlorotic mottle with severe leaf roll was observed in another seed sample of pea cv. Dun from Willunga. Infectivity assay on *Chenopodium amaranticolor* showed that pea cv. Dundale had 3%

PSbMV infection while cv. Dun had an average of 2.4%. With DAS-ELISA, there was a similar low rate of virus detection (2 and 3%) in both the seed and seedlings.

3.4.2 Relationship between seed size and rate of seed transmission

The seed transmissibility of all isolates is summarized in Table 3.4 (see 2.2.3.2). Infected seed in each of the three largest size classes was lighter than the corresponding uninfected seed, and infected seed in all classes had a significantly lower germination rate than uninfected seed. The highest rate of seed transmission was detected in the largest

Table 3.3. Detection of PSbMV in 100 pea seeds by infectivity assay and DAS-ELISA. Numbers represent positive identification from 100 seeds tested.

Pea cultivar	Seed source	Infectivity assay*	DAS-ELISA		Symptom
			Seed	Seedling	
Dundale	Willunga	3	2	2	m, lr
Dun	Willunga	3	2	2	cm, lr
Dun	Mc Laren Vale	3	2	2	m, lr
Dun	Maitland	1	2	2	m, lr
Dun	Keith	2	2	2	m, lr
Dun	Maslins Beach	3	3	3	m, lr

* Extracts from individual seeds were mechanically inoculated to *Chenopodium amaranticolor*.

seed and the lowest was in the smallest. Transmission rates to seedlings were essentially the same as rates of PSbMV detection in seed and this appeared to be independent of variations in the rates of germination. This shows that the germination rate of infected seeds was not reduced by infection.

Conclusion

The isolates compared could be distinguished by their host range and symptoms.

The South Australian isolates (S4 and S6) were classified into one pathotype and group, and had high rates of transmission both in seed and seedlings of pea cv. Dundale.

The largest seed had the highest rate of transmission which was in contrast with the findings of Stevenson and Hagedorn (1973b) that the smallest seed contained more virus. Infectivity assay and DAS-ELISA showed that PSbMV was present in 5 areas of South Australia, but at a low incidence. It is not known whether PSbMV originated from infected seed, or by aphid transmission from reservoirs.

Table 3.4. Characteristics of seed transmission of 5 PSbMV isolates in *P. sativum* cv. Dundale

Isolate	Seed diameter (mm)	Average seed weight (mg)	% seed virus positive[a]	% germination	% seedlings virus positive[a]
US	7-8	240	92	93	91
	6-7	157	87	89	87
	5-6	117	83	85	81
	4-5	76	40	43	40
Q	7-8	277	86	92	85
	6-7	197	85	87	84
	5-6	117	80	79	79
	4-5	77	40	41	38
S4	7-8	297	88	92	87
	6-7	196	82	87	81
	5-6	157	79	80	78
	4-5	77	38	41	35
S6	7-8	277	83	93	82
	6-7	198	82	85	80
	5-6	158	77	81	76
	4-5	118	40	43	39
T	7-8	317	85	93	84
	6-7	237	81	85	79
	5-6	158	75	75	74
	4-5	78	29	31	27
Uninfected					
Seed	7-8	404	0	97	0
	6-7	341	0	96	0
	5-6	282	0	95	0
	4-5	85	0	91	0

[a]Assay by DAS-ELISA

Chapter 4 Purification of PSbMV

Introduction

Highly purified virus preparations are essential for chemical, physical, and certain biochemical studies, and many biological investigations are dependent on the availability of at least partially purified preparations (Francki, 1972). A high degree of purification is rarely a goal in itself; usually a specific use is intended for the purified virus, and that will determine the degree of purity required. If the virus is intended for the production of antiserum then the main concern will be that it is free of all host-plant antigens. On the other hand, if it is for studying the properties of the viral protein (s) or nucleic acid, then care must be taken to ensure freedom from analogous host-plant derivatives (Francki, 1972).

The use of sucrose density gradients has achieved the successful partial purification of several potyviruses in adequate yields, with a high degree of purity and without significant aggregation or change in the particle size distribution (Moghal and Francki, 1976). Knesek *et al* (1974) investigated several procedures for the purification of PSbMV and found that best results were obtained when infected roots were first extracted in 10 mM sodium diethyldithiocarbamate (NaDIECA) + 10 mM cysteine, and leaf was first extracted in NaDIECA + cysteine containing 10 mM EDTA and clarified with 0.5 volume of chloroform.

The purification of PSbMV together with 12 other viruses using isopycnic centrifugation in density gradients of Nycodenz, a non-ionic triiodinated derivative of benzoic acid with three aliphatic hydrophilic side chains, showed no significant losses of particles; they appeared intact, and the degree of purity was increased considerably (Gugerli, 1984). Wang *et al* (1992) purified ten PSbMV isolates using isopycnic caesium sulphate gradients which gave high virus yields.

This chapter describes the development of a purification method for PSbMV. This method has been published (Ligat *et al.*, 1991).

Results and discussion

4.1 Development of a purification schedule

4.1.1 Time course of increase of PSbMV in pea

The minimum and optimum times for harvesting infected material for virus purification were determined by DAS-ELISA. PSbMV-US was propagated in 10 pea cv. Dundale because of its high level of susceptibility to the virus (see Table 3.1). The inoculated and systemically infected leaves were sampled at 7-days intervals. At each harvest a composite sample was triturated in PBS buffer containing 2% polyvinyl pyrollidone (PVP M. W. 40,000) (1:1 w/v) and tested by DAS-ELISA. The virus concentration in the inoculated leaves reached a maximum after 14 days (Figure 4.1) and appeared to decrease thereafter. The virus reached significantly higher concentration (Moghal and Francki, 1976) in systemically infected leaves, the maximum being reached between 14-21 days after inoculation. Systemically infected leaves harvested 2 weeks after inoculation were used routinely for purification of the 4 PSbMV isolates.

4.1.2 Effect of extraction buffers

Phosphate and sodium borate buffers at different ionic strengths and pH values were evaluated for the extraction of PSbMV. There was a significant effect of the various buffers tested on the amount of virus extracted (Table 4.1). The number of local lesions tended to increase with higher molarities up to a certain level and decreased with a further increase of molarity. Sodium borate buffer at 500 mM concentration was finally selected as suitable for extraction of the virus.

4.1.3 Effect of reducing agents

Three reducing agents were tested for their effect on the infectivity of PSbMV extracts. Each was added to 500 mM sodium borate buffer, pH 8.1, before extraction. Maximum infectivity was recovered when thioglycollic acid was added to the buffer (Table 4.2). Thioglycollic acid at 0.15% was then used in the extraction medium in subsequent experiments.

Fig. 4.1 Assessment of virus concentration with time in *Pisum sativum* cv. Dundale. Ten healthy three leaf stage seedlings were mechanically inoculated with isolate US. The inoculated and systemically infected leaves were sampled at 7-days intervals. Each sample was triturated in phosphate buffered saline containing 2% polyvinyl pyrollidone (1:1 w/v) and tested by DAS-ELISA.

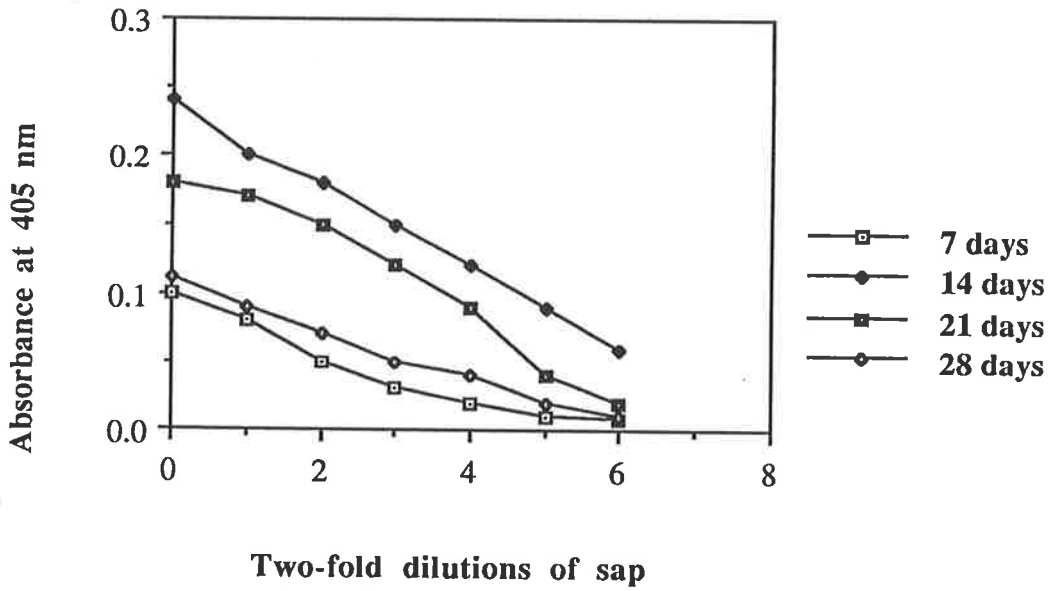

Absorbance at 405 nm
0.3
0.2
0.1
0.0
0
2
4
6
8
Two-fold dilutions of sap
7 days
14 days
21 days
28 days

Table 4.1. Effect of various buffers on the extraction of infectious PSbMV*

Extraction buffer	pH	Local lesions per half-leaf** Dilution 1:10	1:20	1:40	1:80
200 mM phosphate	7.6	31	14	7	2
500 mM phosphate	7.6	35	16	11	6
300 mM sodium borate	8.1	38	17	9	4
400 mM sodium borate	8.1	47	20	11	7
500 mM sodium borate	8.1	65	29	19	13
600 mM sodium borate	8.1	49	22	13	9
700 mM sodium borate	8.1	28	12	7	2

* Systemically infected Dundale leaves were triturated in each buffer (1:1 w/v). The extract was centrifuged at 10,000 *g* for 10 min. Supernatants were diluted with double distilled water before bioassay.

** Eight half-leaves of *Chenopodium amaranticolor* were used for each assay.

Table 4.2. Effect of adding reducing agents to buffer on the infectivity of PSbMV extracts*

Reducing agent	Concentration	Local lesions per half-leaf**
Control	-	14
Thioglycollic acid	0.15%	68
Sodium-DIECA	0.1%	21
2-mercaptoethanol	1%	29

* Systemic infected Dundale leaves were triturated in 500 mM sodium borate buffer, pH 8.1, (1:1 w/v) with or without the reducing agents shown. The extracts were centrifuged at 10,000 *g* for 10 min. The supernatants were diluted to 1/10 with double distilled water before bioassay.

** Two half-leaves of *Chenopodium amaranticolor* were used for each assay.

4.1.4 Effect of pH of the extraction buffer

Solutions of sodium borate buffer at 500 mM, containing 0.15% thioglycollic acid, were adjusted to different pH values ranging from 6.1 to 9.5, and were used to extract PSbMV. Results in Table 4.3 show that infectivity was low at pHs below 7.1. Moghal and Francki (1976) reported that low pH might result in aggregation of the virus or

Table 4.3. Effect of pH of the extraction buffer on the infectivity of PSbMV*

pH of extraction buffer	Local lesions per half-leaf
6.1	0
6.3	0
6.5	0
6.7	2
6.9	6
7.1	14
7.3	21
7.5	27
7.7	33
7.9	37
8.1	72
8.3	58
8.5	35
8.7	24
8.9	13
9.1	5
9.3	0
9.5	0

* Systemically infected leaves of pea cv. Dundale were triturated in 500 mM sodium borate buffer (1:1 w/v) at the pH values shown and bioassayed as in Table 4.2.

coprecipitation with its host materials. Infectivity was also decreased at pHs above 8.9, and the extracts became discolored probably due to oxidation. Infectivity was not recovered at pHs below 6.5 and above 9.3. In the succeeding experiments, the pH of the extraction buffer was maintained at 8.1.

4.1.5 Clarification of extracts

Chloroform, carbon tetrachloride and their combinations were compared for the emulsification of PSbMV extracts prepared in 500 mM sodium borate buffer, pH 8.1, containing 0.15% thioglycollic acid. Each of the organic solvents was added to the extract and the mixture was shaken for 10 min. After breaking the emulsion by centrifugation, the aqueous phase was gently withdrawn and assayed. There was relatively little effect of the 2 solvents which were used singly or in combination on the amount of virus extracted (Table 4.4). Chloroform was selected because of its efficient clarification and the better recovery of virus. In succeeding experiments, infected material (1 g) was homogenized in

Table 4.4. Effect of organic solvents on the infectivity of PSbMV*

Treatment	Volume added	Local lesions per half-leaf** Dilution 1:10	1:20	1:40	1:80
Untreated	-	67	28	13	4
Chloroform	1/2	62	25	13	5
Chloroform	1	65	29	15	6
Carbon tetrachloride	1/2	51	24	9	2
Chloroform + carbon tetrachloride	1/2 + 1/2	68	33	15	9

* Systemically infected pea cv. Dundale leaves were homogenized in 500 mM sodium borate buffer, pH 8.1, 0.15% thioglycollic acid. The extracts were emulsified with organic solvent, shaken for 10 min and centrifuged at 10,000 *g* for 10 min. The aqueous phase from each treatment was assayed after diluting with double distilled water.

** Eight half-leaves of *Chenopodium amaranti color* were inoculated.

a mixture containing one volume (w/v) of 500 mM sodium borate, pH 8.1, 0.15% thioglycollic acid, and one half volume of (w/v) chloroform. The resulting emulsion was broken by centrifugation at 10,000 *g* for 15 min and the aqueous phase was collected.

Triton X-100, a non-ionic detergent was added to the clarified extracts, stirred for 30 min and the virus was concentrated by one cycle of differential centrifugation.

4.1.6 Differential centrifugation

4.1.6.1 Resuspension of virus pellet

Moghal and Francki (1976) reported that the addition of EDTA to the resuspension medium decreased the aggregation of PVY (Delgado-Sanchez and Grogan , 1966), probably by acting as either a surface active or chelating agent (Brakke, 1959). Therefore, the virus was resuspended in 50 mM sodium borate buffer, pH 8.1, containing 5 mM EDTA for 12 h and clarified by centrifugation at 10,000 *g* for 10 min. A sample from the supernatant was examined under the electron microscope and it was observed that there was no lateral aggregation of the viral particles even with the presence of host impurities.

4.1.6.2 Sucrose cushion

To remove some of the host impurities, the supernatant was centrifuged through a cushion of 20% sucrose at 372,000 *g* for 20 min in the TLA 100.3 rotor of a Beckman TL 100 centrifuge. It was observed that the visible host impurities were left on top of the sucrose cushion and that the virus pellet accumulated at the bottom of the tube. The pellet was resuspended in 50 mM sodium borate buffer, pH 8.1, for 2 h and then clarified by centrifugation at 10,000 *g* for 10 min. A sample from the supernatant was examined under the electron microscope and it was noted that there were many virus particles with only traces of visible host impurities.

4.1.6.3 Rate zonal centrifugation

Half ml suspensions of partially purified PSbMV were layered on 10-40% (w/v) linear sucrose density gradients in 50 mM sodium borate buffer, pH 8.1, and centrifuged in an SW 41 rotor at 165,000 *g* for 1.5 h. Gradients were fractionated and the fractions were dialysed against the same buffer as above for 12 h. A sample of 500 µl from each fraction was used for infectivity assays.

Two light scattering bands 2-3 mm wide were clearly visible in the gradient tubes about 49 and 52 mm below the meniscus (Figure 4.2a). When the gradients were fractionated, two ultraviolet absorbing zones were detected but the infectivity was associated with only one band (Figure 4.3). This shows that the top zone consisted of host impurities. PSbMV recovered from the gradient tubes was concentrated by centrifugation using a TLA 100.3 rotor of a Beckman TL 100 centrifuge at 372,000 *g* for 20 min. Pellets were resuspended in the same buffer as above.

4.1.6.4 Isopycnic centrifugation

To remove the host impurities associated with the preparations produced from sucrose density gradients, 50 to 100 µl suspensions of the purified PSbMV were layered on 30-60% Nycodenz density gradients in 50 mM sodium borate buffer, pH 8.1, and centrifuged in a TLA 100.3 rotor of a Beckman TL 100 centrifuge at 264,000 *g* for 4 h. The virus zone was withdrawn with a sterile syringe, diluted and centrifuged through a 2 ml cushion of 30% sucrose at 372,000 *g* for 20 min. The sucrose cushion was used to remove some traces of Nycodenz. The virus pellets were resuspended with the same buffer as above.

Only a single light scattering band 1 mm wide was visible in the gradient tube about 10 mm below the meniscus (Figure 4.2b). The UV absorption spectrum of purified PSbMV

Table 4.5. Range of yields of virus from pea cv. Dundale infected with the 4 isolates of PSbMV*

Isolate	Yield (mg/Kg)
US	10-12
Q	8-10
S4	8-10
S6	8-10

* Virus from systemically infected leaves of pea cv. Dundale was purified using both sucrose and Nycodenz density gradients.

Fig. 4.2 Zone of PSbMV in tube: following rate zonal 10-10% sucrose (a) and isopycnic 30-60% Nycodenz (b) density gradients centrifugation.

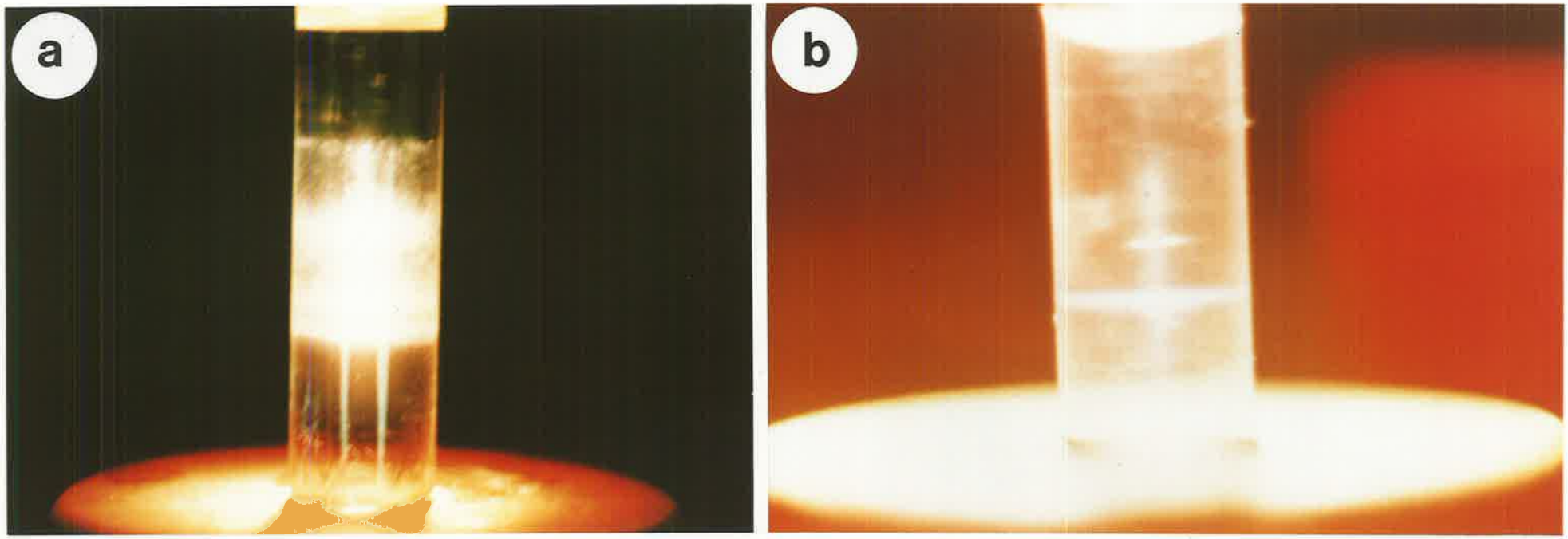
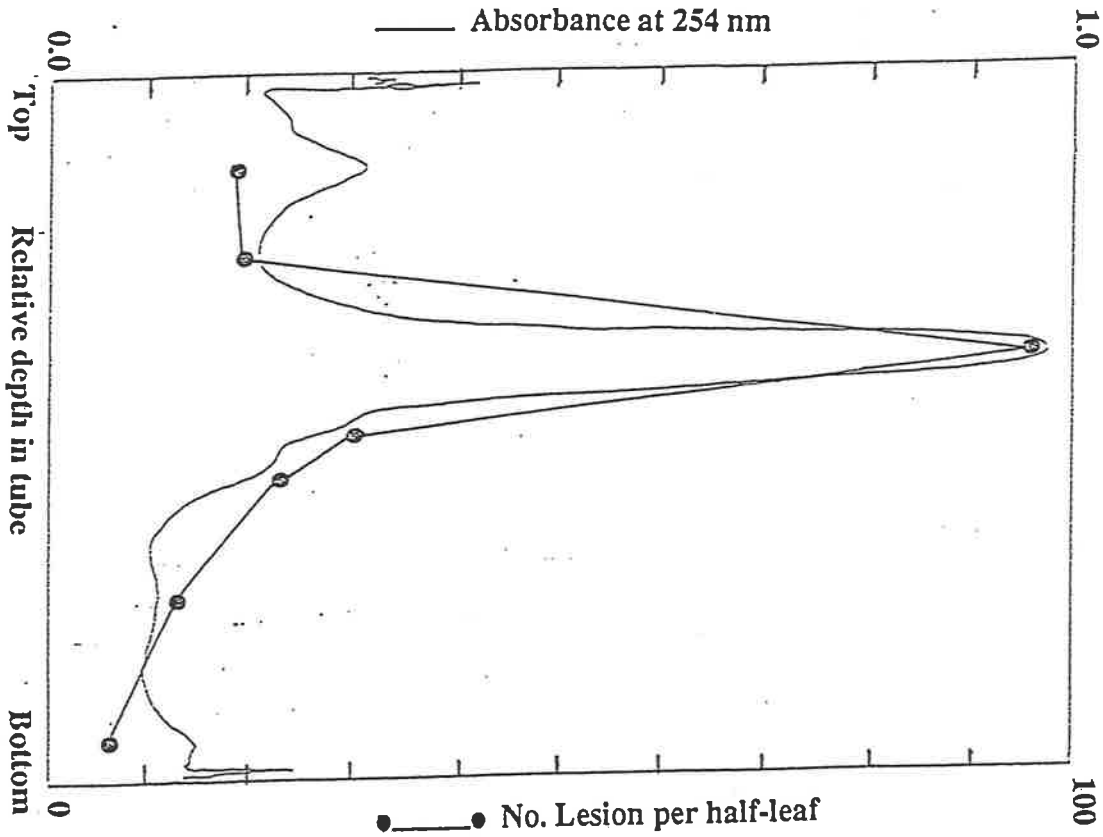

a
b

Fig. 4.3 Ultraviolet absorption profile and distribution of infectivity of PSbMV in 10-40% sucrose density gradients in 50 mM sodium borate buffer, pH 8.1. Five hundred µl suspensions of partially purified PSbMV were layered on the gradients and centrifugation was in an SW 41 rotor at 165,000 *g* for 1.5 h. The tubes were analysed with an ISCO and 500 µl fractions were collected and assayed on 14 half-leaves of *Chenopodium amaranticolor*.


Absorbance at 254 nm
0.0
1.0
Top
Relative depth in tube
Bottom
0
100
No. Lesion per half-leaf

(Figure 4.4) had a peak at 260 nm, which was similar to that of other viruses of the potyvirus group (Hill *et al.*, 1973). Using an extinction coefficient of $E^{0.1\%}_{260\ nm} = 3$ (Brunt, 1970) to estimate the concentration, the above purification methods yielded a range of about 10-12 mg (isolate US) and 8-10 mg (isolates Q, S4 and S6) virus per Kg of infected leaf material (Table 4.5).

4.1.6.5 Final method adopted for the purification of PSbMV

The experiments described above led to the development of an efficient purification procedure which is summarized in Table 4.6. In summary, the Triton method of Moghal and Francki (1976) was further modified and the virus recovered was subjected to isopycnic centrifugation using Nycodenz density gradients (see 2.4). It was observed under the electron microscope that there was a greater recovery of virus (Table 4.5) and the purity was improved (Figure 4.5c) compared to the rate zonal centrifugation. When RNA was extracted from this preparation and cDNA was made, there was no reaction with healthy plant RNA in DHA.

4.2 Storage of preparations

Preparations that were used in a few hours were kept at 4°C while the viruses intended for future use were mixed with 50% glycerol and stored at -15°C.

4.3 Assessment of preparations

4.3.1 Infectivity assay

Purified virus from the 4 PSbMV isolates was inoculated to *Chenopodium amaranticolor*. The US isolate had the highest specific infectivity while S6 isolate had the lowest (Figure 4.6). Isolates Q and S4 were not significantly different from each other.

4.3.2 Electron microscopy

The preparations at various stages of purification were examined under the electron microscope to assess the purity, state of fragmentation and aggregation of virus particles (Moghal and Francki, 1976). Host impurities were always associated with partially purified preparations,but purified virus preparations were devoid of visible amounts of contaminating material.

Table 4.6 Procedure adopted for the purification of PSbMV

Infected material

Homogenize infected leaves in 500 mM sodium borate buffer, pH 8.1, containing 0.15% thioglycollic acid and 0.5 volume of chloroform. Centrifuge emulsion at 10,000 *g* for 15 min.

Supernatant 1

Discard pellet

Add Triton-X 100 to 5% and stir for 30 min at 4°C. Centrifuge in 70 Ti rotor at 311,000 *g* for 1 h.

Pellet 2

Discard supernatant

Resuspend in 50 mM sodium borate buffer, pH 8.1, containing 5 mM EDTA for 12 h at 4°C. Centrifuge 10,000 *g* for 10 min.

Supernatant 3

Discard pellet

Centrifuge through a 2 ml cushion of 20% sucrose at 372,000*g* for 20 min in the TLA 100.3 rotor of a Beckman TL 100 centrifuge.

Pellet 4

Discard supernatant

Resuspend in 50 mM sodium borate buffer (200 µl), pH 8.1, for 2 h, centrifuge 10,000 *g* for 10 min.

Supernatant 5

Discard pellet

Layer on 10-40% sucrose density gradients, centrifuge in SW 41 rotor at 165,000 *g* for 1.5 h. Fractionate gradients. Dialyse fractions with virus against 50 mM sodium borate buffer, pH 8.1, for 12 h; concentrate virus by centrifugation at 372,000 *g* for 20 min.

Virus pellet 6

Discard supernatant

Resuspend in 50 mM sodium borate buffer, pH 8.1, layer on 30-60% gradient of Nycodenz, centrifuge in TLA 100.3 rotor at 264,000 *g* for 4 h. Withdraw virus zone, dilute and centrifuge through a 2 ml cushion of 30% sucrose at 372,000 *g* for 20 min.

Virus pellet 7

Discard supernatant

Resuspend virus in 50 mM sodium borate buffer, pH 8.1.

Purified virus 8

Fig. 4.4 Ultraviolet absorption profile of purified PSbMV preparations from step 8 in Table 4.6.

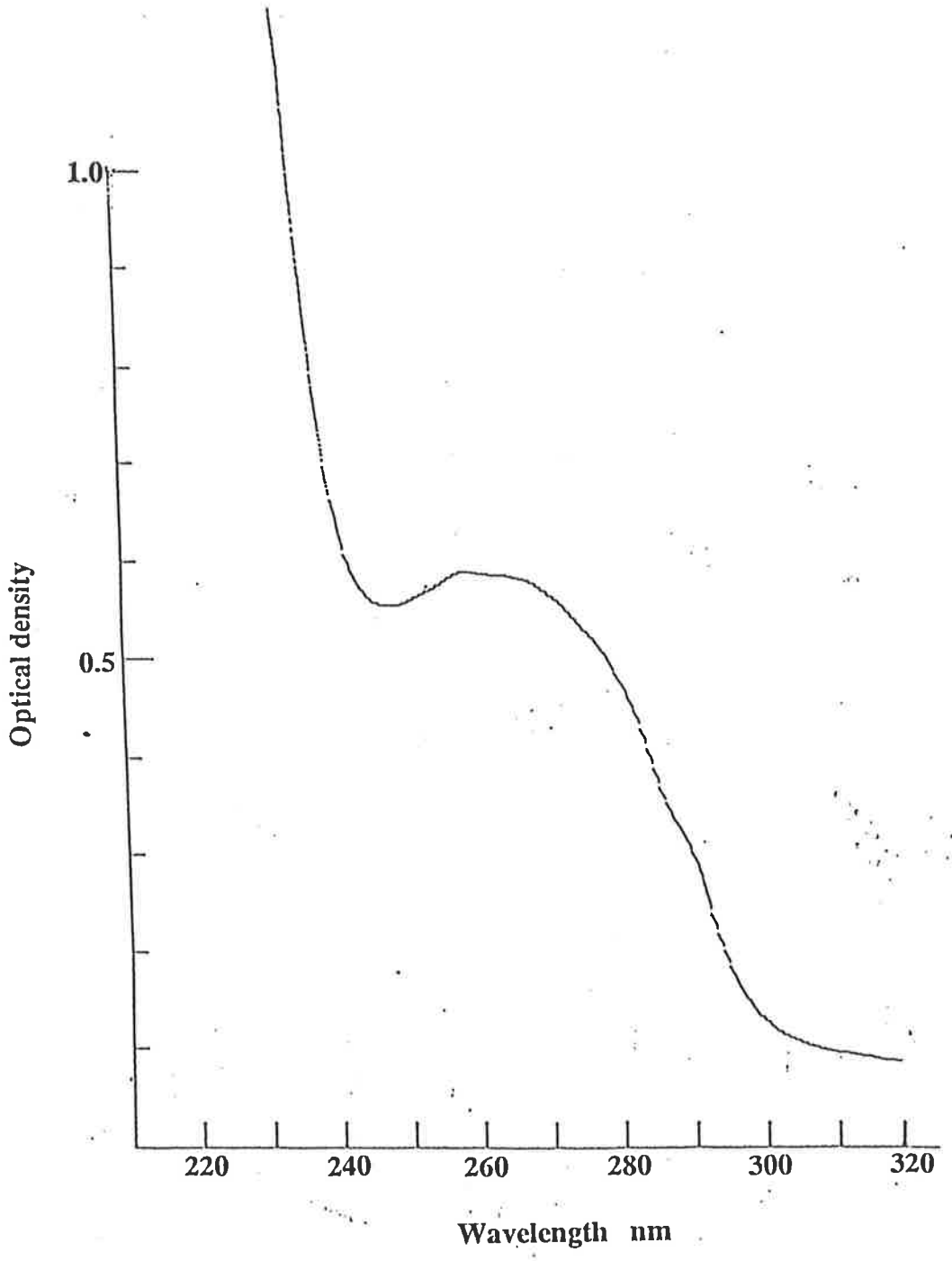

1.0
0.5
Optical density
220
240
260
280
300
320
Wavelength nm

Fig. 4.5 Electron micrographs of particles of PSbMV-US : (a) In sap from leaf. Using the leaf dip method (Noordam, 1973). Leaf pieces were cut and the edge touched to a drop of 2 % phosphotungstic acid (PTA), pH 4.5 (Hatta and Francki, 1984) for about 30 seconds; (b) following rate zonal centrifugation through 10-40% sucrose density gradients; (c) following isopycnic centrifugation in Nycodenz gradients; (d) following immunosorbent electron microscopy. Bar represent 250 nm.

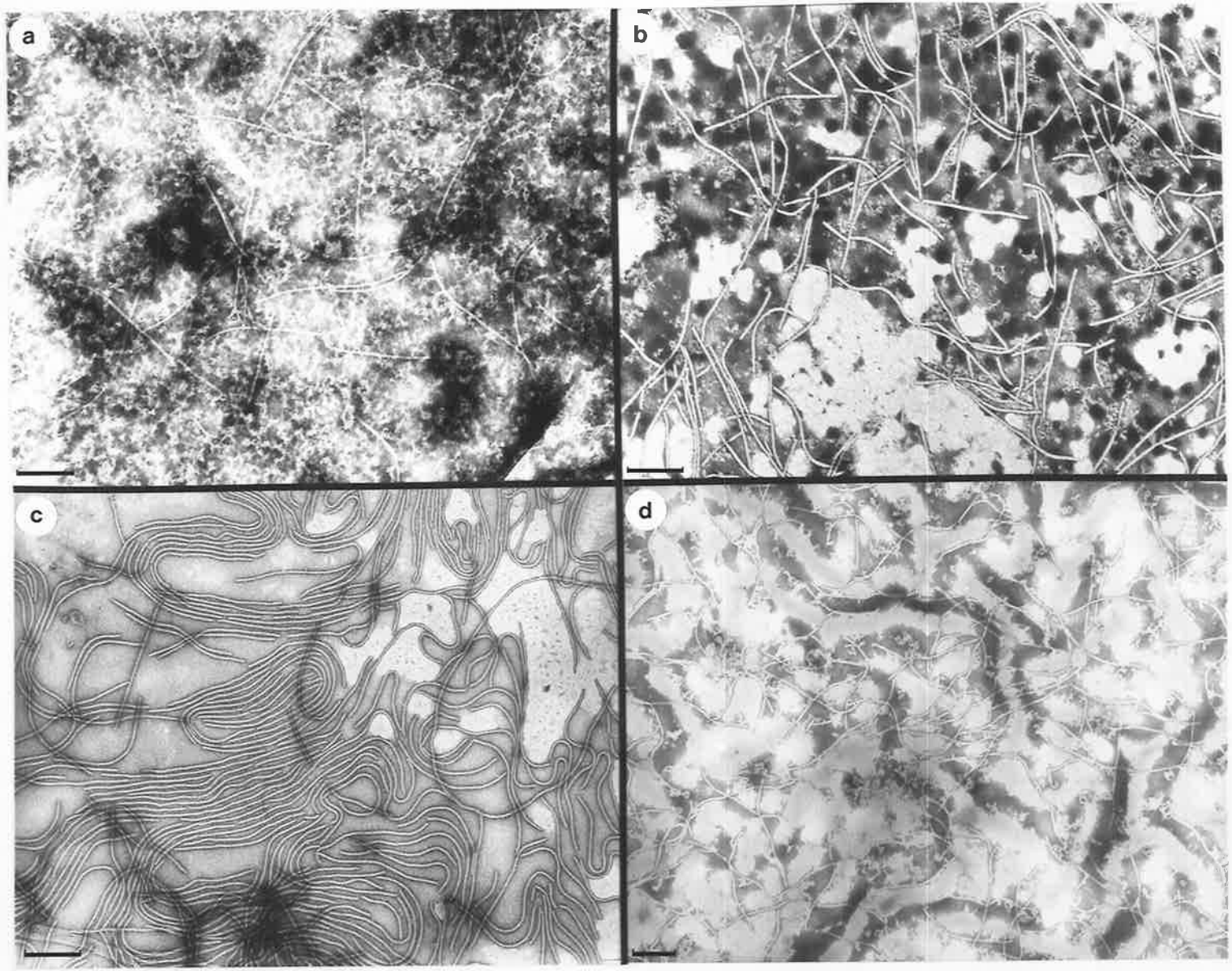
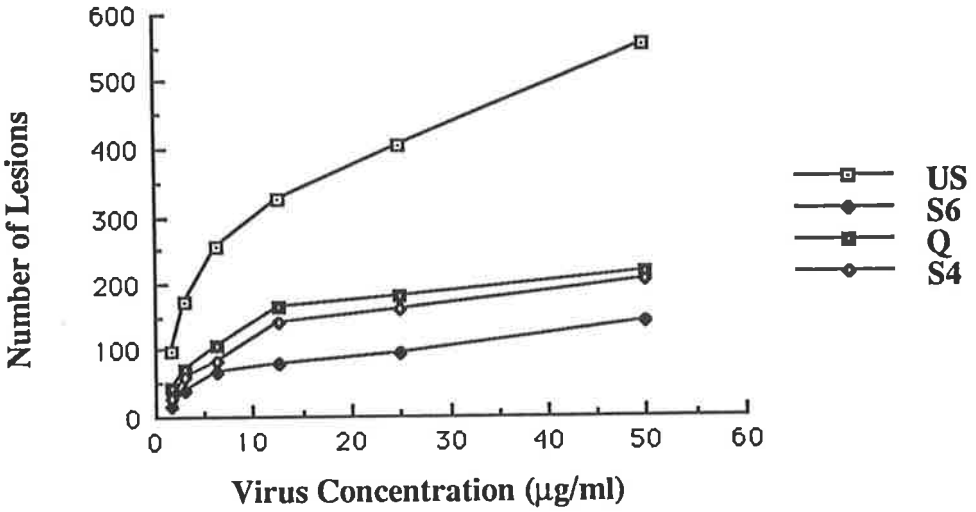

a
b
c
d

Fig. 4.6 Specific infectivity of 4 PSbMV isolates on*Chenopodium amaranticolor*. Preparations (50 µg/ml), after sucrose density gradients centrifugation, from each isolate were diluted six times (two-fold) in 50 mM sodium borate buffer, pH 8.1 before bioassay. Four leaves of *Chenopodium amaranticolor* were used for each assay. Two weeks after inoculation, local lesions were counted and expressed as mean of four leaves.


Number of Lesions
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Virus Concentration (µg/ml)
US
S6
Q
S4

4.3.3 Serology

Antisera prepared against purified virus was checked in two-dimensional immunodiffusion test (TDIT) for the presence of antibodies to the host plant antigens. The US and S6 antisera did not react to the healthy plant antigen (Figure 5.2 A and B).

Conclusion

By studying individual purification steps, through a series of preliminary experiments, a method was developed for the purification of PSbMV. The combination of sucrose and Nycodenz density gradients did not result in significant particle breakage or end to end aggregation as judged by electron microscopy (Figure 4.5 c). When the preparation was used to immunize the rabbits, the antiserum produced did not react with healthy plant sap in TDIT. All preparations were infectious by infectivity assay.

The final method developed and its modifications, which were time-saving (35 h) and efficient for the recovery of virus, were routinely used to purify the 4 PSbMV isolates.

Chapter 5 Serology

Introduction

Most indexing programs for the presence of PSbMV in pea involve the visual inspection of individual plants for characteristic symptoms followed by infectivity assays on pea cultivars and *Chenopodium amaranticolor*, a local lesion host. However, pea cultivars vary in their symptom expression and strains vary in the severity of symptoms they induce. Therefore, screening programs based primarily on symptoms will not be satisfactory for detecting infected plants (Hamilton and Nichols, 1978). Biological indexing has the disadvantages of requiring a number of different hosts and insect vectors, large amounts of space in the glasshouse and long experimental times. Direct assays based on serological detection of virus have a number of advantages (Matthews, 1991): (i) the specificity of the reaction allows virus to be measured in the presence of host material or other impurities; (ii) results are obtained in a few hours or overnight compared with days for infectivity assays; (iii) some serological detection and assay procedures are more sensitive than infectivity measurements; and (iv) serological tests are particularly useful with viruses that have no good local lesion host or that are not sap transmissible.

Wang and Maule (1992) used indirect ELISA for the detection of PSbMV in the reproductive tissues of several pea genotypes. They showed that the virus multiplied to a maximum in mature fresh embryos and that amounts of virus did not decrease during seed dessication. Although ELISA is sensitive, it requires large volumes of reagents, long test times and some expensive equipment. Therefore, Graddon and Randles (1986) have developed a single antibody dot immunoassay (SADI) which was used for the detection of subterranean clover mottle virus in small amounts of infected tissue and purified preparations. The technique was found to be more sensitive than ELISA in terms of total antigen detected. DIBA was developed by Hibi and Saito (1985) for the rapid detection of plant viruses in infected tissues by avoiding non-specific reactions and minimizing the amounts of specific antibodies used. Also, common conjugate is used. With this method, less than 1 ng of tobacco mosaic virus could be detected in several milligrams of infected

tobacco leaves. This chapter describes the development of a rapid and sensitive DIBA suitable for use in the studies described in this thesis.

Results and discussion

5.1 Production of antisera

Antisera were prepared in rabbits (see 2.2.5.1). Table 5.1 shows the titres of the antisera of each isolate against their homologous antigen in TDIT. Antisera to the US isolate had higher titres than antisera from the Australian isolates. None of the antisera reacted with healthy sap in these tests. As shown in Figure 5.1c, unsonicated PSbMV-US did not react with the antiserum to the US isolate in the region between the wells whereas sonicated preparations showed a precipitin line against the homologous antiserum dilutions.

Table 5.1. Homologous titres of antisera to 4 PSbMV isolates in two-dimensional immunodiffusion tests*

Isolate	Rabbit number	Titre
US	1	128
	2	512
Q	1	32
S4	1	16
S6	1	64
	2	64

* The immunization schedule is shown in 2.2.5.2. All the antisera were from the first bleed. Titrations were done against sonicated purified homologous antigen (1 mg/ml).

5.2 Comparison of isolates in TDIT

A precipitin reaction was observed in heterologous tests between the antiserum to the US isolate and the Q, S4 and S6 isolates and between the antiserum to the S6 isolate and the US, Q and S4 isolates (Figure 5.2). Precipitin lines between homologous and

Fig. 5.1. Electron micrographs of purified PSbMV-US particles which were untreated (a) or sonicated (b). Figure 5.1c is a two-dimensional immunodiffusion showing a reaction of sonicated PSbMV particles (top) with polyclonal homologous antiserum dilutions (centre) and untreated particles (bottom) of the same isolate. Bar represents 250 nm.

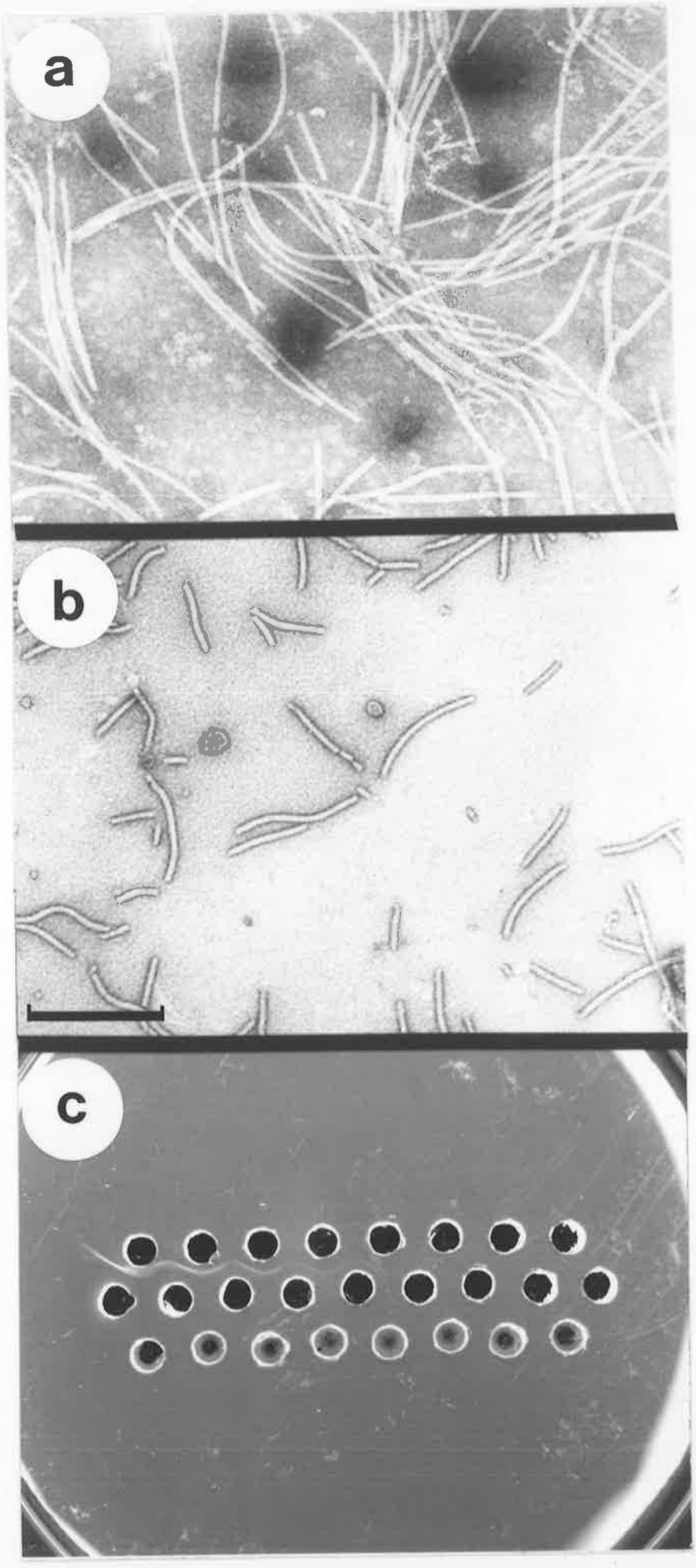
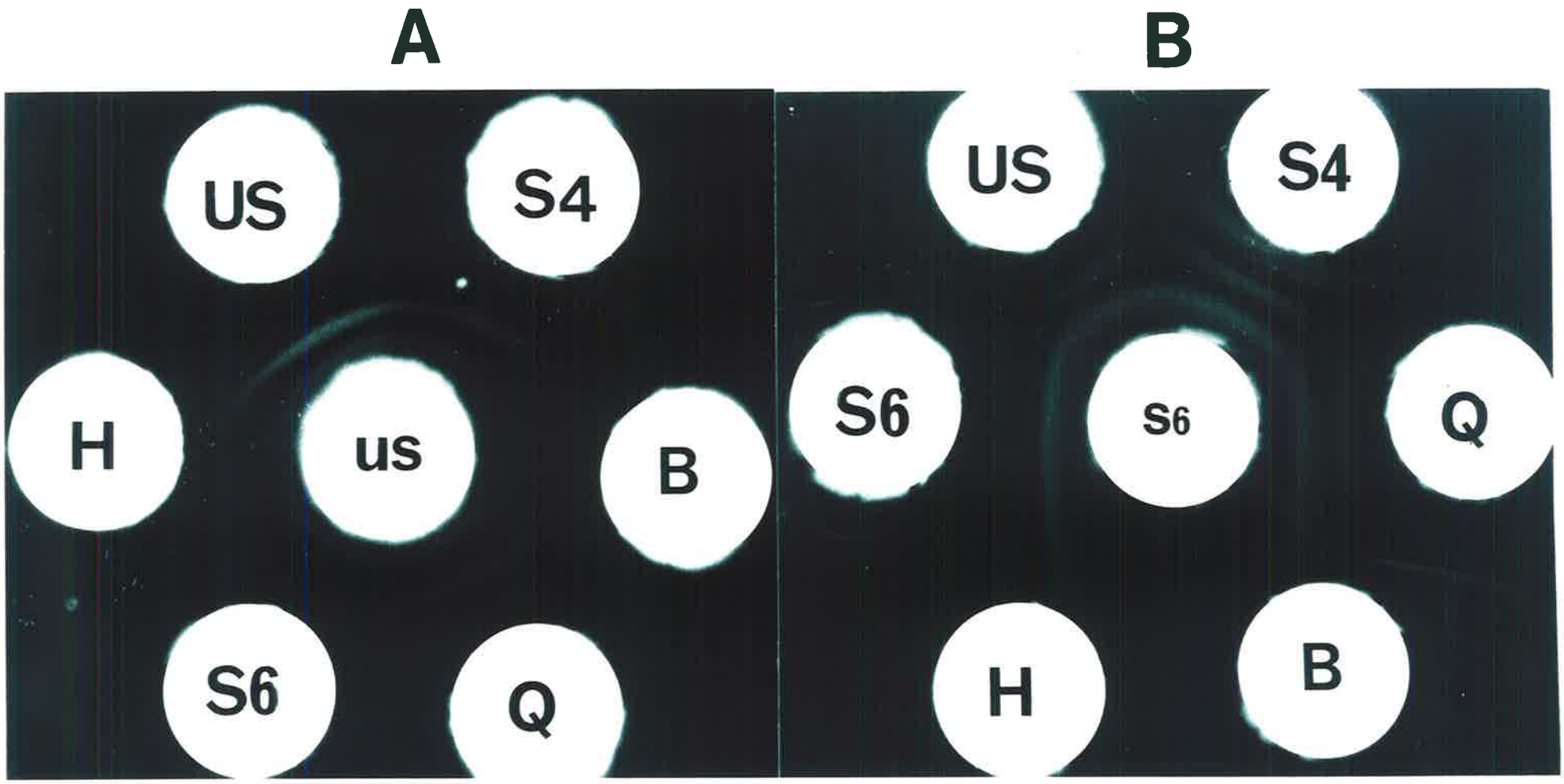

a
b
c

Fig. 5.2. Two-dimensional immunodiffusion test showing confluent precipitin reactions between each of the 4 PSbMV isolates and (A) US antiserum or (B) S6 antiserum. Antigens Q, US, S4 and S6 were sonicated purified virus at a concentration of 100 µg/ml, controls were healthy sap (H) or 50 mM sodium borate buffer, pH 8.1 (B). Antisera were used undiluted.


A
US
S4
H
us
B
S6
Q
B
US
S4
S6
S6
Q
H
B

heterologous antigens were confluent, and without spurs. The development of several precipitin lines may have been due to different lengths of particles resulting from the sonication treatment of the antigen. A serological differentiation index of between 2 and 3 was observed between the US and S6 isolates by titration of antiserum to the US isolate against homologous and heterologous antigens in TDIT. Because the results showed that the isolates US and S6 could not be differentiated serologically, they are considered to belong to the same serotype. Antiserum to the US isolate was used in subsequent experiments.

5.3 Quantitative DAS-ELISA

DAS-ELISA was used to detect PSbMV in seeds or seedlings, and to study the relationship between seed size and rate of seed transmission (see 3.3.1 and 3.3.2).

DAS-ELISA was also used to compare the virus concentrations in sap from leaves of peas infected with each of the 5 PSbMV isolates. Using the antiserum to the isolate US , the homologous antigen reached the highest concentration whereas the T isolate had the lowest (Figure 5.3). The isolates S4 and S6 were not significantly different from each other. Other tests with antiserum to the isolate S6 also showed that the isolate US had the highest concentration (results not shown).

5.4 Development of DIBA

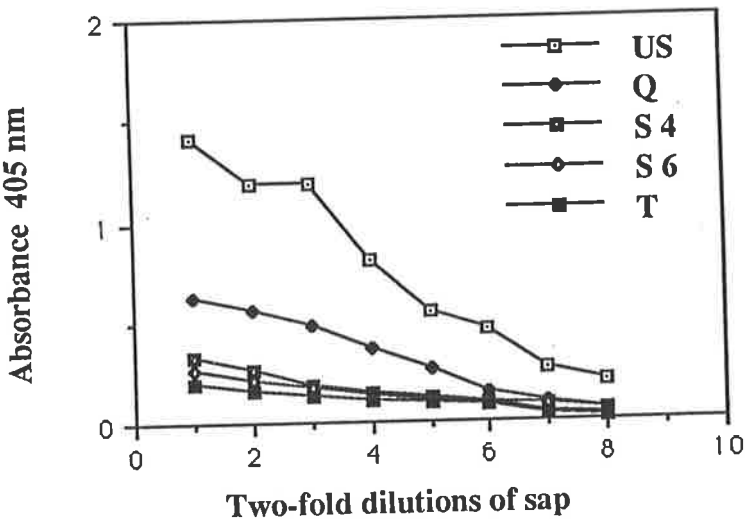
DIBA differs from ELISA in that antigen is bound to nitrocellulose, and the specific antibody is sandwiched between the antigen and the conjugate.

DIBA was done following the procedure of Hibi and Saito (1985). BSA at 2% concentration was used as the blocking agent. The assay was found to be unsatisfactory. There was a strong colour background in the nitrocellulose which is described here as membrane background (MB). Non-specific binding (NSB) of conjugate to sample dots was also observed when healthy leaf sap or seed extract was used as the antigen. A series of trials were done to eliminate these non-specific reactions.

5.4.1 Dilution of antiserum for removal of MB

The MB was thought to be due to the high titre of the antiserum used for the assay. The antiserum was diluted with PBS buffer from 1/1000 to 1/8000 and it was observed that dilutions above 1/4000 gave reduced background, but did not remove it. This shows

Fig. 5.3. Comparison of concentrations of homologous and heterologous antigens in sap of *Pisum sativum* cv. Dundale at 14 days post inoculation. Infected leaf samples (1 g/ml, w/v) from each of the 5 PSbMV isolates were triturated in 50 mM sodium borate buffer , pH 8.1, and two-fold dilutions were tested by DAS-ELISA against both γ–globulin (coating antibody) and conjugate from the US antiserum.


Absorbance 405 nm
2
1
0
0
2
4
6
8
10
US
Q
S 4
S 6
T
Two-fold dilutions of sap

that the MB was not solely due to the high titre of the antiserum. A dilution of 1/5000 was selected for use in subsequent experiments.

5.4.2 Increasing the time of washing for the removal of MB

To determine whether the slight MB was due to inadequate washing, the test sheet was washed for 40 min, 1 h, 1.5 h and 2 h. It was found that from 1 h and above, the MB was removed. However, prolonged washing tended to decrease the sensitivity of the assay. A one hour wash was selected for use in subsequent experiments.

5.4.3 Cross absorption of antiserum for removal of NSB of healthy antigen

Although the MB was removed, the NSB of conjugate to the healthy antigen was still strong. This was thought to be due to healthy antibody in the antiserum. The antiserum was cross absorbed with healthy plant sap and seed extract, but the colour reaction of the healthy antigen was not removed by this treatment.

5.4.4 Use of pre-immune serum and mouse serum to determine the nature of NSB healthy antigen

To test whether the NSB occurred with non-immune serum, the antiserum was substituted with pre-immune serum. Mouse serum was also tested as substitution for the antiserum. However, the NSB of conjugate to the healthy antigen was not removed.

5.4.5 Use of monosaccharides in buffer

A trial was done to determine whether the NSB was due to lectin, which is a carbohydrate-binding protein or glycoprotein of non-immune origin with 2 or more binding sites that recognize a specific sugar (s) or sequence of sugars (Higgins et al., 1983). To test whether saturation of sites with monosaccharides would remove the NSB, an experiment was done with different concentrations of mannose and glucose dissolved in PBS buffer and added to all the other buffers used in the assay. Healthy leaves from pea cv. Dundale were triturated in PBS buffer (1:1 w/v) and samples dotted onto nitrocellulose membrane. BSA was used as the blocking agent and conjugate were allowed to react with the dot directly before colour development. Table 5.2 shows that concentrations from 50 to 300 and 700 to 800 mM of sugars had high NSB. A concentration of mannose and glucose at 400-600 mM showed the minimum NSB. Mannose and glucose at 500 mM was selected for use in subsequent experiments.

5.4.6 Comparison of healthy leaf sap and seed extract with other blocking agents

To test whether the blocking agent had an effect on the slight NSB of conjugate to the healthy antigen, an experiment was done to compare 3 different blocking agents.

5.4.6.1 Bovine serum albumin

BSA at different concentrations (Table 5.3) was initially used as a blocking agent , but the NSB of conjugate to the healthy antigen was still strong.

5.4.6.2 Skim milk

Table 5.3 shows the different concentrations of skim milk which were incorporated

Table 5.2. Effect of different concentrations of mannose and glucose on NSB*

Sugar	Concentration (mM)	Colour reaction
Mannose + Glucose	50	+++
	100	+++
	200	+++
	300	+++
	400	+
	500	+
	600	+
	700	+++
	800	+++
Mannose	500	+++
Glucose	500	+++

* The mannose and glucose were dissolved in PBS buffer and added to all the other buffers used in the assay.

with PBS buffer and used to block the NSB of conjugate to the healthy antigen. However, the NSB was not removed.

5.4.6.3 Healthy leaf sap or seed extract

Healthy leaf sap, seed extract or both (1:1) at different dilutions (Table 5.3) were used as the blocking agent. It was found that when the dilutions at 1/10 and 1/20 were used, the slight NSB of the healthy antigen was removed (Figure 5.4 a). A dilution of 1/10 was selected for use in subsequent experiments.

Leaf sap was used as the blocking agent when membranes contained only samples from roots, leaves and flowers.

A seed extract was used for blocking membranes containing samples from immature to mature dry seed.

With membranes containing both tissue and seed samples, blocking was done with a

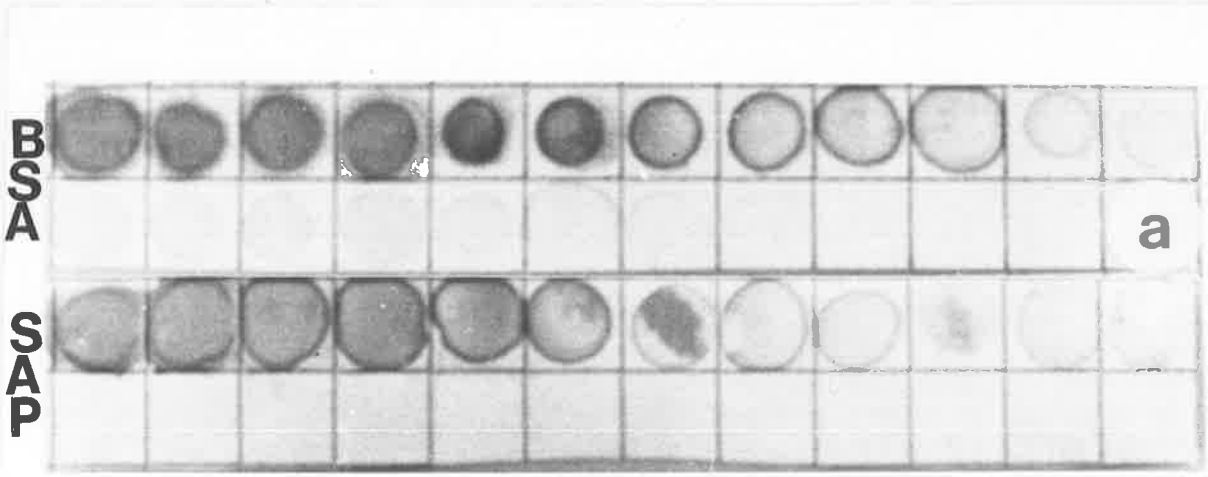
Table 5.3. Comparison of blocking agents in DIBA*

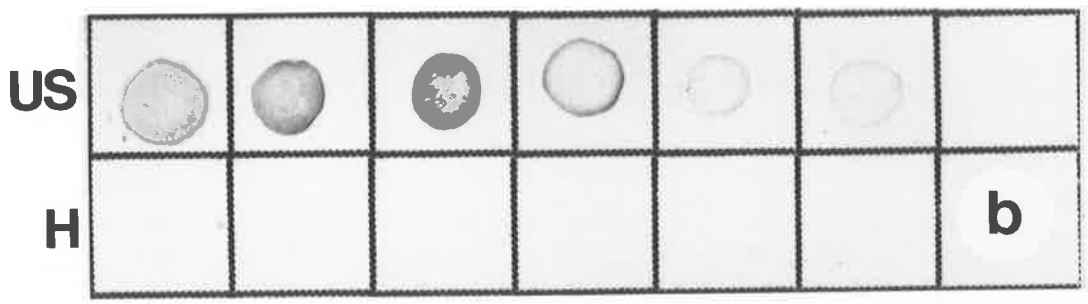
Sample	Concentration	Colour reaction
Skim milk	0.5%	+
	1.0%	+
	1.5%	+
Bovine serum albumin	0.5%	+
	1.0%	+
	1.5%	+
Healthy plant sap	1/5	+
	1/10	-
	1/20	-
Healthy seed extract	1/5	+
	1/10	-
	1/20	-

* Skim milk and BSA were dissolved in PBS buffer. Healthy plant leaf or seed was triturated in PBS buffer (1:9, w/v).

Fig. 5.4. a. A comparison of 2% BSA and healthy pea sap (1/2 dilution) as blocking agents in DIBA . Homologous reactions are shown for PSbMV-US. The first and third rows are a two-fold dilution series of sap from infected pea; the second and fourth are a two-fold dilution series from healthy pea: range from 1/2 to 1/4096.

b. DIBA showing dilution endpoint of 1 *u*l dots of purified PSbMV-US ranging from 32 ng/ml to 1 µg/ml. Row H is a control series of healthy pea sap ranging from 1/2 to 1/128 dilution. Blocked with healthy sap as in a.


BSA
SAP
a


US
H
b

mixture of leaf sap (1/10) and seed extract (1/10) in the ratio 1:1.

5.4.7 Final procedure adopted for DIBA of PSbMV

The experiments described above led to the development of a routine procedure which is summarized in Table 5.4. The method was used for the detection of PSbMV in subsequent experiments.

5.4.8 Dilution end-point of antigen in sap

Virus antigen was detectable in sap by DIBA at dilutions of sap down to 1/4096 (Figure 5.4 a).

5.4.9 Titre of antisera

The titration of antisera against sap from pea infected with any of the 6 isolates of PSbMV showed that all antigens reacted with both antisera within one 2-fold dilution of each other (Figure 5.5). The titre of PSbMV US antiserum was between 128000 and 256000 (Figure 5.5 a), whereas that of PSbMV S6 was between 32000 and 64000 (Figure 5.5 b).

5.4.10 Optimum condition for tests

DIBA detected as little as 32 ng of virus per spot (Figure 5.4b). It was also found that the antibody could be used up to 10 times without loss of sensitivity.

5.4.11 Use of PSbMV antiserum

PSbMV antiserum reacted specifically with purified virus and extracts of infected plant tissue. No reaction was observed in tests using pre-immune serum in place of antiserum.

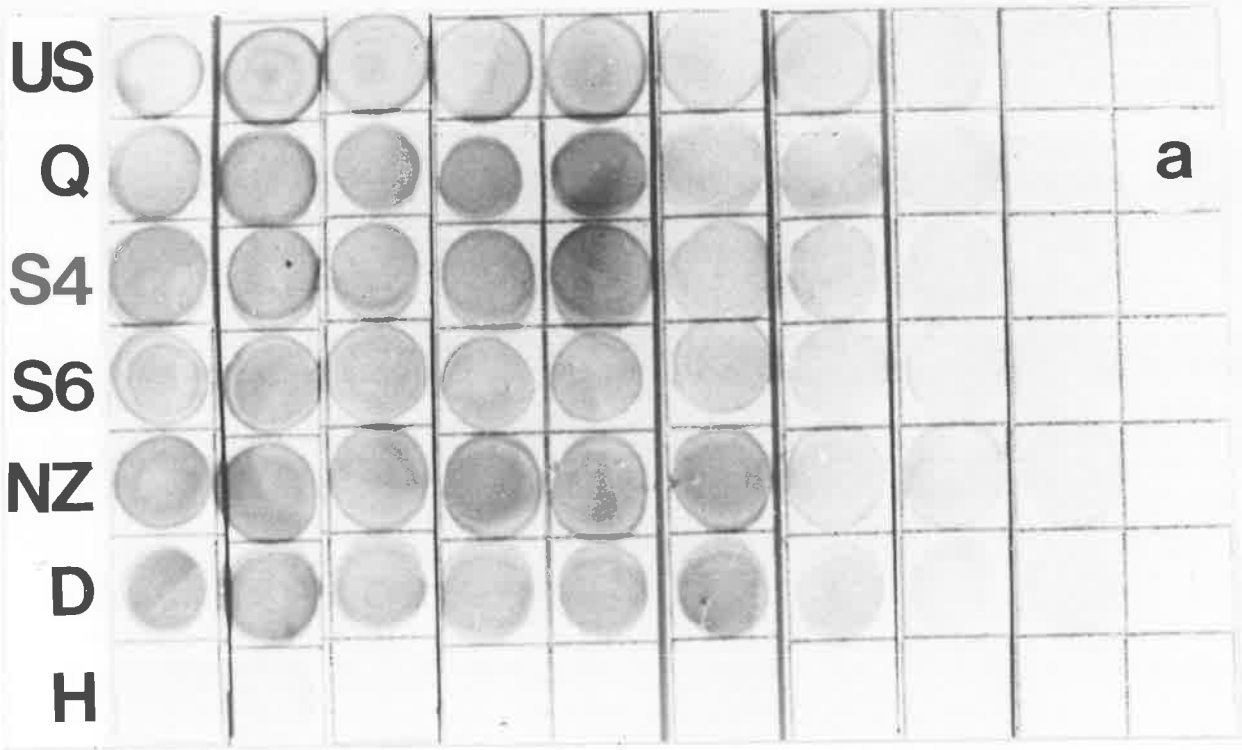
5.4.12 Use of cytoplasmic inclusion protein antiserum

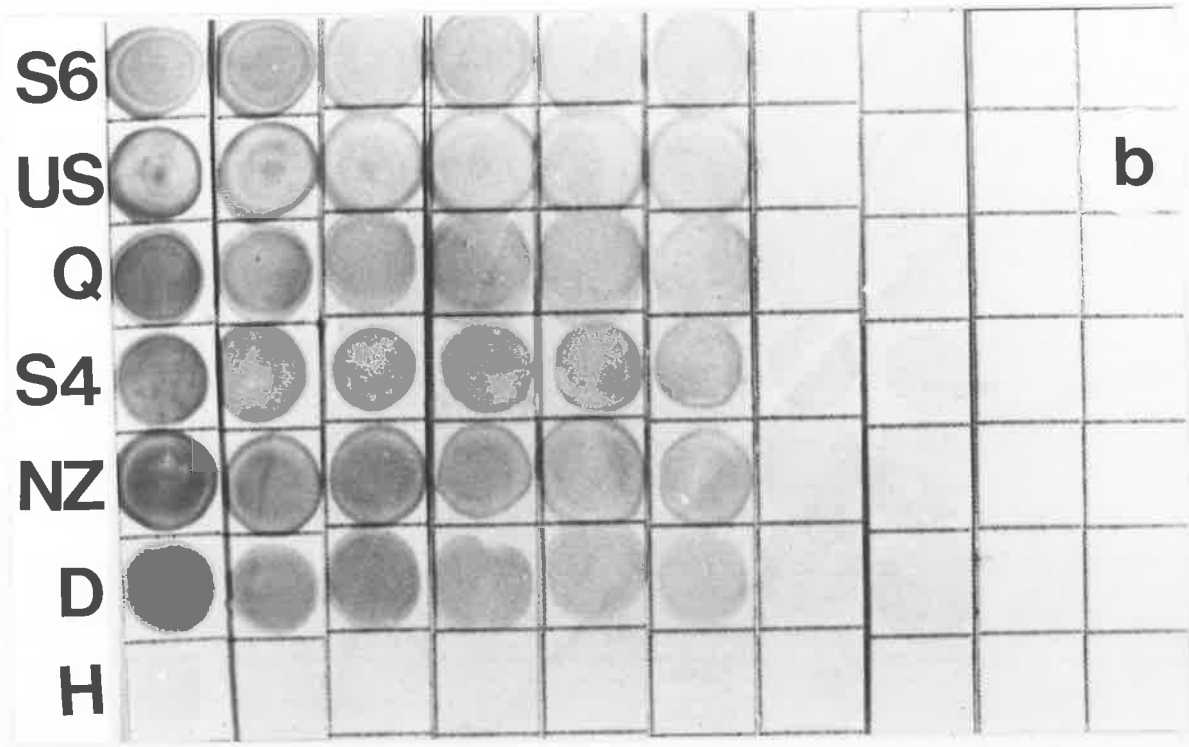
Cytoplasmic inclusion protein (CIP) antiserum did not react with purified virus or extracts of healthy pea tissue. It reacted with infected plant tissue, indicating that it was suitable for detecting the cytoplasmic inclusion bodies of PSbMV under these assay conditions. Therefore, CIP antiserum was used throughout this work to identify tissue in which PSbMV was replicating and in which CIP was translated, as distinct from tests with PSbMV antiserum where only the presence of virus was detectable.

Table 5.4 **Procedure adopted for DIBA of PSbMV**

Infected material	
1	Triturate infected material in PBS buffer (1:1 w/v). Spot 1 µl of each antigen sample onto nitrocellulose membrane and air dry.
Blocking	
2	Incubate the test sheet in PBS buffer containing either healthy leaf sap (1/10), seed extract (1/10) or both (1:1) for 30 min at 25°C.
First antibody	
3	Incubate the test sheet in PBS buffer containing polyclonal antiserum diluted 1/5000 for 1 h at 25°C.
Washing	
4	Incubate the test sheet in wash buffer pH 7.5 on a platform rocker or a shaker for 1 h at room temperature.
Second antibody	
5	Incubate the test sheet in PBS buffer containing goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1/5000 for 1 h at 25°C.
Washing	
6	Incubate the test sheet in wash buffer pH 7.5 on a platform rocker or a shaker for 1 h at room temperature.
Substrate	
7	Detect the alkaline phosphatase with substrate buffer pH 9.5 containing 75 mg/ml nitro blue tetrazolium, 2 mg/ml phenazine methosulphate and 40 mg/ml 5-bromo-4-chloro-3-indolyl phosphate for 2-5 min at 37°C.
Stop buffer	
8	Stop the reaction by incubating the test sheet in stop buffer pH 7.5 containing 10 mM Tris-HCL and 5 mM EDTA for 10-15 min at room temparature.

Fig. 5.5. Titration of antisera to PSbMV-US (a) and -S6 (b) against sap (diluted 1/2) from pea infected with each of 6 PSbMV isolates, and healthy pea (H). Each identical vertical strip was incubated with antiserum diluted two-fold from 1/1000 to 1/512000 (left to right).


US
Q
S4
S6
NZ
D
H
a


S6
US
Q
S4
NZ
D
H
b

5.5 Comparison of DIBA and DAS-ELISA

An experiment was done to compare the reactions obtained with DIBA and DAS-ELISA. Plants from experiments described in 7.1 to 7.3 were sampled and duplicate samples assayed. Table 5.5 summarizes the results from Figures 7.1to 7.3 and Appendices II-1 to II-3. Both assays detected PSbMV in the vegetative and reproductive parts of inoculated plants except in Figure 7.2 where the virus was detected only in the mature green seeds (for full description see Chapter VI). It was found that DIBA was more reliable for the detection of

Table 5.5. Ratio of positive samples detected by DAS-ELISA compared with DIBA

Sample	Leaf	Flower	Seed
Fig. 7.1 (App. II-1)	10/10*	7/10	2/10
Fig. 7.2 (App. II-2)	0/0	0/0	3/9
Fig. 7.3 (App. II-3)	8/8	2/4	3/8

* Number of samples positive by DAS-ELISA over number positive by DIBA.

PSbMV than DAS-ELISA. For example, for leaf, both appeared to be equally reliable, for the seed, DAS-ELISA was not reliable.

5.6 Correlation of symptoms with serological detection of PSbMV

The relationship between the detection of PSbMV by DIBA and DAS-ELISA, and presence of virus symptoms was determined. The development of visible virus symptoms in pea cv. Dundale was recorded after 2 weeks from inoculation until the flowering stage. The results are summarized in Chapter VII, Figures 7.1 to 7.3. For example, in Figure 7.1, from the third leaf until flowering stage, all the plants had mosaic symptoms in the leaves and colour break in the flowers. The virus was constantly detected by both assays. In Figure 7.2, all the plants were symptomless and the virus antigen was negative by serology. In most cases, plants showing symptoms were positive by DIBA, but not always by DAS-ELISA.

Conclusion

DAS-ELISA is laborious, time-consuming, and needs specialized equipment. A need for diagnostic tests applicable to simple laboratory facilities or field use has led to the development of variations of dot-immunobinding assays. Graddon and Randles (1986) found that single antibody dot immunoassay (SADI) is twelve times more sensitive than ELISA in terms of total antigen detected. They showed that DIBA, an indirect dot immunoassay, was about twice as sensitive as SADI. It is a simple, rapid and sensitive assay that can detect a minimum amount of about 30 ng of virus. This method was developed for use with PSbMV. An initial difficulty encountered with DIBA was the occurrence of false positive results possibly due to the non-specific binding of antibodies by plant lectins (Higgins et al., 1983). The problem was partially overcome by incorporating mannose and glucose in all buffers. Extracts of healthy leaf sap, healthy seed extracts or both in combination were demonstrated to be better blocking agents than BSA or milk. A possible explanation is that healthy antigen in sap competes for binding sites to healthy antibody with healthy antigen bound to the nitrocellulose. Because there was a greater amount of the healthy antigen in the blocking buffer than in the nitrocellulose, the healthy antibody would have been preferably removed into the liquid phase and not available for binding to healthy antigen in the dot.

In comparison between DIBA and DAS-ELISA for the detection of PSbMV, the former was a simple, rapid and sensitive assay that can detect a minimum amount of about 30 ng of virus (Ligat *et al.*, 1991). Therefore, DIBA was adopted for use in subsequent experiments.

Chapter 6 Virus distribution in pea

Introduction

Since resistance to PSbMV is conferred by a single recessive gene, whole plant necrosis might be caused by modifier-genes of a unique germplasm that enhances host sensitivity to PSbMV, whereas tendencies towards latent infection or infection with mild symptoms might be caused by modifier-genes that reduced host sensitivity (Hampton and Marx, 1981). Seed-borne pea fizzletop virus (PFV) symptoms were reported to become transient in 6 to 8-week-old plants, and under field conditions most infected plants appeared normal at full bloom stage (Hampton, 1972). Five to 10% of the symptomless, vigorous plants from infected seedlots were found to contain PFV. The virus in these symptomless plants was not detectable by bioassay until plants were 5 to 8 weeks old (Hampton, 1972). Khetarpal and Maury (1990) observed that pea plants considered as healthy after checking by ELISA began to give ELISA positive reactions about 5 to 6 weeks after emergence. Such infected plants usually remained symptomless and rarely exhibited a very slight leaf rolling. They concluded that the frequent observation of such a phenomenon in 3 different pea cultivars was a form of latency in seed transmission of PSbMV.

In the course of a study on PSbMV diagnosis and the development of control measures, it was observed that although primary inoculated plants were symptomatic, only a small proportion of seedlings raised from the seed of the first generation plants were symptomatic. Unexpectedly, however, the symptomless plants from the second generation showed a high incidence of infection in the seed.

DIBA and dot hybridization assay (DHA) with cDNA to PSbMV RNA were used to investigate the transfer of PSbMV between generations by seed. This chapter reports that the virus becomes subliminal in the vegetative stage of the plant , but that it reaches a relatively high concentration in the seed. The implications of these findings for virus epidemiology and control are discussed.

Results and Discussion

6.1 Distribution of symptoms and virus in 5 generations of pea plants

6.1.1 Localisation of PSbMV in pea tissues

Table 6.1 and Figure 6.1 outline the layout of the experiment which was done to localise PSbMV in pea tissues for five generations. In the first generation (G_1), the mechanically inoculated plants showed symptoms on all leaves, for all isolates of PSbMV. In the second generation (G_2), each of the 20 plants raised from seed again showed leaf symptoms. For all isolates, viral and CIB antigen were detectable by DIBA in all tissues assayed, except for the cotyledon and embryo of the mature green seed.

6.1.2 Vertical transmission of PSbMV in five generations

The pattern of detection obtained in G_2 to G_5 with all isolates is shown in Table 6.1. From plants infected with isolate Q, seeds raised from G_2 gave some symptomatic plants whereas the other isolates gave none. For all isolates, plants raised from the seed of G_3, G_4, and G_5 were symptomless and no virus or CIB protein was detectable by DIBA in sap extracts from leaves of these plants. Moreover, no virus was detectable by infectivity assay which probably has a sensitivity similar to that of DIBA. In contrast, all strains were detected in floral parts by DIBA for virus and CIB. For example, with G_3 in all isolates, virus and CIB were detectable in the pod wall as well as in mature green seed (Table 6.1). In G_4 and G_5, whole seed extracts only were tested and were found to be positive for both antigens.

6.2 Distribution and relative amount of virus in seed

The distribution of virus in seed was determined for seed from all isolates, from G_2 to G_5. In G_2, the testas of mature green seeds were positive for both PSbMV and CIB (Table 6.1) indicating that replication had occurred in this tissue. In G_3, both virus and CIB were detected not only in the testa, but also in the cotyledon and embryo of mature green seed. In G_4 and G_5, both antigens were detected in immature, mature green and mature dried seeds. Table 6.2 shows the relative amounts of virus in individual whole seeds at different stages of maturity. Mature green seeds generally had the highest level of extractable virus.

Table 6.1. Detection of PSbMV in vertical transfer through five generations (G1-G5) of *Pisum sativum* cv. Dundale

Seed generation	Tissue tested	Isolate* US	Q	S4	S6
G1	Twenty plants were inoculated mechanically. All were symptomatic. From these, 20 seeds were collected at random and sown.				
G2	Leaf (1-9)	20**	20	20	20
	Flower***	20	20	20	20
	Stipule	20	20	20	20
	Peduncle	20	20	20	20
	Pod wall	20	20	20	20
	MGS testa	20	20	20	20
	cotyledon	0	0	0	0
	embryo	0	0	0	0
G3	Leaf (1-9)	0 (0)****	3 (2)	0 (0)	0 (0)
	Calyx	0 (0)	0 (0)	0 (0)	0 (0)
	Stipule	0 (0)	0 (0)	0 (0)	0 (0)
	Peduncle	0 (0)	0 (0)	0 (0)	0 (0)
	Pod wall	3 (2)	6 (2)	3 (1)	1 (3)
	MGS testa	18 (19)	16 (20)	18 (19)	18 (19)
	cotyledon	20 (20)	16 (20)	20 (20)	20 (20)
	embryo	20 (20)	16 (20)	20 (20)	20 (20)
G4	Leaf (1-9)	0	0	0	0
	Calyx	0	0	0	0
	Peduncle	0	0	0	0
	MGS (whole)	20	20	20	20
	MDS (whole)	20	20	20	20
G5	Leaf (1-9)	0	0	0	0
	MGS (whole)	20	20	20	20
	MDS (whole)	20	20	20	20

* See materials and methods for allocation to pathotypes and groups.

** Number of positive samples out of 20 as tested by DIBA.

*** Flower parts tested include: standard, wing, calyx, keel, pollen grain, anther, filament, stigma, style, stamen tube, ovary, ovule and receptacle.

**** Figures in parentheses are for plants raised from G_2 seed which have had the testa removed before sowing.

MGS Mature green seed.

MDS Mature dry seed.

Fig. 6.1 A schematic diagram of pea cv. Dundale showing the pattern of sampling of the plant parts.

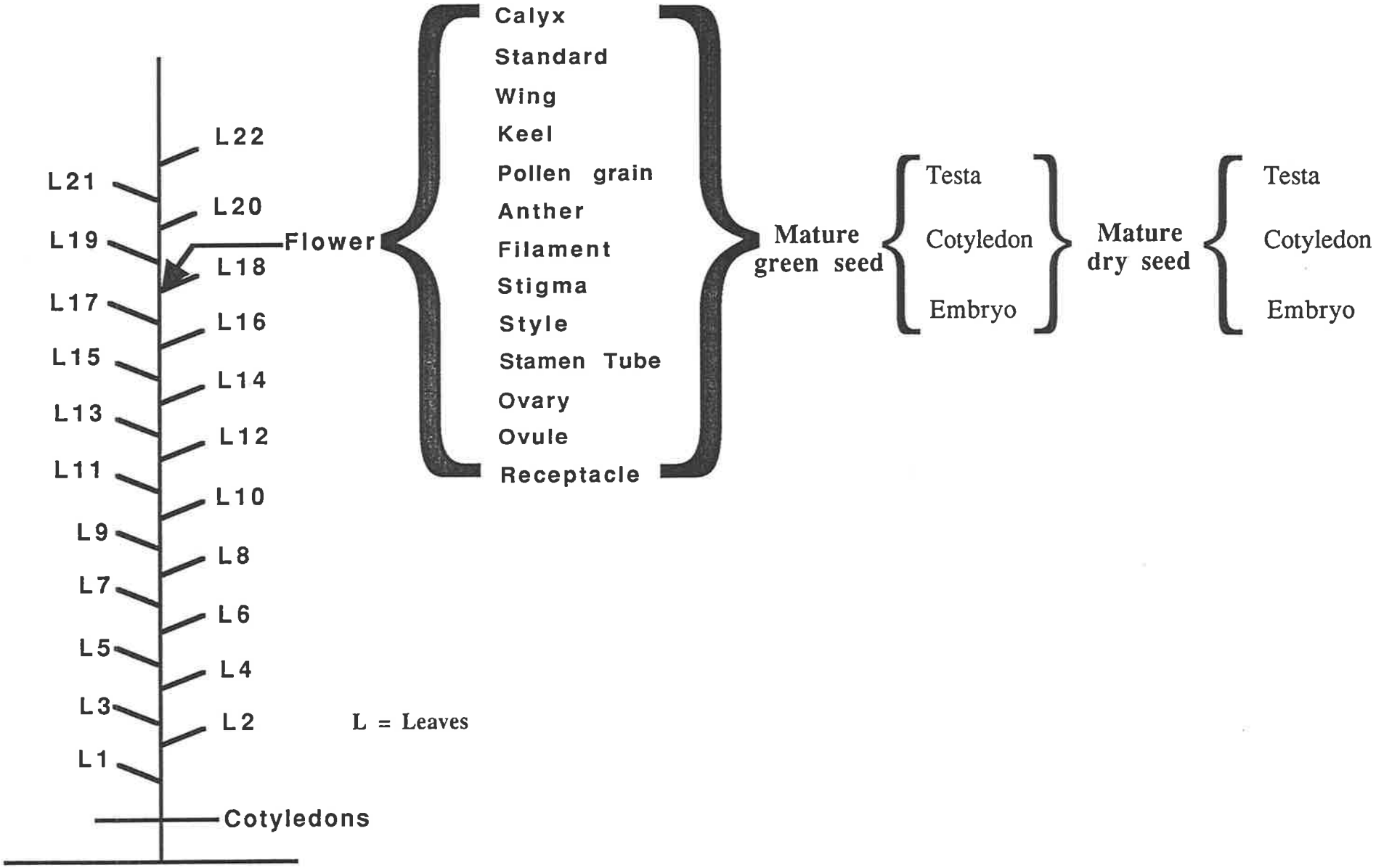

L22
L21
L20
L19
Flower
L18
L17
L16
L15
L14
L13
L12
L11
L10
L9
L8
L7
L6
L5
L4
L3
L2
L1
Cotyledons
L = Leaves
Calyx
Standard
Wing
Keel
Pollen grain
Anther
Filament
Stigma
Style
Stamen Tube
Ovary
Ovule
Receptacle
Mature green seed
Testa
Cotyledon
Embryo
Mature dry seed
Testa
Cotyledon
Embryo

Table 6.2. Relative concentration of PSbMV in seed from G_5 at different stages of maturity. Three seeds were used for each test

Stage of Seed Development	Seed Diameter (mm)	ISOLATE US	Q	S4	S6
1 (immature)	4-5	1[a]	2	2	2
		1	2	2	2
		3	2	1	2
2 (immature)	5-6	2	2	3	1
		2	2	2	1
		2	2	1	1
3 (immature)	6-7	4	2	3	4
		2	2	4	3
		3	2	4	2
4 (mature green)	7-8	4	4	2	2
		4	4	4	2
		4	4	4	3
5 (mature dry)	7-8	4	2	2	2
		2	2	2	2
		2	2	2	2

[a] Number indicates $\log_{10}$ of dilution end-point of PSbMV in infected tissue

6.3 Comparison of transmission of PSbMV through seeds with and without a testa

As described above, in G_2, PSbMV was detected in the seed testa (Table 6.1). To test whether virus was carried between generations in the testa, an experiment was done in which plants were raised from G_2 seed which was either intact, or had the testa removed. As shown in Table 6.1, the plants raised in G_3 from seed with or without a testa had the same level of infection in their seed, indicating that the testa was not involved in vertical transmission of virus.

6.4 Detection of separate products of virus infection in vegetative and reproductive tissue

Both vegetative and reproductive tissue from plants in G4 were compared for content of virus, CIB and RNA. Table 6.3 shows that PSbMV-RNA was detectable in both types of tissue, whereas antibody to both virus and CIB detected antigen in reproductive but not vegetative tissue.

Table 6.3. Detection of separate virus components in vegetative and reproductive tissue of pea at G4

Isolate	Growth stage	PSbMV (DIBA)	CIB (DIBA)	RNA (DHA)
US	V	0[a]	0	8
	R	10	10	7
Q	V	0	0	8
	R	10	10	8
S_4	V	0	0	7
	R	10	10	7
S_6	V	0	0	5
	R	10	10	10

a Number positive out of 10 samples

V Vegetative tissue (leaf)

R Reproductive tissue (seed)

6.5 The detection of infectious virus in seed of G5

Table 6.2 shows that PSbMV antigen was recovered from the seed of symptomless plants after 4 generations of seed transmission. To determine whether the antigen detected was associated with infected cells and infectious virus, seed from G5 was assayed for the presence of CIB by DIBA, and for virus particles by both infectivity assay (Table 6.4) and immunosorbent electron microscopy (Figure 4.5 d). All tests detected the virus in the immature, mature and dry seeds, indicating that infectious PSbMV was being carried vertically from generation to generation despite the absence of detectable virus in the

vegetative parts of the plants.

Table 6.4. Infectivity of G5 on *Pisum sativum* cv. Dundale

Replicate	*P. sativum*	*C. amaranticolor*
I	4[a]	4[b]
II	2	0
III	4	4
IV	7	4

[a] Number positive by DIBA using PSbMV and CIP polyclonal antisera, out of 25 pea cv. Dundale seedlings mechanically inoculated with extract from individual G5 seeds. These seeds were produced from plants which had been mechanically inoculated with isolate US at G1, and propagated through 4 generations by seed only.

[b] Number of plants with necrotic local lesions out of 25 *Chenopodium amaranticolor* seedlings mechanically inoculated as above.

Conclusion

In this study, the second generation (G_2) raised from seed infected with each of the 4 isolates (G1) had symptoms and PSbMV was detectable by DIBA in all the plant parts assayed, except the cotyledons and embryos. However, infection was transferred through the seed to G_3, and I conclude that the level of antigen in the embryo was below the threshold of detection by DIBA.

These studies have identified a previously undescribed "eclipse" of detectable PSbMV in the vegetative tissue of plants in generations G_3 to G_5. The "eclipse" was "partial" in G_3, with isolate Q (Table 6.1), but complete for all other isolates in G_3, and all isolates in G_4 and G_5. In the "eclipse" phase, the virus was latent for the whole duration of the vegetative stage and became detectable by DIBA only at the reproductive stage (see Figure 7.2). Because virus was detectable in vegetative tissue only by cDNA, we cannot determine whether infection persists in the vegetative stage of the plant as RNA, as subliminal levels of virus, or as normal levels of virus but only in a few cells. This aspect needs further study. The only previous report of a latent phase of PSbMV is that of Khetarpal & Maury (1990) who found that infection was latent by ELISA for 5 weeks after germination and then again after 9

weeks. The infected pea seed contained both infectious virus, and intermediates of replication at G5.

An important implication of this result is that I have shown that PSbMV can be maintained in the seed at a high incidence without secondary transmission.

If the testa is not involved in vertical transmission of the virus, transfer is presumably via the embryo.

Symptomless plants negative by serological tests may produce infected seed, and it appears that dot hybridization assays may be necessary for indexing plants. However, DIBA is shown to be reliable for indexing seeds.

Chapter 7 Cross protection study on PSbMV

Introduction

Interference between mixedly inoculated viruses has been ascribed to competition for infectible sites. and the requirement for the interfering agent to be biologically active indicates that at least correct expression of the interfering RNA is necessary for the inhibition to occur (Sterk and De Jager, 1987).

Cross protection is the reduced susceptibility of a plant infected with a mild strain of a given virus to infection with a virulent strain of the same virus (Hamilton, 1980; Fulton, 1982). It is generally accepted that protection of a plant by one strain of a virus against infection with a second depends on the presence of the protecting virus in the protected tissue (Matthews, 1991). Wang *et al* . (1991) have reported that mild strains selected from local severe strains provide better cross protection and their effectiveness was good under different levels of disease pressure.

Although cross protection was first described over 50 years ago, the mechanism of protection remains poorly understood (Register III and Beachy, 1988). There are two popular hypotheses: (1) coat protein (CP) synthesized by the protecting strain inhibits superinfection by reencapsidating or preventing uncoating of the challenge strain (DeZoeten and Fulton, 1975; Sherwood and Fulton, 1982; Dodds *et al.*, 1985) or (2) interactions between the RNAs of the two virus strains inhibit replication of the challenge strain (Palukaitis and Zaitlin, 1984). This phenomenon can be mimicked in transgenic plants that have been engineered to accumulate viral coat protein (Van Dun and Bol, 1988). The expression of the virus capsid protein gene in plants has been shown to be associated with a delay in disease development and diminished invasion by viruses from a range of different groups (Beachy *et al.*, 1990). Angenent *et al.* (1990) have reported that plants expressing CP were resistant to infection with virions of the homologous strain but susceptible to infection with RNA of the homologous strain or nucleoprotein of a heterologous strain. On the other hand, Lindbo and Dougherty (1992) found that RNA-mediated untranslatable TEV RNA protection results in plants which (1) do not display symptoms, even at high virus inoculum levels; (2) show resistance in very young plants; (3) are not protected against challenge inoculation with

heterologous viruses; and (4) show reduced virus replication. The high degree of resistance obtained by this method makes it an attractive alternative to CP-mediated resistance.

The phenomenon of resistance induced by CP transgenesis seems to be analogous to cross protection. It suggests that cross protection can occur in plants challenged with the same strain as the protecting strain. Therefore, I decided to use interference in a biological test to determine whether the pea plants from G3 to G5, which were RNA positive were susceptible to superinfection with PSbMV of the same isolate. If they were infected, it would be expected from the above reasoning that they would be protected against superinfection. The G5 plants were germinated, inoculated on leaves 1 and 2 , and susceptibility to infection was determined by assaying leaves and flower parts as described in Chapter VI. The experiment was done with PSbMV-US only.

Results and Discussion

7.1 Healthy seedlings mechanically inoculated with PSbMV (stage G1)

To determine the response of healthy plants to mechanical inoculation with PSbMV, Dundale seedlings were mechanically inoculated on leaves 1 and 2 immediately after expansion . Plants were allowed to develop to maturity. As each new leaf developed, a leaflet was removed for immediate serological assay. Symptoms were recorded at the same time. Figure 7.1 shows that all leaves above the inoculated leaves of all infected plants had typical symptoms. Both DIBA and DAS-ELISA detected antigen in all leaves, whole flowers and whole mature green seeds.

7.2 Distribution of PSbMV antigen in plants at stage G5

Seedlings from G5 which had been mechanically inoculated at G1, and propagated through 4 generations by seed only , were used as an uninoculated control treatment.. Whole plants were assayed as in 7.1 to determine whether the pattern of symptoms and distribution of virus antigen was as previously observed (Table 6.1). The results in Figure 7. 2 show that the leaves and flowers were negative for both symptom and virus antigen, but DIBA detected the virus in 9 out of 10 of the whole mature green seeds. ELISA detected virus in 3 of these seeds. These results were similar to that described in Table 6.1.

7.3 Distribution of PSbMV in G5 plants mechanically inoculated on leaves 1 and 2

Seedlings from G5 were germinated as in 7.2 above and were inoculated with sap from pea infected with the homologous US isolate. Whole plants were assayed as in 7.1 and 7.2 above.

7.3.1 Susceptibility of inoculated plants

Figure 7.3 shows that 4 plants of 10 inoculated plants had symptoms, 5 were infected, but symptomless, and one was free of symptoms and virus antigen (also see Appendix Table II-3). All leaves with symptoms were positive by DIBA, but not all were positive by ELISA.

7.3.2 Detection of virus in inoculated leaf

Of the 10 inoculated plants, only two contained detectable antigen in the inoculated leaves (Figure 7.3). Of these, one had virus in leaves 1 and 2 (plant 5). The other only in leaf 2 (plant 8).

7.3.3 Detection of virus in uninoculated parts of plants

7.3.3.1 Leaves

There was an uneven distribution of antigen in vegetative parts of the plants (Figure 7.3). The appearance of symptoms and detection of virus was delayed in two plants (plants 2 and 7) until the fourth leaves. In plant 7, there was no symptom and virus detection in the ninth leaf until the fourteenth leaf.

In two symptomless plants (plants 3 and 9), the virus was detected by both assays only in the third and fourth leaves.

In another two plants (plants 5 and 6), although symptomless, the virus was detected by DIBA in leaves 1-4 (plant 5) and 9-14 (plants 5 and 6), but ELISA was unable to detect the antigen in all of the leaves positive by DIBA.

7.3.3.2 Whole flower

Two plants without symptoms (plants 5 and 6) had corollas with colour break. DIBA detected the virus antigen in the whole flowers of 4 plants (plants 1, 2, 5 and 6) and ELISA in only 2 of the 4 plants (plants 5 and 6) .

7.3.3.3 Whole mature green seed

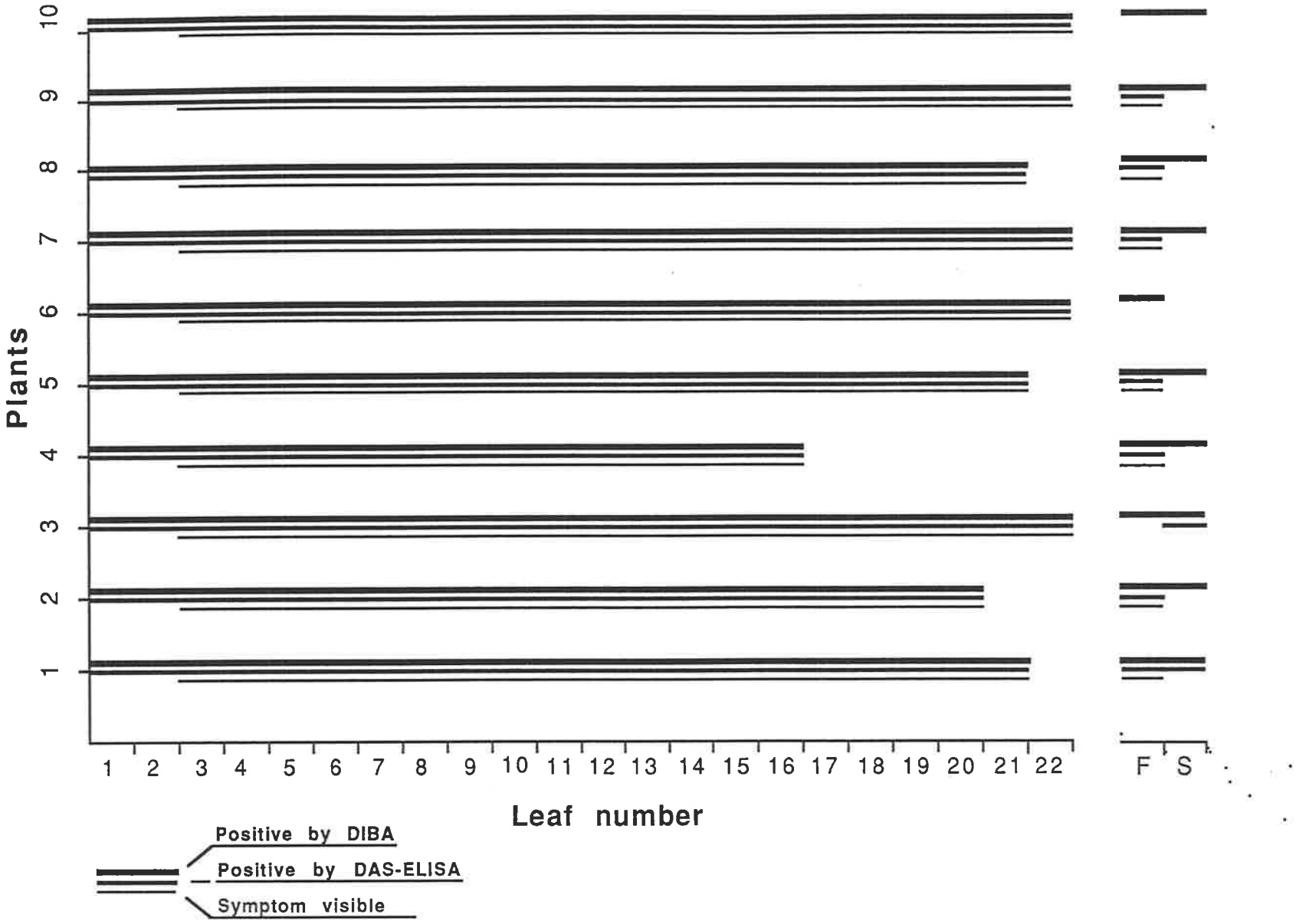
DIBA detected the virus antigen in whole mature green seeds of 8 plants. ELISA detected virus in only 3 of these seeds.

Conclusion

DAS-ELISA was unable to detect the virus antigen in some leaflets of the seedlings from G5 inoculated with the homologous isolate US, which were symptomatic and positive by DIBA. In 7.1, all the plants had symptoms and virus antigen was detected by DIBA and ELISA. The uninoculated seedlings from G5 were symptomless and virus antigen was not detected by serology, but the virus was detected in the mature green seeds by both assays.

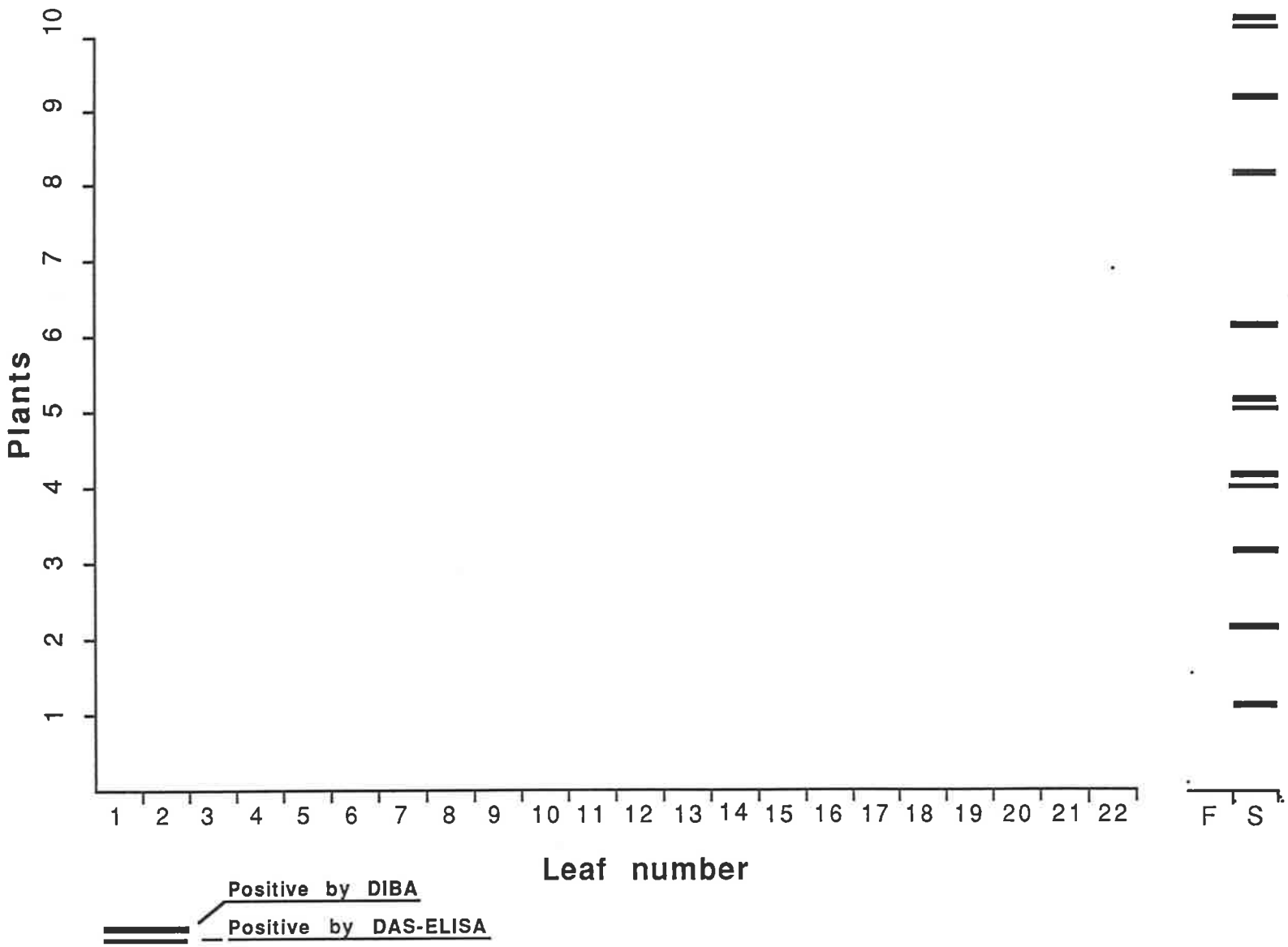
The biological test used in this study showed an uneven distribution of antigen when plants from G5 were challenged with the homologous US isolate. When antigen was present, symptoms were frequently absent, compared with plants in Figure 7.1. Leaves of plants kept until maturity remained asymptomatic, and were not reassayed by DIBA. Therefore, plants in the eclipse phase in G5 exhibit an apparent resistance to infection by systemic movement. It is apparently incomplete. This resistance is probably a form of cross protection. It supports the evidence that peas in eclipse phase are subliminally infected as shown by DHA in Chapter VI. It also shows that parts of plants can vary in virus content. Thus, the systemically infected region, and those with higher virus content, may be zones free of protecting virus infection.

Fig. 7.1 Distribution of PSbMV-US in *Pisum sativum* cv. Dundale. Plants were mechanically inoculated on leaves 1 and 2. Two weeks after inoculation, plants were assessed visually for symptoms and a leaflet from each node was assayed by DIBA and DAS-ELISA. Samples were taken from each new leaf as it developed, and sampling continued to the 22nd node, or earlier if plants were stunted (see Figure 6.1 for a diagram of plant structure). Most plants initiated flowering on the 18th node. The whole first flower was cut off and assayed as above. The first mature green seed (closest to the peduncle) from the pod of the second flower was also tested by both assays.



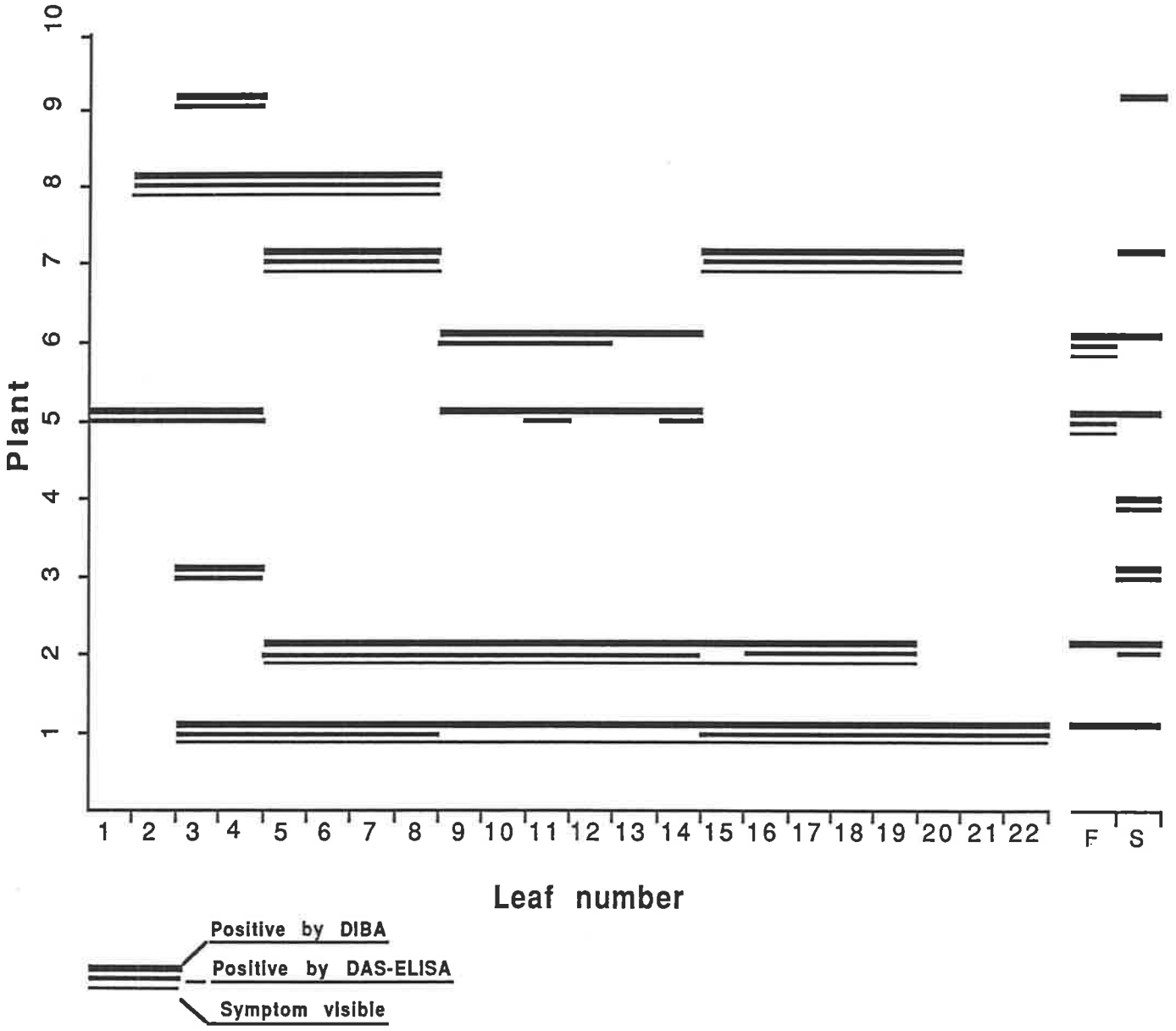
F—Flower (whole)
S—Mature green seed (whole)

Fig. 7.2 Distribution of PSbMV in symptomless seedlings from G5. Seeds from G5 (Table 6.1) were germinated as in 7.1, but not inoculated. Whole plants were assayed as in 7.1.



F—Flower (whole)
S—Mature green seed (whole)

Fig. 7.3 Distribution of PSbMV in seedlings from G5 mechanically inoculated on leaves 1 and 2 with the US isolate. Seeds from G5 were germinated and inoculated as in 7.1. Whole plants were assayed as in 7.1.



F — Flower (whole)

S — Mature green seed (whole)

Chapter 8 General Discussion

8.1 PSbMV in Australia

These studies have shown that PSbMV has become established in commercial crops in South Australia. Infectivity assays and DAS-ELISA have shown that PSbMV is present in 5 areas of South Australia, but at a low incidence (2-3%). It is not known whether PSbMV originated from infected seed, or by aphid transmission from reservoirs.

Seed samples of five pea breeders lines from the South Australian Department of Agriculture were tested by DIBA for PSbMV. It was found that one line had 90-100% infection while two others had 7 and 9% infection (results not shown). No screening for PSbMV is incorporated into the Australian pea-breeding program. This observation is similar to that for breeders lines in other countries (Khetarpal and Maury, 1987) where PSbMV incidence reaches high levels when no screening is done.

A possible explanation for the ability of PSbMV to accumulate to high levels in breeders lines is that mild strains may be selected by continuous selection of the population for yield. Thus, those plants which are infected with strains not significantly affecting yield will be maintained in the population and the incidence of PSbMV infection in seed may rise to levels of the order of 90-100%.

In addition, self-rogueing of symptomatic plants may lead to selection of mild strains. Self-rogueing is the reduction of infected germplasm as a result of reduced vigor or seed-setting in infected plants. Thus, if the plants were infected with a range of strains of PSbMV, those with symptoms would be expected to be less productive and be overgrown, whereas those which are symptomless would mature and produce seeds. There is a possibility that some of the seedlings raised from these seeds will be symptomless while those with symptoms would be uncompetitive and would be removed. If this selection is allowed to continue, there would be an accumulation of mild or latent strains instead of virulent strains. This would be analogous to artificial rogueing where the effect would be as described by Matthews (1949), who reported that mild strains of potato virus X in potato have become more prevalent than virulent strains as a result of rogueing.

Another explanation for high incidence of PSbMV could be the rapid and efficient secondary spread of virus by aphids. No studies on the epidemiology of PSbMV have been described and so it is not known whether it can reach high levels of incidence in a growing season. However, none of these explanations satisfactorily show how PSbMV incidence in seed can reach and be maintained at such high levels.

8.2 The technical development of serological assays and their application to assay of PSbMV in small tissue samples

This thesis describes the development of an effective purification method for PSbMV. The combination of sucrose and Nycodenz density gradients led to very good recovery and a high level of purity for all isolates of the virus as judged by electron microscopy. RNA was extracted from this preparation, and the cDNA that was made was specific and did not react to RNA extracts from healthy plants in DHA.

When preparations from this method were used to immunize rabbits, the antisera produced were of high titre and showed only minor crossreactions with healthy antigen in ELISA and DIBA.

This thesis also describes the development of an indirect DIBA which requires only simple laboratory facilities and can therefore be used in the field with PSbMV and also allows very small samples to be assayed. It is a simple, rapid and sensitive assay that can detect a minimum amount of about 32 ng of virus (Ligat *et al.*, 1991). An initial difficulty encountered with DIBA was the occurrence of false positive results, possibly due to the non-specific binding of antibodies by plant lectins (Higgins et al., 1983). The problem was partially overcome by incorporating mannose and glucose in all buffers. Extracts of healthy leaf sap, healthy seed extracts or both in combination were demonstrated to be better blocking agents than BSA or milk. Healthy antigen was always used as a basis for comparison in the assay. DAS-ELISA was found to be less reliable for the detection of PSbMV in small samples than was DIBA (see Figure 7.3).

The PSbMV specific cytoplasmic inclusion body protein (CIB), was also detected by DIBA simply by substituting CIB antiserum for the virus antiserum. As described by Calder and Ingerfeld (1990), the cylindrical inclusion body protein functions both in facilitating cell-to-cell spread of the virus and in the induction of vesicles from rough endoplasmic reticulum.

The vesicles are the sites of virus-specific RNA synthesis and encapsidation of viral RNA (Caliguiri and Tamm, 1970; Bienz et al., 1980; Yin, 1977).

Virus specific proteins of potyviruses are produced by proteolytic cleavage of a polyprotein translation product (Matthews, 1991). Thus, detection of CIB indicates that virus RNA was present in the cell and that polyprotein has been translated from it. Consequently, these cells would be sites of viral RNA replication. Thus, the detection of both virus and CIB in tissue sample was an indication that virus replication and accumulation of particles was occurring in the tissue.

The inability of DIBA to detect virus in tests where DHA detected virus RNA can probably be explained on the basis of differences in sensitivity. DHA would be expected to detect levels down to 2 pg. Assuming that dots contained about 100 pg of RNA, this would be associated with 2 ng of protein, a level which is below the detection threshold (32 ng) of the DIBA in this study. Levels above 2 ng of RNA would be expected to be reflected by a positive DIBA.

8.3 Demonstration of an eclipse phase in pea cv. Dundale

The most important finding reported in this thesis is the demonstration of a latent or "eclipse" phase for PSbMV in pea cv. Dundale.

This is defined as an inability to detect PSbMV in the vegetative tissue of plants in generations G3 to G5 by DIBA or ELISA, although virus RNA was detected by DHA. The "eclipse" was "partial" in G3 with isolate Q, but complete for all other isolates in G3, and all isolates in G4 and G5 (Table 6.1). In the "eclipse" phase, the virus was latent for the duration of the vegetative stage and became detectable by DIBA only at the reproductive stage. Although virus RNA was detectable in vegetative tissue by cDNA, it is not known whether PSbMV infection persisted in the vegetative stage of the plant either as RNA, as a subliminal level of virus, or as a normal level of virus but only in a few cells. This needs further study. The only previous report of a latent phase for PSbMV is that of Khetarpal & Maury (1990), who found that infection was latent for 5 weeks after germination and then again after 9 weeks. To my knowledge, a full eclipse in the vegetative phase has not been previously reported for any seed-borne virus, but an analogous situation has been described for avocado

sunblotch viroid (Desjardins, 1978), where the symptomless state was associated with a high level of seed transmission in the range 90-100%.

The cycle of PSbMV transmission between plant and seed involves the infection of the embryo probably via embryo sac fluid (Wang & Maule, 1992). The infected embryo produces an infected seedling. Transmission of virus into the embryo through either pollen or ovule appears not to occur and Wang & Maule (1992) attributed seed transmission to direct invasion of immature pea embryos.

In this study, the second generation (G2) raised from seed infected with each of the 4 isolates had symptoms, and PSbMV was detectable in all the plant parts assayed, including the pollen grains and ovules. This was in contrast to the results of Wang & Maule (1992), who did not detect virus in the pollen grains or ovules by either ELISA or electron microscopy. It is possible that DIBA allowed the detection of virus in samples which failed to show virus by ELISA, but the study also used different PSbMV isolates and a different host plant cultivar from those of Wang & Maule (1992).

Khetarpal and Maury (1990) reported a late detection of the virus antigen up to 5 weeks after germination of infected seed and again after 9 weeks. They conducted sampling by collecting the leaves from the upper and middle or lower parts of each of the seedlings or plants and ground them together in the extraction buffer before carrying out ELISA tests. In this study, the plants were tested by sampling the leaflets from leaf one to twenty-two (see Figure 6.1) of each plant. Sap was extracted from each leaflet (see 2.2.5.3 and 2.2.5.4) and tested for PSbMV by DIBA and DAS-ELISA. Under these experimental conditions, ELISA was not as reliable in the detection of virus antigen as DIBA. However, these discrepancy between results and those of Khetarpal and Maury (1990) could be related to the different isolate of PSbMV and host pea cultivar used.

8.4 Implications for control of PSbMV

Generally, seedborne virus diseases are self-limiting because they reduce seed production. For example, strains of CMV which can be seedborne reach a maximum incidence of 18% (Jones, 1987) and EAMV reaches a maximum of 15% (Gibbs and Paul, 1970). PSbMV differs in that it reaches levels of about 90% in commercial seedlots (Knesek & Mink, 1970). The seed transmission observed in this study ranged from 83-92% (Ligat *et*

al., 1991) as described above (8.1). It is probable that these high rates of seed transmission can be maintained because the virus infection becomes latent, with the result that plants are symptomless in crops. Infection would therefore have little effect on the number of viable seeds produced.

The experiments reported in this thesis have demonstrated that such a situation can be demonstrated experimentally. Thus, PSbMV was shown to be transmitted through seed from generation to generation at a high rate, with no horizontal transmission by vectors being required to maintain a high level of infection, and no symptoms or antigen being expressed in the infected plants. Since no secondary spread is required to maintain high virus incidence in seed, and since virus is readily detectable in seeds, the control of PSbMV should be directed towards the production of virus free seed. The results of this work show that serology may not give an accurate indication of infection in growing crops due to subliminal levels of virus. However, serology can detect the virus accurately in seed, and therefore seed should be tested by serology prior to multiplication and distribution to growers. Tissue samples which are negative by serology should be further tested by hybridization assay if necessary.

8.5 Implications of this work for resistance breeding

Breeding for resistance is a possible means of control for PSbMV (Fletcher *et al.*, 1989), but the biological variability in virus isolates described here shows that any Australian breeding program should include challenge inoculations with a range of biologically different PSbMV isolates.

For example, lines to be tested could be inoculated mechanically with PSbMV isolates at the three leaf stage and kept in a glasshouse with controlled temperature and continuous light as described in the materials and methods. Two weeks after inoculation, plants could be reinoculated with the same isolate to reduce the possibility of escape. After two weeks, plants could be regularly tested serologically on sucessive leaves for PSbMV until maturity. Seeds of inoculated plants could be assayed by DIBA and sown again for further observation . Selected lines could then be tested in the field.

Resistance screening needs to take account of the possibility that plants in eclipse may be resistant to mechanical inoculation, due to cross protection. Therefore, seeds should be

tested for virus before use in a screening program, so that subliminal infection does not induce resistance by cross protection.

8.6 Further work to be done

Further studies are needed to determine whether infection persists in the vegetative stage of the plant either as RNA, as subliminal levels of virus, or as normal levels of virus but only in a few cells.

The relationships of PSbMV isolates US, Q, S4 and S6 need to be determined by a comparison of nucleotide sequence homology.

Further experiments on the vertical transmission and distribution of PSbMV in the plant should be done with other pea cultivars. These plants could be grown in a range of environments, including varying temperatures, to determine if environment affects virus replication and the expression of symptoms in vegetative tissue.

Glasshouse and field experiments on the assessment of yield of different susceptible pea cultivars inoculated with PSbMV would contribute to knowledge of the economic importance of the disease, and whether yield losses occur during the eclipse phase.

The model for cross protection described would allow the interaction between identical and variant isolates of PSbMV to be studied at the molecular level. It would therefore be of interest to conduct a detailed study on cross protection with PSbMV.

Appendix I-1: Main biochemicals used in this study

Biochemical	Source
*d*ATP	Boehringer-Mannheim, Germany
*d*CTP	" "
*d*GTP	" "
*d*TTP	" "
^{32}P-*d*CTP (3000 Ci/mmol)	Bresatec, Australia
M-MLV RNA reverse transcriptase	Bethesda Research Laboratory, U.S.A.
Hexanucleotide random DNA primer	Sigma, U.S.A

Appendix I-2: Some chemicals used in this study

Biochemical	Source
2-Mercaptoethanol	BDH Chemicals Ltd, Australia
Acrylamide (99.9%)	Bio-Rad Laboratories, U.S.A.
Agarose (Type II)	Sigma, U.S.A.
Bromophenol blue	BDH Chemicals, England
Ethidium bromide	Sigma, U.S.A.
Formaldehyde solution (formalin)	Ajax Chemical Ltd. Australia
Fuji X-ray film (RX)	Fuji Photo Film Co. Ltd., Japan
G50 Sephadex (fine)	Sigma, U.S.A.
Ilford rapid fixer	Ilford Pty Ltd., Australia
Kodak developer	Kodak Pty Ltd.
N,N'-Methylene-bis-acrylamide (Bis)	Bio-Rad Laboratories, U.S.A.
N,N,N'-N'-tetramethyl ethylenediamine (TEMED)	Sigma, U.S.A.
Polyethylene glycol (PEG_{6000})	ACE Chemical Co., Australia
Silver nitrate	Ajax Chemical Ltd.
Sodium diethyldithiocarbamate	Sigma, U.S.A.
Sodium dodecyl sulphate (SDS)	BDH Chemicals, England
Thioglycollic acid (TGA)	Ajax Chemical Ltd.
Triton X-100	BDH Chemicals, England

Appendix II-1. Comparison of DIBA and DAS-ELISA for the detection of PSbMV in *Pisum sativum* cv. Dundale mechanically inoculated with the US isolate

Sample		Plant 1		2		3		4		5		6		7		8		9		10	
		D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E
Leaf	1*	1	.155	1	.013	1	.162	1	.120	1	.040	1	.039	1	.194	1	.050	1	.067	1	.050
	2*	1	.141	1	.020	1	.155	1	.170	1	.052	1	.040	1	.196	2	.468	2	.090	2	.098
	3	2**	.513	2	.387	3	.845	3	.499	3	.807	3	.692	3	.845	3	.624	3	.733	2	.133
	4	2	.470	2	.349	2	.115	3	.625	3	.610	3	.453	3	.702	3	.582	3	.593	2	.112
	5	3	.322	3	.265	3	.282	3	.422	3	.270	3	.217	3	.216	3	.334	3	.139	3	.242
	6	3	.273	3	.309	3	.259	3	.194	3	.234	3	.218	3	.218	3	.256	3	.259	3	.150
	7	3	.283	3	.278	3	.280	3	.170	3	.297	3	.194	3	.276	3	.455	3	.278	3	.255
	8	3	.270	3	.237	3	.337	3	.160	3	.355	3	.188	3	.325	3	.336	3	.235	3	.169
	9	3	.224	3	.226	3	.215	3	.080	3	.107	3	.112	3	.123	3	.136	3	.130	3	.143
	10	3	..223	3	.217	3	.112	3	.078	3	.152	3	.101	3	.112	3	.145	3	.123	3	.122
	11	3	.114	3	.075	3	.097	3	.067	3	.176	3	.098	3	.145	3	.167	3	.154	3	.112
	12	3	.112	3	.217	3	.087	3	.065	3	.122	3	.085	3	.133	3	.161	3	.163	3	.113
	13	3	.098	3	.221	3	.067	3	.056	3	.123	3	.059	3	.137	3	.156	3	.138	3	.098
	14	3	.112	3	.092	3	.056	3	.047	3	.161	3	.155	3	.122	3	.224	3	.094	3	.080
	15	3	.239	3	.304	3	.158	3	.107	3	.173	3	.359	3	.232	3	.379	3	.216	3	.301
	16	3	.221	3	.226	3	.130	3	.120	3	.186	3	.262	3	.206	3	.322	3	.240	3	.296
	17	3	.266	3	.236	3	.181	na	na	3	.215	3	.307	3	.227	3	.365	3	.261	3	.334
	18	3	.261	3	.232	3	.169			3	.185	3	.284	3	.229	3	.385	3	.258	3	.302
	19	3	.255	3	.240	3	.163			3	.204	3	.271	3	.227	3	.391	3	.266	3	.331
	20	3	.242	3	.244	3	.167			3	.229	3	.289	3	.243	3	.368	3	.275	3	.327
	21	3	.283	na	na	3	.187			3	.221	3	.258	3	.287	3	.357	3	.220	3	.314
	22	na	na			3	.093			na	na	3	.307	3	.321	na	na	3	.248	3	.354
Flower (whole)		3	.025	3	.013	3	0	3	.009	3	.023	3	0	3	.012	3	.016	3	.044	3	.002
Seed (whole)		3	.008	3	0	3	.006	3	0	3	0	na	na	3	0	3	0	3	0	3	0

D DIBA; reaction: 1 - weak; 2 - mild; 3 - strong; 0 - negative.
E DAS-ELISA . A_{405} value given.
na Not available.

* Indicates inoculated leaves.
** Underlining indicates presence of symptoms.

Appendix II-2. Comparison of DIBA and DAS-ELISA for the detection of PSbMV in uninoculated seedlings from G5

Sample		Plant 1		2		3		4		5		6		7		8		9		10	
		D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E
Leaf	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	21	na	na	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	0	0
	22			na	na	0	0	0	0	0	0	na	na	na	na	0	0			0	0
Flower (whole)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Seed (whole)		3	.003	3	0	3	0	3	.006	3	.011	3	0	na	na	3	0	3	.002	3	.009

Footnotes as in Appendix II-1.

Appendix II-3. Comparison of DIBA and DAS-ELISA for the detection of PSbMV in seedlings from G5 mechanically inoculated with the US isolate

Sample		Plant 1		2		3		4		5		6		7		8		9		10	
		D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E	D	E
Leaf	1*	0	0	0	0	0	0	0	0	1	.099	0	0	0	0	0	0	0	0	0	0
	2*	0	0	0	0	0	0	0	0	3	.371	0	0	0	0	3	.583	0	0	0	0
	3	3**	.486	0	0	3	.107	0	0	3	.080	0	0	0	0	3	.443	3	.510	0	0
	4	3	.015	0	0	3	.038	0	0	3	.217	0	0	0	0	3	.431	3	.088	0	0
	5	3	.187	3	.189	0	0	0	0	0	0	0	0	3	.165	3	.113	0	0	0	0
	6	3	.210	3	.204	0	0	0	0	0	0	0	0	3	.148	3	.167	0	0	0	0
	7	3	.235	3	.045	0	0	0	0	0	0	0	0	3	.230	3	.176	0	0	0	0
	8	3	.268	3	.140	0	0	0	0	0	0	0	0	3	.215	3	.185	0	0	0	0
	9	3	0	3	.356	0	0	0	0	3	0	3	.053	0	0	na	na	0	0	0	0
	10	3	0	3	.201	0	0	0	0	3	0	3	.082	0	0			0	0	0	0
	11	3	0	3	.183	0	0	0	0	3	.008	3	.099	0	0			0	0	0	0
	12	3	0	3	.138	0	0	0	0	3	0	3	.029	0	0			0	0	0	0
	13	3	0	3	.103	0	0	0	0	3	0	3	0	0	0			0	0	0	0
	14	3	0	3	.157	0	0	0	0	3	.041	3	0	0	0			0	0	0	0
	15	3	.171	3	0	0	0	0	0	0	0	0	0	3	.227			0	0	na	na
	16	3	.192	3	.043	na	na	0	0	0	0	0	0	3	.307			0	0		
	17	3	.221	3	.041			0	0	0	0	0	0	3	.269			0	0		
	18	3	.196	3	.047			0	0	0	0	0	0	3	.278			0	0		
	19	3	.196	3	.049			0	0	0	0	0	0	3	.288			0	0		
	20	3	.206	na	na			0	0	0	0	0	0	3	.338			0	0		
	21	3	.241					0	0	0	0	na	na	na	na			na	na		
	22	3	.214					na	na	0	0										
Flower (whole)		3	0	3	0	0	0	0	0	3	.005	3	.017	0	0	0	0	0	0	0	0
Seed (whole)		3	0	3	.011	3	.018	3	.013	3	0	3	0	2	0	na	na	2	0	na	na

Footnotes as in Appendix II-1.

Appendix III. Published work

Ligat, J. S., Cartwright, D., and Randles, J. W. (1991). Comparison of some pea seed-borne mosaic virus isolates and their detection by dot-immunobinding assay, *Aust. J. Agric. Res.* **42**, 441-451

Ligat, J. S., and Randles, J. W. (1993). An eclipse of pea seed-borne mosaic virus in vegetative tissue of pea following repeated transmission through the seed, *Ann. Appl. Biol.* In press

Ligat, J. S., Cartwright, D. & Randles, J. W. (1991). Comparison of some pea seed-borne mosaic virus isolates and their detection by dot-immunobinding assay. *Australian Journal of Agricultural Research, 42*(3), 441-451.

Ann. appl. Biol. (1993), 122, 000–000
Printed in Great Britain

An eclipse of pea seed-borne mosaic virus in vegetative tissue of pea following repeated transmission through the seed

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(Accepted 11 September 1992)

Summary

Four isolates of pea seed-borne mosaic virus (PSbMV) representing pathotypes P1 (isolate US) and P4 (isolates Q, S4 and S6), and groups III (US and Q) and V (S4 and S6) have been used in a study of the survival and partitioning of PSbMV under conditions of continuous seed transmission in the commercial pea cultivar Dundale.

Assays suitable for detecting virus in small tissue samples were developed, and included dot-immunobinding assay with antisera to both PSbMV and cytoplasmic inclusion body (CIB) protein, and dot hybridisation assay (DHA) with cDNA transcribed from virus RNA. Under the conditions of our experiments, seed transmission occurred at rates exceeding 90% for all virus isolates.

Virus was detectable by serology and symptoms in inoculated plants, and in all vegetative tissue of second generation plants raised from seed of the inoculated plants. However, in the third, fourth and fifth sequential generations raised from seed, all plants were symptomless. Neither virus nor CIB were detectable in leaf, stem or roots by serology, but both were readily detectable in some floral parts, and in immature and mature seed. Mature seed contained virus and CIB antigen in the testa, cotyledon and embryo. Inoculum prepared from whole seeds was infectious. The testa was shown not to be involved in transmission between generations, thus implicating the embryo alone in vertical transmission. Virus antigen could not be detected in the emerging cotyledons of germinating seed and all true leaves by serology, but the leaves contained PSbMV RNA detectable by DHA.

These results show that PSbMV infection can be transferred through the vegetative phase at a subliminal level, and reaches relatively high concentrations in floral parts and seeds. Thus PSbMV may be maintained at a high level of infection in seed in the absence of any apparent symptoms in the plant, and without a requirement for horizontal transmission between plants by vectors. Such a mechanism may explain the high levels of infection commonly reported in pea breeding lines.

Key words: Dot-immunobinding assay, cytoplasmic inclusion body, dot-hybridisation assay, subliminal infection

Introduction

Pea seed-borne mosaic virus (PSbMV, Hampton & Mink, 1975) is a typical potyvirus encapsidating a single stranded, positive-sense RNA containing an open reading frame of 9618 nucleotides (Johansen, Rasmussen, Heide & Borkhardt, 1991).

PSbMV is economically important both because of its high rate of seed transmission and because the virus can reduce pod yield by 63% and seed yield by 84% (Anon., 1985). The virus is spread in the field by 21 species of aphids (Khetarpal & Maury, 1987), and secondary spread is thought to account for high levels of infection in crops and seed (Hampton & Mink, 1975). The main method of control is the use of serological tests to ensure that less than 0.1% of seed are virus positive (Stace-Smith & Hamilton, 1988). Quarantine restrictions on PSbMV further increase the costs of transfer of seed between countries.

The symptoms induced by PSbMV vary according to virus isolate and host genotype. Differential genotypes of *Pisum* have been used for standardising isolates and strains of PSbMV (Hampton, 1980). Symptoms induced in these differential hosts by PSbMV range from very rapid development of whole-plant necrosis at one extreme to very slight leaf rolling and/or vein clearing at the other. The pattern of resistance to these differential hosts, and the symptoms produced, have allowed isolates to be classed into either one of three pathotypes (P1, P4 and L1; Alconero, Provvidenti & Gonsalves, 1986) or one of five groups (I-V; Hampton *et al.*, 1981).

PSbMV was first reported in Australia by Munro (1978) in an imported pea line undergoing quarantine in Tasmania. It has since been regularly intercepted in imported pea breeding material in post-entry quarantine in South Australia, Tasmania and Queensland. Ligat, Cartwright & Randles (1991) found that maximum seed transmission rates for five PSbMV isolates on pea cv. Dundale occurred with the largest seed and ranged from 83% to 92%. The smallest seeds had the lowest rate of seed transmission, in the range from 29% to 40%. A rapid and sensitive indirect dot-immunobinding assay (DIBA) for studies on PSbMV was developed with a detection limit of about 32 ng per 1 μl sample (Ligat *et al.*, 1991).

In the course of a study on PSbMV diagnosis and the development of control measures, it was observed that although primary inoculated plants were symptomatic, only a small proportion of seedlings raised from the seed of the first generation plants were symptomatic. Unexpectedly, however, the symptomless plants from the second generation showed a high incidence of infection in the seed.

We have used DIBA and molecular hybridisation with cDNA to PSbMV RNA to further investigate the transfer of PSbMV between generations by seed. This paper reports that the virus becomes subliminal in the vegetative stage of the plant, but that it reaches a relatively high concentration in the seed. The implications of these findings for virus epidemiology and control are discussed.

Materials and Methods

Virus and plants

The American (US) and Australian (Q, S4 and S6) isolates of PSbMV were maintained both in plants and seed of *Pisum sativum* cv. Dundale (Ligat *et al.*, 1991). This is a commercially available field pea cultivar widely grown in South Australia. Inoculum from leaf or seed was prepared by triturating tissue with a pestle and mortar in 1 volume (w/v) of 50 mM sodium borate buffer, pH 8.1, and clarifying the extract at 10000 *g* for 10 min. *Chenopodium amaranticolor* Coste and Reyn. and Dundale pea seedlings were mechanically inoculated and maintained at 25°C under continuous light at 2000 lx. At 2 wk after inoculation, the pea seedlings were transferred to an insect-free glasshouse (20 ± 2°C) and maintained until maturity. Mature dry pods were harvested and the dry seeds stored at 4°C. Subsequent generations were raised by germinating seed in the same glasshouse.

Classification of PSbMV isolates with pea differentials

Six pea differentials: PIs 272171, 269774, 193836, 347484, 347422 and 347329 (originating from the USDA germplasm collection and kindly provided by Dr J. Fletcher, DSIR, New Zealand) were used to classify the PSbMV isolates. Four groups of 10 seeds from each line were sown. At the three leaf stage, each group was mechanically inoculated with isolates US, Q, S4 or S6. All plants were tested individually by DIBA at 2 wk after inoculation.

The US isolate was classified as pathotype P1 and Q, S4 and S6 as pathotype P4. Using the grouping system, US and Q were in group III, while S4 and S6 were in group V.

Preparation of PSbMV

PSbMV was prepared by the method of Ligat *et al.* (1991) which had been modified as follows: leaf was blended in 1 volume (w/v) of 500 mM sodium borate buffer, pH 8.1; virus was sedimented at 372000 *g* for 20 min in the TLA 100.3 rotor of a Beckman TL 100 centrifuge; and virus was fractionated in a 300–600 g/litre isopycnic gradient of Nycodenz (Nycgaard; Gugerli, 1984; dissolved in 50 mM borate buffer, pH 8.1) by centrifugation for 4 h at 264000 *g* in the TLA 100.3 rotor. The single light scattering band three-quarters of the way down the gradient was withdrawn with a sterile syringe and sedimented through a 300 g/litre (w/v) sucrose cushion to remove the Nycodenz.

Preparation of total plant nucleic acids

Seed and leaf samples from healthy or infected pea were triturated with a pestle and mortar in 2 volumes (w/v) each of 50 mM Tris-HCl, pH 7.5, 10 g/litre sodium dodecyl sulphate (SDS) and aqueous phenol (900 g/litre containing 1 g/litre 8-hydroxyquinoline). The mixture was shaken for 45 min, centrifuged at 10000 *g* for 10 min and the aqueous supernatant fraction was extracted once with 0.5 volumes each of phenol and chloroform. Nucleic acids were precipitated with three volumes of ethanol in the presence of 200 mM sodium acetate.

Preparation of virus RNA

PSbMV-RNA was prepared by incubating virus in 50 mM Tris-HCl, pH 7.5, 10 g/litre, SDS, containing 200 mM sodium acetate and protease type VI (2 mg/ml; Sigma) for at least 12 h at 25°C or 3 h at 37°C. Mixtures were extracted once with a 0.5 volume of phenol-cresol (9:1 ratio) and RNA was precipitated with ethanol.

Synthesis of cDNA

Virus RNA (approximately 1 μg) in water was incubated in the presence of hexadeoxynucleotide primers (62.5 μg/ml; Pharmacia), 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 50 μM-*d*ATP, -*d*GTP, and -*d*TTP, 30 μCi[^{32}P]*d*CTP (3000 Ci/mmol; Amersham), 500 μM 2-mercaptoethanol, and MMuLV reverse transcriptase (400 units; Bethesda Research Laboratories). The volume of the reaction mixture was 31 μl, with incubation for 5 h at 37°C. The transcribed probe was separated on a G-50 Sephadex column, boiled in TE buffer, and added to hybridisation buffer to give between 0.5×10^6 and 1.0×10^6 cpm/ml (Randles & Rohde, 1990).

Hybridisation assays

Nucleic acid extracts dissolved in water were applied as 1 μl dots to nylon membrane (Zeta-probe, Biorad). The nylon was baked at 80°C for 1.5 h, prehybridised at 42°C for 16 h in 20× SSC (3 M NaCl, 0.3 M sodium citrate) containing 5 mM-EDTA, 2 g/litre SDS, 50

mM sodium phosphate, pH 6.5, 0.2 g/litre each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolidone (PVP, Mr 40000), 50% deionised formamide and boiled herring sperm DNA (0.25 mg/ml). Hybridisation buffer was as above but contained 2 g/litre each of BSA, Ficoll 400, and PVP 40000, 50% dextran sulphate, 1 mg/ml boiled carrier DNA and boiled cDNA. Incubation was at 42°C for 16 h. Membranes were washed at 65°C in 1 × SSC with 1 g/litre SDS for 1 h, then in 0.1 × SSC with 1 g/litre SDS for 1 h before autoradiography at −70°C using intensifying screens.

cDNA detected purified PSbMV RNA to a minimum level of 100 pg per 1 μl dot, and was used for assays of virus RNA in plant tissue.

Dot-immunobinding assay (DIBA)

Extracts of plant parts were tested against PSbMV or cytoplasmic inclusion body (CIB) polyclonal antisera using DIBA as described by Ligat *et al.* (1991). Antiserum to PSbMV cytoplasmic inclusion body protein was kindly supplied by Dr M. Albrechtsen, Plantevaerns Centret, Lottenborgget, Lyngby, Denmark. Healthy leaf or dry seed was extracted by crushing in nine volumes of phosphate buffered saline, and used as the blocking agent. Leaf sap was used as the blocking agent when membranes contained only samples from roots, leaves and flowers. Seed extracts were used for membranes containing samples from immature to mature dry seed. With membranes containing both tissue and seed samples, blocking was done with a mixture of leaf and seed extracts in the ratio 1:1. Both antiserum and goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) were diluted 1/5000.

PSbMV antiserum reacted specifically with purified virus and extracts of infected plant tissue. No reaction was observed in tests using pre-immune serum in place of antiserum.

CIB antiserum did not react with purified virus or extracts of healthy pea tissue. It reacted with infected plant tissue, indicating that it was suitable for detecting the cell inclusion fraction of PSbMV under these assay conditions. Therefore, CIB antiserum was used throughout this work to identify tissue in which PSbMV was replicating as distinct from tests with PSbMV antiserum where only the presence of virus was detectable.

DIBA detected purified virus to a minimum level of 32 ng per 1 μl sample. Virus and CIB antigen was detectable in sap at dilutions down to 1/4096.

Immunosorbent electron microscopy (ISEM)

Leaf and seed extracts were tested using a modification of the method of Roberts & Harrison (1979). Carbon-coated grids were floated film side down on 5 μl drops of antiserum diluted 1:100000 in phosphate buffered saline for 1 h at 25°C. The grids were then drained briefly by touching to a filter paper. Grids coated with antiserum were then floated on 20 μl drops of plant extract placed on aluminium foil in Petri dishes containing moist filter paper, for 12 h at 4°C. Grids with attached virus were washed once with double distilled water, then negatively stained with either neutralised 20 g/litre phosphotungstic acid or 20 g/litre uranyl acetate and examined under the electron microscope.

Studies on virus distribution in pea

Groups of 20 *P. sativum* cv. Dundale seedlings at the three leaf stage were each mechanically inoculated with isolates US, Q, S4 or S6. Two weeks after inoculation, plants were tested for PSbMV by DIBA using homologous polyclonal antisera. For each virus isolate 20 seeds were collected at random from infected mature plants and sown to produce the second vegetative generation (G_2). The first true leaves were assayed immediately after expansion by DIBA for both PSbMV and CIB. As the plants grew, the leaves, stipule and peduncle were sampled as soon as they became available and tested as above. At the

reproductive stage, the flowers were excised and dissected and the floral parts described in Table 1 were individually tested by DIBA. Some pods with green seeds were also dissected and the pod wall, midrib plus funicle, testa, cotyledon and embryo separated and tested. The remaining pods were left to mature.

At the completion of the second generation, 40 seeds were again taken for each of the four isolates and divided into two groups of 20 seeds. For one group the seeds were left intact, whereas in the other the testas were removed. Seeds were sown to give the third generation (G_3) and leaves one to nine of each plant were tested. After pod set, pods with green seeds were again dissected and tested.

Twenty mature dry seeds were collected from the plants raised from intact seed and these were sown to provide the fourth generation (G_4). All leaves of fully grown plants, and green and dry mature seeds were then tested by both DIBA and dot hybridisation assay using ^{32}P-cDNA specific for PSbMV RNA. This was repeated for the fifth generation (G_5).

Results

Distribution of symptoms and virus in five generations of pea plants

Table 1 outlines the layout of the experiment which was done to localise PSbMV in pea tissues, and to follow its vertical transmission for five generations. In the first generation (G_1), the mechanically inoculated plants showed symptoms on all leaves, for all isolates of PSbMV. In the second generation (G_2), each of the 20 plants raised from seed again showed leaf symptoms. For all isolates, viral and CIB antigen were detectable by DIBA in all tissues assayed, except for the cotyledon and embryo of the mature green seed. Table 1 shows the pattern of detection obtained in G_2 to G_5 with all isolates. From plants infected with isolate Q, seeds raised from G_2 gave some symptomatic plants whereas the other isolates gave none. For all isolates, plants raised from the seed of G_3, G_4 and G_5 were symptomless and no virus or CIB protein was detectable by DIBA in sap extracts from leaves of these plants. Moreover, no virus was detectable by infectivity assay which probably has a sensitivity similar to that of DIBA. In contrast, all strains were detected in floral parts by DIBA for virus and CIB. For example, with G_3 in all isolates, virus and CIB were detectable in the pod wall as well as in mature green seed (Table 1). In G_4 and G_5, whole seed extracts only were tested and were found to be positive for both antigens.

Distribution and relative amount of virus in seed

The distribution of virus in seed was determined for seed from plants infected with all isolates, from G_2 to G_5. In G_2, the testas of mature green seeds were positive for both PSbMV and CIB (Table 1) indicating that replication had occurred in this tissue. In G_3, both virus and CIB were detected not only in the testa, but also in the cotyledon and embryo of mature green seed. In G_4 and G_5, both antigens were detected in immature, mature green and mature dried seeds. Table 2 shows the relative amounts of virus in individual whole seeds at different stages of maturity. Mature green seeds generally had the highest level of extractable virus.

Comparison of transmission of PSbMV through seeds with and without a testa

As described above, in G_2, PSbMV was detected in the seed testa (Table 1). To test whether virus was carried between generations in the testa, an experiment was done in which plants were raised from G_2 seed which was either intact, or had the testa removed.

Table 1. *Detection of PSbMV in vertical transfer through five generations (G1-G5) of* Pisum sativum *cv. Dundale*

Seed generation	Tissue tested	Isolate* US	Q	S4	S6
G1	Twenty plants were inoculated mechanically. All were symptomatic. From these, 20 seeds were collected at random and sown.				
G2	Leaf (1–9)	20**	20	20	20
	Flower***	20	20	20	20
	Stipule	20	20	20	20
	Peduncle	20	20	20	20
	Pod wall	20	20	20	20
	MGS testa	20	20	20	20
	cotyledon	0	0	0	0
	embryo	0	0	0	0
G3	Leaf (1–9)	0 (0)****	3 (2)	0 (0)	0 (0)
	Calyx	0 (0)	0 (0)	0 (0)	0 (0)
	Stipule	0 (0)	0 (0)	0 (0)	0 (0)
	Peduncle	0 (0)	0 (0)	0 (0)	0 (0)
	Pod wall	3 (2)	6 (2)	3 (1)	1 (3)
	MGS testa	18 (19)	16 (20)	18 (19)	18 (19)
	cotyledon	20 (20)	16 (20)	20 (20)	20 (20)
	embryo	20 (20)	16 (20)	20 (20)	20 (20)
G4	Leaf (1–9)	0	0	0	0
	Calyx	0	0	0	0
	Peduncle	0	0	0	0
	MGS (whole)	20	20	20	20
	MDS (whole)	20	20	20	20
G5	Leaf (1–9)	0	0	0	0
	MGS (whole)	20	20	20	20
	MDS (whole)	20	20	20	20

* See materials and methods for allocation to pathotypes and groups.
** Number of positive samples out of 20 as tested by DIBA.
*** Flower parts tested include: standard, wing, calyx, keel, pollen grain, anther, filament, stigma, style, stamen tube, ovary, ovule and receptacle.
**** Figures in parentheses are for plants raised from G_2 seed which had the testa removed before sowing.
MGS—Mature green seed; MDS—Mature dry seed.

As shown in Table 1, the plants raised in G_3 from seed with or without a testa had the same level of infection in their seed, indicating that the testa was not involved in vertical transmission of virus.

Detection of separate products of virus infection in vegetative and reproductive tissue

Both vegetative and reproductive tissue from plants in G_4 were compared for content of virus, CIB and RNA. PSbMV-RNA was detectable in both types of tissue, whereas antibody to both virus and CIB detected antigen in reproductive but not vegetative tissue.

The detection of infectious virus in seed of G_5

Table 2 shows that PSbMV antigen was recovered from the seed of symptomless plants after four generations of seed transmission. To determine whether the antigen detected was associated with infected cells and infectious virus, seed from G_5 was assayed for the presence

Table 2. *Relative concentration of PSbMV in seed from G5 at different stages of maturity*

Stage of seed Development	Seed Diameter (mm)	ISOLATE US	Q	S4	S6
1 (immature)	4–5	1*	2	2	2
		1	2	2	2
		3	2	1	2
2 (immature)	5–6	2	2	3	1
		2	2	2	1
		2	2	1	1
3 (immature)	6–7	4	2	3	4
		2	2	4	3
		3	2	4	2
4 (mature green)	7–8	4	4	2	2
		4	4	4	2
		4	4	4	3
5 (mature dry)	7–8	4	2	2	2
		2	2	2	2
		2	2	2	2

* Number indicates $\log_{10}$ of the dilution end-point of PSbMV in infected whole seed, by DIBA. Three seeds were used for each test.

of CIB by DIBA, and for virus particles by both infectivity assay and immunosorbent electron microscopy. All tests detected the virus in the immature, mature and dry seeds, indicating that infectious PSbMV was being carried vertically from generation to generation despite the absence of detectable virus in the vegetative parts of the plants.

Discussion

Using plants equivalent to those from generation G_1 as described in this paper, Wang & Maule (1992) showed that the cycle of PSbMV transmission between plant and seed involves the infection of the embryo probably via the embryo sac fluid. The infected embryo produces an infected seedling. They concluded that transmission of virus into the embryo through either pollen or ovule did not occur and attributed seed transmission to direct invasion of immature pea embryos.

In this study, the second generation (G_2) raised from seed infected with each of the four isolates had symptoms and PSbMV was detectable by DIBA in all the plant parts assayed, except the cotyledons and embryos. However, infection was transferred through the seed to G_3, and we conclude that the level of antigen was below the threshold of detection by DIBA.

Our results have identified a previously undescribed 'eclipse' of detectable PSbMV in the vegetative tissue of plants in generations G_3 to G_5. The 'eclipse' was partial in G_3, with isolate Q (Table 1), but complete for all other isolates in G_3, and all isolates in G_4 and G_5. In the 'eclipse' phase, the virus was latent for the whole duration of the vegetative stage and became detectable by DIBA only at the reproductive stage. Because virus was detectable in vegetative tissue only by cDNA, we cannot determine whether infection persists in the vegetative stage of the plant as RNA, as subliminal levels of virus, or as normal levels of virus but only in a few cells. This aspect needs further study. The only previous report of a latent phase of PSbMV is that of Khetarpal & Maury (1990) who found that infection was latent for 5 weeks after germination and then again after 9 weeks. The infected pea seed contained both infectious virus, and intermediates of replication at G_5.

Generally, seedborne virus diseases are self-limiting because they reduce seed production. For example CMV reaches a maximum of 18% (Jones, 1987) and EAMV reaches a maximum of 15% (Gibbs & Paul, 1970). PSbMV differs in that it reaches levels of about 90% in commercial seedlots (Knesek & Mink, 1970). It seems that such a high rate could be maintained as a result of a high incidence of symptomless plants in crops and infection having little effect on the number of viable seeds produced.

This study shows that PSbMV can be transmitted through seed from generation to generation at a high rate without a requirement for horizontal transmission by vectors. Therefore, if no secondary spread is required to maintain high virus incidence in seed, the control of PSbMV should be directed towards the production of virus free seed. Since our results show that serology may not give an accurate indication of infection in crops due to subliminal level of virus, seeds should be tested by serology prior to multiplication and distribution to growers.

Acknowledgements

We thank Dr J. Fletcher for seed and pea differential lines, and Dr M. Albrechtsen for CIB antiserum. J. S. Ligat received an award from the Australian International Development Assistance Bureau.

References

Anon 1985. Diseases of legumes. *Rothamsted Experimental Station Report for 1985*:123–124.

Alconero R, Provvidenti R, Gonsalves D 1986. Three pea seedborne mosaic virus pathotypes from pea and lentil germplasm. *Plant Disease* 70:783–786.

Gibbs A J, Paul H L 1970. Echtes Ackerbohnenmosaik-Virus. *Association of Applied Biologists Descriptions of Plant Viruses*. Commonwealth Mycological Institute. No. 20.

Gugerli P. 1984. Isopycnic centrifugation of plant viruses in Nycodenz density gradients? *Journal of Virological Methods* 9:249–258.

Hampton R O. 1980. Pea seedborne mosaic symptom variation among *Pisum* plant introduction accessions: expression and variation in symptom expression among different genotypes of *Pisum* and the pathological implications. *Research Reports* 12:29. Oregon, USA: USDA.

Hampton R O, Mink G I. 1975. Pea seed-borne mosaic virus. *Association of Applied Biologists Descriptions of Plant Viruses*. Commonwealth Mycological Institute. No. 146.

Hampton R O, Mink G I, Bos L, Inouye T, Musil M, Hagedorn D J. 1981. Host differentiation and serological homology of pea seed-borne mosaic virus isolates. *Netherlands Journal of Plant Pathology* 87:1–10.

Johansen E, Rasmussen, O F, Heide M, Borkhardt B. 1991. The complete nucleotide sequence of pea seed-borne mosaic virus RNA. *Journal of General Virology* 72:2625–2632.

Jones R A C. 1987. Cucumber mosaic virus in lupins. *Western Australian Journal of Agriculture, Series IV* 28:50–54.

Khetarpal R K, Maury Y. 1987. Pea seed-borne mosaic virus: a review. *Agronomie* 7:215–224.

Khetarpal R K, Maury Y. 1990. Seed transmission of pea seed-borne mosaic virus in peas: early and late expression of the virus in the progeny. *Journal of Phytopathology* 129:265–270.

Knesek J E, Mink G I. 1970. Incidence of a seed-borne virus in peas grown in Washington and Idaho. *Plant Disease Reporter* 54:497–498.

Ligat J S, Cartwright D, Randles J W. 1991. Comparison of some pea seed-borne mosaic virus isolates and their detection by dot-immunobinding assay. *Australian Journal of Agricultural Research* 42:441–451.

Munro D. 1978. Pea seed-borne mosaic virus in quarantine. *Australian Plant Pathology Society Newsletter* 7:10.

Randles J W, Rohde W 1990 *Nicotiana velutina* mosaic virus: evidence for a bipartite genome comprising 3 Kb and 8 Kb RNAs. *Journal of General Virology* 71:1019–1027.

Roberts I M, Harrison B D. 1979. Detection of potato leafroll and potato mop-top viruses by immunosorbent electron microscopy. *Annals of Applied Biology* 93:289–297.

Stace-Smith R, Hamilton R I. 1988. Inoculum thresholds of seedborne pathogens, viruses. *The American Phytopathological Society* 78:875–880.

Wang D, Maule A J. 1992. Early embryo invasion as a determinant in the seed transmission of pea seed-borne mosaic virus in pea. *Journal of General Virology*. In press.

(Received 29 June 1992)

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References

Aapola, A. A., Knesek, J. E., and Mink, G. I. (1974). The influence of inoculation procedure on the host range of pea seed-borne mosaic virus, *Phytopathology* **64**, 1003-1006

Alconero, R., Weeden, N. F., Gonsalves, D., and Fox, D. T. (1985). Loss of genetic diversity in pea germplasm by the elimination of individuals infected by pea seed-borne mosaic virus, *Ann. of Applied Biology* **106**, 357-364

Alconero, R., Provvidenti, R., and Gonsalves, D (1986). Three pea seed-borne mosaic virus pathotypes from pea and lentil germplasm, *Plant Disease* **70**, 783-786

Alconero, R., and Hoch, J. G. (1989). Incidence of pea seed-borne mosaic virus pathotypes in the US National *Pisum* germplasm collection, *Annals of Applied Biology* **114**, 311-315

Ali-Khan, S. T., and Zimmer, R. C. (1979). Screening of field pea breeding lines for pea seed-borne mosaic virus, *Canadian Journal of Plant Science* **59**, 171-175

Angenent, G. C., Van Den Ouweland, J. M. W., and Bol, J. F. (1990). Susceptibility to virus infection of transgenic tobacco plants expressing structural and nonstructural genes of tobacco rattle virus,*Virology* **175**, 191-198

Anon. (1985). Diseases of legumes, *Rothamsted Experimental Station Report for 1985*, 123-124

Bagget, J. R., and Hampton, R. O. (1972). Plant Introduction lines of *Pisum sativum* resistant to pea fizzletop disease, *Plant Disease Reporter* **56** 2, 131-132

Barker, H., Reavy, B., Kumar, A., Webster, K. D., and Mayo, M. A. (1992). Restricted virus multiplication in potatoes transformed with the coat protein gene of potato leaf roll luteovirus: similarities with a type of host gene-mediated resistance, *Annals of applied Biology* **120**, 55-64

Beachy, R. N , Loesch-Fries, S., and Tumer, N. E. (1990). Coat protein mediated resistance against virus infection, *Annual Reniew of Phytopathology* **28**, 451-474

Bertioli, D. J., Cooper, J. I., Edwards, M. L., and Hawes, W. S. (1992). Arabis mosaic

nepovirus coat protein in transgenic tobacco lessens disease severity and virus replication, *Annals of Applied Biology* **120**, 47-54

Bienz, K., Egger, D., Rasser, Y., and Bossart, W. (1980). Kinetics and location of poliovirus macromolecular synthesis in correlation to virus-induced cytopathology, *Virology* **100**, 390-399

Bos, L. (1970). The identification of three new viruses isolated from Wisteria and *Pisum* in the Netherlands and the problem of variation within potato virus Y group, *Netherlands Journal of Plant Pathology* **76**, 8-46

Brakke, M. K. (1959). Dispersion of aggregated barley stripe mosaic virus by detergents, *Virology* **9**, 506-521

Brunt, A. A. (1970). Virology - Gladiolus, *Glasshouse Crops Research Institute, Annual Report 1970*, 150

Calder, V. L., and Ingerfeld, M. (1990). The roles of the cylindrical inclusion protein of a potyvirus in the induction of vesicles and cell-to-cell spread, *Journal of Structural Biology* **105**, 62-66

Caliguiri, L. A., and Tamm, I. (1970). The role of cytoplasmic membranes in poliovirus biosynthesis, *Virology* **42**, 100-111

Chang, C. A., Chen, C. M., Chen, H. C., Deng, T. C., and Huang, C. H. (1989). Pea seed-borne mosaic virus causing pea mottling in Taiwan, *Plant Protection Bulletin, Taiwan* **314**, 366-676

Chiko, A. W., and Zimmer, R. C. (1978). Effect of pea seed-borne mosaic virus on two cultivars of field pea grown in Manitoba, *Canadian Journal of Plant Science* **58**, 1073-1080

Clark, M. F., and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses, *Journal of General Virology* **34**, 475-483

Clark, M. F. (1981). Immunosorbent assays in plant pathology, *Annual Review of Phytopathology* **19**, 83-106

Delgado-Sanchez, S., and Grogan, R. G. (1966). Purification and properties of potato virus Y, *Phytopathology* **56**, 1397-1404

Desjardins, P. R. (1987). Avocado sunblotch, In: *The Viroids* (T. O. Diener, Ed.), p. 304. Plenum Press, New York

DeZoeten, G. A., and Fulton, R. W. (1975). Understanding generates possibilities, *Photopathology* **64**, 221-222

Dodds, J. A., Lee, S. Q., and Tiffany, M. (1985). Cross-protection between strains of cucumber mosaic virus: Effect of host and type of inoculum on accumulation of virions and dsRNA of the challenge strain,*Virology* **144**, 301-309

Fletcher, J. D., Goulden, D. S., Russel, A. C., and Scott, R. E. (1989). Breeding for resistance to pea seedborne mosaic virus, *Proceedings of the 7th Australasian Plant Pathology Society Conference* 122

Francki, R. I. B. (1972). Isolation and characterization of viruses, In: *Principles and Techniques in Plant Virology* (C. I. Kado and H. O. Agrawal, Eds.), pp. 295-325. Van Nostrand Reinhold Company, New York

Fry, P. R., and Young, B. R. (1980). Pea seed-borne mosaic virus in New Zealand, *Australian Plant Pathology* **9** (1), 10-11

Fulton, R. W. (1982). In: *Active Defence Mechanisms in Plants* (R. K. S. Wood, Ed.), pp. 231-245. Plenum, New York

Gibbs, A. J., and Paul, H. L. (1970). Echtes Ackerbohnenmosaik-Virus, *Association of Applied Biologists. Descriptions of plant viruses* . Commonwealth Mycological Institute. No. 20.

Gonzales, L. C., and Hagedorn, D. J. (1970). Aphid transmission of pea seed-borne mosaic virus, *Phytopathology* **60**, 1293

Goodell, J. J., and Hamton, R. O. (1983). Interactions of *Pisum* genes *sbm* and *mo* with pea seed-borne mosaic virus (PSbMV): Symptom expression and immunity to three variant strains, *Phytopathology* **73**, 789

Goodell, J. J., and Hampton, R. O. (1984). Ecological characteristics of the lentil strain of pea seed-borne mosaic virus, *Plant Disease* **68**, 148-150

Gould, A. R., and Symons, R. H. (1983). A molecular biological approach to relationships among viruses, *Annual Review of Phytopathology* **21**, 179-199

Graddon, D. J., and Randles, J. W. (1986). Single antibody dot immunoassay- a simple technique for rapid detection of a plant virus, *Journal of Virological Methods* **13**, 63-69

Gugerli, P. (1984). Isopycnic centrifugation of plant viruses in nycodenz density gradients, *Journal of Virological Methods* **9**, 249-258

Hagedorn, D. J., and Gritton, E. T. (1973). Inheritance of resistance to the pea seed-borne mosaic virus, *Phytopathology* **63**, 1130-1133

Hamilton, R. I., and Nichols, C. (1978). Serological methods for detection of pea seed-borne mosaic virus in leaves and seeds of *Pisum sativum*, *Phytopathology* **68**, 539-543

Hamilton, R. I. (1980). In: *Plant Disease*, vol. **5**., pp. 279-303 *An Advanced Treatice* (J. G. Horsfall and E. B. Crowling, Eds.). Academic Press, New York

Hampton, R. O. (1969). Characteristics of virus particles associated with seed-borne pea fizzletop disease, *Phytopathology* **59**, 1029

Hampton, R. O., and Baggett, J. R. (1970). Host effects and diagnostic symptoms of pea fizzletop disease, *Plant Disease Reporter* **54**(5), 355-358

Hampton, R. O. (1972). Dynamics of symptom development of the seed-borne pea fizzletop virus, *Phytopathology* **62**, 268-272

Hampton, R. O., Phillips, S., Knesek, J. E., and Mink, G. I. (1973). Ultrastructural cytology of pea leaves and roots infected by pea seed-borne mosaic virus, *Archiv fur die gesamte Virusforchung* **42**, 242-253

Hampton, R. O., Knesek, J. E., and Mink, G. I. (1974). Particle-length variability of the pea seed-borne mosaic virus, *Phytopathology* **64**, 1358-1363

Hampton, R. O., and Mink, G. I. (1975). Pea seed-borne mosaic virus, *Association of Applied Biologists. Descriptions of plant viruses*, Commonwelath Mycological Institute. No. 146

Hampton, R. O., Mink, G. I., Hamilton, R. I., Kraft, J. M., and Muehlbauer, F. J. (1976). Occurrence of pea seed-borne mosaic virus in North American pea breeding lines, and procedures for its elimination, *Plant Disease Reporter* **60**(6), 455-459

Hampton, R. O., and Muehlbauer F. J. (1977). Seed transmission of the pea seed-borne mosaic virus in lentils, *Plant Disease Reporter* **61**(3), 235-238

Hampton, R. O., and Braverman, S. W. (1979). Occurrence of pea seed-borne mosaic virus-immune germplasm in the plant introduction collection of *Pisum sativum*, *Plant Disease Reporter* **63**(2), 95-99

Hampton, R. O. (1980). Pea seed-borne mosaic symptom variation among *Pisum* plant introduction accessions: expressions and variation in symptom expression among different genotypes of *Pisum* and the pathological implications, *Pisum Newsletter* **12**, 29-30

Hampton, R. O., and Marx, G. A. (1981). Immunity to pea seed-borne mosaic virus: A re-assessment, *Pisum Newsletter* **13**, 16-17

Hampton, R. O., Mink, G. I., Bos, L., Inouye, T., Musil, M. and Hagedorn, D. J. (1981). Host differentiation and serological homology of pea seed-borne mosaic virus isolates, *Netherlands Journal of Plant Pathology* **87**, 1-10

Hatta, T., and Francki, R. I. B. (1984). Differences in the morphology of isometric particles of some plant viruses stained with uranyl acetate as an aid to their identification, *Journal of Virological Methods* **9**, 237-247

Hibi, T., and Saito, Y. (1985). A dot immunobinding assay for the detection of tobacco mosaic virus in infected tissues, *Journal of General Virology* **66**, 1191-1194

Higgins, T. S. V., Chandler, P. M., Zurawski, G., Button, S. C. and Spencer, D. (1983). The biosynthsis and primary structure of pea seed lectin,*The Journal of Biological Chemistry* **258** (15), 9544-9549

Hill, J. H., Ford, R. E., and Benner, H. (1973). Purification and partial characterization of maize dwarf mosaic virus, *Journal of General Virology* **20**, 327-339

Huang, E. S., and Pagano, J. S. (1977). Nucleic acid hybridization technology and detection of proviral genomes, *Methods in Virology* **6**, 457-497

Huttinga, H. (1973). Properties of viruses of the potyvirus group. 1. A simple method to purify bean yellow mosaic virus, pea mosaic virus, lettuce mosaic virus and potato virus Y^N, *Netherlands Journal of Plant Pathology* **79**, 125-129

Huttinga, H. (1975). Properties of viruses of the potyvirus group. 3. A comparison of bouyant density, S value, particle morphology, and molecular weight of the coat protein subunit of 10 viruses and virus isolates, *Netherlands Journal of Plant Pathology* **81**, 58-63

Inouye, T. (1967). A seed-borne mosaic virus of pea, *Ann. Phytopath. Soc. Jpn*, **33**, 38-42

Inouye, T. (1971). Cytoplasmic inclusion in plant cells infected with pea seed-borne mosaic virus, *Nogaku, Kenyu, The Japanese Report of the Institute for Agricultural and Biological Sciences, Okayama University* **53**(4), 189-196

Johansen, E., Rasmussen, O. F., Heidi, M., and Borkhardt, B. (1991). The complete nucleotide sequence of pea seed-borne mosaic virus RNA, *Journal of General Virology* **72**, 2625-2632

Jones, R. A. C. (1987b). Cucumber mosaic virus in lupins,*Western Australian Journal of Agriculture*. Series IV **28**, 50-54.

Kamei, T., Honda, Y., and Matsui, C. (1969). Intracellular appearance of turnip mosaic and bean yellow mosaic virus particles, *Phytopathology* **59**, 139-144

Karl, E., and Schmidt, H. E. (1978). Untersuchungen zum Artenspektrum der Aphidenvektoren des Erbsenblattrollmosaik-virus (pea leafrolling mosaic virus), *Arch. Phytopath. Pflanzensch., Berlin* **14**, 367-372

Khetarpal, R. K., and Maury, Y. (1987). Pea seed-borne mosaic virus: a review, *Agronomie* **7** (4), 215-224

Khetarpal, R. K., Bossennec, J. M., Burghofer, A., Cousin, R., and Maury, Y. (1988). Effect of pea seed-borne mosaic virus on yield of field pea, *Agronomie* **8** (9), 811-815

Khetarpal, R. K., and Maury, Y. (1990). Seed transmission of pea seed-borne mosaic virus in peas: early and late expression of the virus in the progeny, *Journal of Phytopathology* **129**, 265-270

Khetarpal, R. K., Maury, Y., Cousin, R., Burghofer, A., and Varma, A. (1990). Studies on resistance of pea to pea seed-borne mosaic virus and new pathotypes, *Annals of Apllied Biology* **116**, 297-304

Knesek, J. E., and Mink, G. I. (1970). Incidence of a seedborne virus in peas grown in Washington and Idaho, *Plant Disease Reporter* **54** (6), 497-498

Knesek, J. E., Mink, G. I., and Hampton, R. O. (1974). Purification and properties of pea seed-borne mosaic virus, *Phytopathology* **64**, 1076-1081

Koenig, R., An, D., and Burgermmeister, W. (1988). The use of filter hybridization techniques for the identification, differentiation and classification of plant viruses, *Journal of Virological Methods* **19**, 57-68

Kowalska, C. (1979). Viruses infecting pea (*Pisum L.*) in Poland, *Genet. Pol.* **20**, 211-215

Kraft, J. M., and Hampton, R. O. (1980). Crop losses from pea seed-borne mosaic virus in six processing pea cultivars, *Plant Disease* **64**, 922-924

Ligat, J. S., Cartwright, D., and Randles, J. W. (1991). Comparison of some pea seed-borne mosaic virus isolates and their detection by dot-immunobinding assay, *Australian Journal of Agricultural Research* **42**, 441-451

Lindbo, J. A., and Dougherty, W. G. (1992). Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts,*Virology* **189**, 725-733

Lindsten, K., Brishhammar, S., and Tomenius, K. (1976). Investigations on relationship and variation of some legume viruses within the potyvirus group, *Medd. Statens Vaxtskyddsanstalt*. **16**, 289-322

Lister, R. M. (1978). Application of the enzyme-linked immunosorbent assay for detecting viruses in soybean seed and plants, *Phytopathology* **68**, 1393-1400

Matthews, R. E. F. (1949). Studies on potato virus X. II. Criteria of relationships between strains, *Annals of Applied Biology* **36**, 460-474

Matthews, R. E. F. (1991). In: *Plant Virology* (Third edition), pp. 197-509. Academic Press, Inc., San Diego, California

Matthews, P., Dawson, J. R. O., and Hills, G. H. (1981). Pea seed-borne mosaic virus (PSbMV), *John Innes Seventy-first Annual Report (1980)*, 31-32

Maury, Y., Bossennic, J. M., Boudazin, G., Hampton, R. O., Pietersen, G., and Maguire, J. (1987). Factors influencing ELISA evaluation of transmission of pea seed-borne mosaic virus in infected pea seed: seed group size and seed decortication, *Agronomie* **7**(4), 225-230

McLaughlin, M. R., Barnett, O. W., Burrows, P. M., and Baum, R. H. (1981). Improved ELISA conditions for detection of plant viruses, *Journal of Virological Methods* **3**, 13-25

Milicic, D., and Grbelja, J. (1977). Pea seed-borne mosaic virus in some pea cultivars in Yugoslavia, *Zastila Bilja* **28**, 147-154

Milicic, D., and Plavsic, B. (1978). Contribution to the knowledge of pea seed-borne mosaic virus, *Acta Botanica Croatica* **37**, 9-19

Mink, G. I., Kraft, J., Knesek, J., and Jafri, A. (1969). A seed-borne virus of pea, *Phytopathology* **59**, 1342-1343

Mink, G. I., Inouye, T. Hampton, R. O., and Knesek, J. E. (1974). Relationships among isolates of pea seed-borne mosaic virus from the United States and Japan, *Phytopathology* **64**(4), 569-570

Mink, G. I., and Parsons, J. C. (1978). Detection of pea seed-borne mosaic virus in pea seed by direct-seed assay, *Plant Disease Reporter* **62**, 249-253

Moghal, S. M., and Francki, R. I. B. (1976). *Studies on some potyviruses with special reference to those infecting legumes in Australia*, PhD Thesis, The University of Adelaide, South Australia

Munro, D. (1978). Pea seed-borne mosaic virus in quarantine, *Australian Plant Pathology Society Newsletter* **7**, 10

Musil, M. (1966). Uber das Vorkommen des Virus des Blattrollens der Erbse in der Slowakei (Vorlaufige Mitteilung), *Biologia (Bratislava)* **21**, 133-138

Musil, M. (1970). Pea leaf rolling mosaic virus and its properties, *Biologia (Bratislava)* 25, 379-392

Musil, M. (1980). Seed transmission of pea leaf rolling mosaic virus, *Sameniibertragbarkeit des Blattrollmosaik Virus der Erbse* **184**, 345-352

Naim, Z., and Hampton, R. O. (1979). *Pisum sativum* seed characteristics and assay methodology as factors affecting detection of pea seed-borne mosaic virus, *Allahabad Farmer* **50**(1), 25-28

Noordam, D. (1973). *Identification of Plant Viruses: Methods and Experiments*. Pudoc, Wageningen

Palukaitis, P., and Zaitlin, M. (1984). A model to explain the "cross protection" phenomenon shown by plant viruses and viroids, In "*Plant-Microbe Interactions. Molecular and Genetic Perspectives*" (T. Kosuge and E. N. Nester, Eds.), pp. 420-429. Macmillan, New York

Pelet, F. (1980). Pea seed-borne mosaic virus (La mosaique hereditaire du pois), *Rev. Suisse Vitic. Arboric. Hortic.* **12**, 281

Ponz, F., and Bruening, G. (1986). Mechanisms of resistance to plant viruses, *Annual Review of Phytopathology* **24**, 355-381

Provvidenti, R., and Alconero, R. (1988). Inheritance of resistance to a third pathotypes of pea seed-borne mosaic virus in *Pisum sativum*, *The Journal of Heredity* **79**(1), 76-80

Provvidenti, R., and Alconero, R. (1988). Sources of resistance to pathotypes of pea seed-borne mosaic virus in the US Plant Introductions of *Pisum sativum*, *Research Reports PNL* **20**, 30-31

Purcifull, D. E., and Edwardson, J. R. (1967). Watermelon mosaic virus: Tubular inclusions in pumpkin leaves and aggregates in leaf extracts,*Virology* **32**, 393-401

Randles, J. W., and Rohde, W. (1990). *Nicotiana velutina* mosaic virus: evidence for a bipartite genome comprising 3 Kb and 8 Kb RNAs, *Journal of General Virology* **71**, 1019-1027

Register III, J. C., and Beachy, R. N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection, *Virology* **166**, 524-532

Rezende, J. A. M., Urban, L., Sherwood, J. L., and Melcher, U. (1992). Host effect on cross protection between two strains of tobacco mosaic virus, *Journal of Phytopathology* **136**, 147-153

Roberts, I. M., and Harrison, B. D. (1979). Detection of potato leaf roll and potato mop-top viruses by immunosorbent electron microscopy, *Annals of Applied Biology* **93**, 289-297

Rocha-Pena, M. A., Lee, R. F. and Niblett, C. L. (1991). Development of a dot-immunobinding assay for detection of citrus tristeza virus, *Journal of Virological Methods* **34**, 297-309

Sanford, J. C., and Johnston, S. A. (1985). The concept of pathogen derived resistance: Deriving resistance genes from the parasite's own genome, *J. Theor. Biol.* **113**, 395-405

Sela, I. Reichman, M., and Weissbach, A. (1984). Comparison of dot molecular hybridization and ELISA for detecting tobacco mosaic virus in plant tissues and protoplasts, *Phytopathology* **74**, 385-389

Sherwood, J. L., and Fulton, R. W. (1982). The specific involvement of coat protein in tobacco mosaic virus cross protection,*Virology* **119**, 150-158

Sterk, P., and De Jager, C. P. (1987). Interference between cowpea mosaic virus and cowpea severe mosaic virus in a cowpea host immune to cowpea mosaic virus, *Journal of General Virology* **68**, 2751-2758

Stevenson, W. R., and Hagedorn, D. J. (1969). A new seed-borne virus of peas, *Phytopathology* **59**, 1051-1052

Stevenson,W. R., and Hagedorn, D. J. (1971). Reaction of *Pisum sativum* to the pea seed-borne mosaic virus, *Plant Disease Reporter* **55**(5), 408-410

Stevenson, W. R., and Hagedorn, D. J. (1973). Antiserum preparation and serodiagnosis of pea seed-borne mosaic virus, *Phytopathology* **63**, 1467-1469

Stevenson, W. R., and Hagedorn, D. J. (1973). Further studies on seed transmission of pea seed-borne mosaic virus in *Pisum sativum*, *Plant Disease Reporter* **57**(3), 248-252

Stevenson, W.R., and Hagedorn, D. J. (1973). Partial purification of the pea seed-borne mosaic virus, *Phytopathology* **63**, 1346-1352

Thakur, V. S., Thakur, M. S., and Khurana, S. M. P. (1984). The pea seed-borne mosaic virus of pea in Himachal Pradesh, *Indian Journal of Plant Pathology* **2**, 156-160

Thottappilly, G. E., and Schmutter, H. (1968). Zur Kenntnis eines mechanisch Samen-, Pilz- and Insektenubertragbaren neuen Virus der Erbse, *Z. Pflanzenkr. Pflanzen pathol. Pflanzensch* **74**, 1-8

Timmerman, G. M., Calder, V.L., and Bolger, L. E. A. (1990). Nucleotide sequence of the coat protein gene of pea seed-borne mosaic potyvirus, *Journal of General Virology* **71**, 1869-1872

Van Dun, C. M. P., and Bol, J. F. (1988). Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus,*Virology* **167**, 649-652

Van Regenmortel, M. H. V. (1978). Applications of plant virus serology, *Annual Review of Phytopathology* **16**, 57-81

Van Regenmortel, M. H. V. (1982). *Serology and immunochemistry of plant viruses*, p. 302. Academic, New York

Yen, D. E., and Fry, P. R. (1956). The inheritance of immunity to pea mosaic virus, *Australian Journal of Agricultural Research* **7**, 272-280

Yin, F. H. (1977). Involvement of viral procapsid in the RNA synthesis and maturation of poliovirus, *Virology* **82**, 299-307

Wang, H. L., Gonsalves, D., Provvidenti, R., and Lecoq, H. L. (1991). Effectiveness of cross protection by a mild strain of zucchini yellow mosaic virus in cucumber, melon, and squash, *Plant Disease* **75**, 203-207

Wang, D., and Maule, A. J. (1992). Early embryo invasion as a determinant in pea of the seed transmission of pea seed-borne mosaic virus, *Journal of General Virology* **73**, 1615-1620

Wang, D., Woods, R. D., Swaby, A. G., and Cockbain, A. J. (1991). Detection of pea seed-borne mosaic virus virion and pinwheel inclusion body proteins in leaf and seed tissues of pea (*Pisum sativum* L.) by immunogold labelling, *Agronomie* 11, 387-394

Wang, D., Hayes, I. M., and Maule, A. J. (1992). Procedures for the efficient purification of pea seed-borne mosaic virus and its genomic RNA, *Journal of Virological Methods*. In press

Wang, D., Maule, A. J., Biddle, A. J., Woods, R. D., and Cockbain, A. J. (1992). The susceptibility of pea cultivars to pea seed-borne mosaic virus infection and virus seed transmission in the UK, *Plant Pathology*. In press

Zimmer, R. C. (1979). Influence of agar on immunodiffusion serology of pea seed-borne mosaic virus, *Plant Disease Reporter* **63**, 278-282

Zimmer, R. C., and Ali-Khan, S. T. (1976). New seed-borne virus of field peas, *Canadian Agriculture* **21**, 6-8