Studies on virus diseases of Passiflora

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SUMMARY

Three viruses were isolated from *Passiflora* spp. with virus-like symtoms in Sri Lanka. One virus, passion fruit virus 1 (PV1), was isolated from *Passiflora edulis cv. flavicarpe* with mottle symptoms, a second virus, passion fruit virus 2 (PV2), was isolated from *P. caerulea* with yellow flecking symptoms, a third virus passion fruit virus 3 (PV3), was isolated from *P. edulis cv. flavicarpa* with both mottle and chlorotic ringspot symptoms.

The host ranges of the three isolates were restricted to members of the families Passifloraceae, Leguminosae, Chenopodiaceae, Amaranthaceae and Solanaceae. The three isolates differed slightly in the symptoms expressed in some hosts, particularly Passifloraceae. The three isolates showed some differences in physical properties using sap extracts of *Passiflora foetida*.

Leaf 'squash' preparations of *P. foetida* infected with PV1, PV2 or PV3 showed flexuous rod-shaped particles with the range 770-860 nm. Ultrathin sections of *Passiflora* spp. infected with PV1 and PV2 isolates showed various inclusions including 'pinwheels', scrolls and laminated aggregates.

Each of the three isolates was purified by a method which included clarification with Triton X-100, concentration by polyethylene glycol precipitation and caesium sulphate density gradient centrifugation.

Antisera to the three virus isolates were obtained. In indirect enzyme immunoassays, the three isolates cross-reacted with antisera to several known potyviruses. In addition, PV1, PV2 and PV3 cross reacted with each others antiserum and serological differentiation indices for pairs of isolates, and cross-absorption tests, suggested that the three isolates were serologically closely related to each other.

Comparision of band patterns after analysis of infected tissues for double-stranded RNA showed that PV1 and PV3 produced one major species of dsRNA while PV2 produced two major bands. Purified preparations of the three isolates showed at least two molecular weight capsid protein species when electrophoresed in sodium dodecyl sulphate polyacrylamide gels.

Inoculation of a single lesion isolate of PV1 to *P. edulis cv. flavicarpa* and the reproduction of the mottle symptom observed on the original host, strongly indicated a causal relationship between the virus and the disease. The pathogenicity of PV3 in the same species was also confirmed, although the full symptom expression observed on the original host, was not reproduced.

The epidemiology of the passion fruit viruses was studied in passionfruit cultivations. All isolates were transmitted by aphids, in a non persistent manner with acquisition feeds of five minutes or less.

The clarification of PV1, PV2 and PV3 as members of the potyvirus group was discussed, as well as the possibility that these isolates should be considered closely related strains or 'pathotypes'. The implications of ecology and epidemiology in the spread and control of passion fruit viruses were also discussed.

ABBREVIATIONS

Standard abbreviations are not included here

A	=	absorbance (at stated wavelength)
cv.	=	cultivar
cv%	=	coefficient of variation
DEP	=	dilution end point
DNase	=	deoxyribonuclease
dsRNA	=	double-stranded RNA
E	=	extinction coefficient
ELISA	=	enzyme-linked immunosorbent assay
hr	=	hours
λ	=	Lambda
LIV	=	longevity *in vitro*
min	=	minutes
m.w.	=	molecular weight
µE	=	micro Einsteins
PBS	=	phosphate-buffered saline
PEG	=	polyethylene glycol
PVP	=	polyvinyl (poly) pyrrolidone
RNase	=	ribonuclease
SDI	=	serological differentiation index
SDS	=	sodium dodecyl sulphate
spp.	=	species
TIP	=	thermal inactivation point
u.v.	=	ultraviolet
v	=	volume
w	=	weight

CHAPTER 1 LITERATURE REVIEW

1.1 Cultivation of Passiflora in Sri Lanka

Passiflora species (family Passifloraceae) are native evergreen plants of the tropical areas of South America. Fruits produced by these plants vary in size from about 5 - 22 cm in length, according to the species. The fruits contain numerous seeds embedded in a quantity of fragrant, juicy pulp, which is sweet to taste and is often used in the production of fruit juices.

There are two definite cultivars grown in Sri Lanka, one producing yellow fruits *(Passiflora edulis cv. flavicarpa)* and the other purple fruits *(Passiflora edulis)*; besides those two there are a number of intermediate forms which have arisen from natural crosses. The yellow fruited cultivar grows best in the dry-zone, and the low country wet zone (see Fig. 29) while the purple fruited cultivar thrives at elevations over 1,200 metres.

At present, passion fruit is grown in Sri Lanka by smallholder farmers, who own approximately 1 - 25 hectares of land. It is also a very common home garden crop. Passion fruits are climbing plants and are grown on trellises up to two and a half metres high. Vines are trained along a stick, or a string up to the top wire. Then two lateral branches from the main stem are trained on either side and fruiting branches from those two laterals are allowed to grow downward. (Plate 1).

Plate 1: Passion fruit (Passiflora edulis cv.flavicarpa) cultivation in Sri Lanka.

Generally, seedlings are used for propagation, although it is also easy to propagate vegetatively, these however, can carry virus diseases. The minimum economic yield per hectare has been estimated to be some 4,000 - 5,000 Kg. of fruits. An economic yield can be obtained up to 3 - 4 years of age, but plants survive more than 7 years.

1.2 Virus diseases of *Passiflora* worldwide.

Studies on viruses affecting *Passiflora* spp. are of recent origin, although the first report of a virus-like disease in *Passiflora* was over 88 years ago (Cobb, 1901). In addition, virus diseases of passion vine have been recorded in Sumatra (Palm, 1922), England (Bewley, 1923) and Russia (Atanasoff, 1935).

In 1928, R. J. Noble published the first detailed report on 'woodiness' or 'bullet' disease of the passion vine *(P. edulis)* in New South Wales, Australia, which he showed was due to a sap transmissible virus. This disease was further described in 1938 (Noble and Noble, 1938) and shown to be transmissible by four spp. of aphids. Later, Magee (1948) stated the virus concerned to be cucumber virus 1 (syn. cucumber mosaic virus). Infected vines were stunted and fruits unmarketable. The woody character was observed in fruits at all stages of its development and was due to an abnormal thickening of the rind. The thickening also resulted in a small pulp cavity and

restricted the growth of the rind, so that affected fruits were often cracked. Fruits on diseased vines were marked with a series of fine dots or stipples and occasionally showed a range of ring-like patterns, in addition to developing a thickened rind. In some cases projections were present on the rind. McDonald (1937), reported a similar disease causing considerable damage in the plantations of South Africa.

In New Zealand, cucumber mosaic virus (CMV) isolated from *P. edulis* was most pronounced during the winter. Foliage symptoms were absent during summer and the disease could be recognized by fruit abnormalities (Chamberlain, 1954). On the other hand, a strain of CMV causing disease without woodiness symptoms in *Passiflora caerulea* was reported from U.S.A. (Teakle *et al.*, 1963) and Germany (Zschau, 1964).

At the same time, the viral agent reported by Ishii and Pascual (1964) from Hawaii could easily be transmitted by mechanical means from *P. edulis*. Virus-infected plants showed vein-clearing, spotting, chlorotic spots and malformation of the leaves of *P. edulis cv. flavicarpa*. In addition to the above symptoms, premature defoliation was also reported. However, fruit shape and yield were not affected, but the whole plant was stunted. No symptoms were observed on *P. edulis* in the field. At a temperature of 90°F, the infection rate was less than at lower temperatures. These authors suspected that the virus may be related to a strain of

CMV, on the basis of host range and general disease symptoms.

Hirata and Tominaga (1974) reported a CMV strain from passion fruit. According to their studies, sap inoculation of the virus into French bean *(Phaseolus vulgaris)* leaves, resulted in dark brown local lesions of one millimetre diameter without any systemic symptoms. Cross protection against cucumber mosaic virus (CMV)-Y strain on *Zinnia* leaves was 97 - 100%, and the virus was serologically related to CMV-O. Spherical particles 35 nm in diameter were seen in the electron microscope and the virus was concluded to be a strain of CMV, on the basis of biological and serological properties.

Another CMV strain from *P. edulis* was reported by Hollings *et al.* (1977) which was associated with yellow leaf blotches of *P. edulis*. Yonaha *et al.*, (1979) from Japan, isolated a strain of CMV from diseased *P. edulis* and *P. foetida* plants. The three CMV isolates were associated with chlorotic spotting and yellow mottle on passion fruit leaves, and the terminal shoot leaves were often curled and twisted. The isolates reacted to CMV-Y antiserum in agar double-diffusion tests.

Plese and Wrischer (1984), isolated CMV from the ornamental plant *Passiflora caerulea* in Yugoslavia. The host range and symptomatology of the isolated virus corresponded to those of CMV (Francki *et al.*, 1979). In agar double-diffusion tests, the crude sap of *Chenopodium quinoa*

reacted with serum against a strain of CMV from carnation, with the formation of straight precipitation lines characteristic of soluble virus protein.

Nattrass (1939, 1940) and Storey (1939, 1940) reported two types of passion fruit woodiness in Kenya. In the Trans Nzoia isolate, the fruit symptoms varied considerably. The fruit was often misshapen with cracking and splitting of the pericarp, and there was an uneven purple colouration of the fruit marked with a series of small green rings of mottling and mosaic. On seedlings, the first indication of infection was usually a sharp curling downwards and inwards of the youngest leaves, which later showed a distinct mottling, and further growth almost ceased. In contrast to the Trans Nzoia isolate, the Sotic isolate did not induce any symptoms on foliage, but the effect on the fruit was marked. This varied from distortion of the whole fruit, with one or more large swellings, to a reduction of the fruit in size, with a very small internal cavity. Mechanical transmision was not reported, and the transmission of the virus was by graft inoculation. Bakker (1974) suspected woodiness Trans Nzoia isolate was possibly due to an East African strain of CMV.

In Surinam, *P. edulis cv. flavicarpa* was affected by a spherical virus 30 nm diameter, which was mechanically transmissible to *Passiflora* spp. only (Maas and Rossel, 1971). The affected plants showed leaf malformation, had low fruit yield and sometimes died.

However, the virus associated with the disease was not identified.

Another spherical virus was isolated from *Passiflora allardii* (Horticultural cultivar), the red-flowered ornamental plants in Zimbabwe (Gopo and Cavill, 1982). Infected plants showed mosaic and some rugosity of leaves. The particle structure of the purified virus was isometric, about 18 - 20 nm in diameter. Preliminary results of the infectivity assay indicated the virus to be specific to *P. allardii*.

A virus particle of 24 nm diameter was consistently isolated from passion fruit plants in Brazil. Infected plants showed foliar mosaic, vein clearing and irregular chlorotic vein-banding, fruit deformation, hardening and poor fructification. The virus was not serologically related to CMV or transmitted by *Myzus persicae*. Sap transmission occurred to passion fruit and *Passiflora* species only (Chagas *et al.*, 1984).

Crestani *et al.* (1986) reported a virus disease in Brazil which infected *P. edulis f. flavicarpa* (golden passion fruit). Symptoms of yellow net, yellow mosaic and leaf crinkle were expressed. These symptoms were reproduced in about two weeks after graft or mechanical transmission to healthy seedlings. The virus was isometric, 30 nm diameter and belonged to the 'tymovirus' group. The virus was named passion fruit yellow mosaic virus.

Woodiness is an economically important disease of the passion fruit elsewhere, and several authors reported an elongated virus that like also produced woody symptoms. For example, McKnight (1953) and Taylor and Kimble (1964) studied the symptomatology of passion fruit woodiness virus - Queensland (PWV-Q), a mechanically transmissible virus from *P. edulis*, which expressed typical symptoms of mosaic, ringspot, rugosity and distortion of leaves of *P. edulis*. Infected fruit was often distorted and the pericarp hard and thick. The productivity and life span of the plants were found to be greatly decreased. Simmonds (1959) regarded the disease as the most important disease of *P. edulis* in sub-tropical Australia. The virus particles consisted of flexuous rods 730 - 745 nm in length. Teakle and Wildermuth (1967) confirmed that the virus belonged to the 'potyvirus' group.

Several strains of PWV were found, one was the cause of tip blight disease of *P. edulis* (Greber, 1966), another produces a mosaic of *Centrosema pubescens*, but was almost latent in *P. edulis* (Greber, 1971).

Van Velsen (1961) reported a virus associated with chlorotic spots of *P. foetida* in New Guinea. The virus, named passion fruit chlorotic spot (PCSV), was easily transmitted to *Passiflora* spp. and some other plants mechanically, but not to *P. edulis* nor to *P. suberosa*. The host range and symptom patterns were used as the critical criteria in identifying the virus and it

was evident that the host range of PCSV was similar to that of passion fruit woodiness virus (McKnight, 1953).

Passion fruit woodiness in South Africa was shown to be caused by a long flexuous virus 650 nm long (Graca, 1976). The particles resembled those of passion fruit woodiness virus (McKnight, 1953), although were slightly shorter. Bakker (1974), in Kenya, isolated a virus resembling the passion fruit woodiness virus from Queensland, Australia (McKnight, 1953), but different strains may be involved. At the same time, Peregrine (1975), reported a PWV-like disease in Brunei based on general disease symptoms.

A potyvirus was isolated by Chagas *et al.* (1981) from Brazil. Infected passion fruit plants were stunted with leaf mosaic and distortion. Fruits were hardened and malformed and the flowers were often aborted. Yield was also severely reduced. Elongated flexuous rod-shaped particles 700 - 750 nm long were seen in leaf dip preparations. Leaf extracts from infected bean plants reacted positively to antiserum of an Australian isolate of passion fruit woodiness virus.

In addition to CMV, Plese and Wrischer (1984) isolated from *P. caerulea* a 'potyvirus', which seemed to be an isolate of bean yellow mosaic virus (BYMV). Most of the cytological changes of protein crystals in nucleoli and the reaction of the test plants indicated

that it was different from ordinary (BYMV) isolates. Particle length of the virus varied from 400 - 750 nm.

A mechanically transmissible virus causing leaf mottling and ringspotting of *P.edulis cv. flavicarpa* in the Ivory Coast was described by De Wijs (1974a) It had particles of flexuous rods 810 - 830 nm long and 15 nm wide. The virus was serologically related but not identical to passion fruit woodiness virus from Queensland, and virus seemed to belong to the 'potyvirus' group.The virus was named as passion fruit ringspot virus (PRV).

A mosaic of passion fruit similar to one occurring in the Dominican Republic was discovered in the western coast of Puerto Rico (Escudero *et al.*, 1988). Typical potyvirus inclusions as well as flexuous particles were observed in epidermal leaf dips of infected plants when examined in electron microscope. Close serological relationships were found to exist between the Puerto Rican and Dominican passion fruit viruses. Heterologous relationship seems to occur between Puerto Rican passion fruit mosaic virus, dasheen mosaic virus (Fiji and Florida) and watermelon mosaic virus II.

Another elongated virus causing woodiness in Nigeria, passion fruit mosaic virus (PFMV) was described by Martini (1962). The virus did not infect any of the common hosts of CMV, and systemic infections were

obtained in *Passiflora* spp. only. Purified virus preparations contained elongated particles.

In the Philippines, Rosario *et al.* (1964) studied an elongated virus on wild passion vine (*Passiflora foetida* L.). It was mechanically transmissible to *P. foetida* and, with more difficulty, to *P. edulis,* and *C. amaranticolor* which showed local lesions.

On the basis of transmission studies, host range, physical properties and serology the virus which infected *P. cerulea* with mosaic symptoms in India was identified as tobacco mosaic virus (TMV) by Mali and Vyanjane (1980).

More recently, Fribourg *et al.* (1987), isolated a virus with particles typical of a tobamovirus from mosaic-diseased plants of *P. edulis* in Brazil. The virus induced systemic infection in *P. edulis* and *Nicotiana benthamina,* but local lesions were produced in 25 other species from nine different families. The virus had particles 304 nm long in infected cells and formed plate-like aggregates with a thickness equal to one particle length.

Passiflora latent virus (PLV) in *P. caerulea* was mechanically transmissible to *P. caerulea, P. suberosa, C. quinoa,* and *C. album. Chenopodium quinoa* was a local and systemic host which proved suitable for virus multiplication. PLV was serologically related to

potato virus S and M, carnation latent virus, Chrysanthemum virus B, Cactus virus 2 (Brandes and Wetter, 1963), and distantly related to red clover vein mosaic virus (Varma and Gibbs, 1967). *Passiflora* latent virus had rod-shaped particles with a normal length of 647 nm. (Schnepf and Brandes, 1961). In ultrathin sections of infected *C. quinoa*, accumulation of particles attached to the chloroplast, similar to those known for the potato virus S and M, were seen. However, there were no structures characteristic of infections belonging to the 'potyvirus' group.

In addition to typical PWV particles, bacilliform particles 250 x 69 nm were observed by Pares *et al.* (1983) associated with symptoms of passion fruit woodiness infection in New South Wales, Australia. The rhabdovirus-like particles were present in nuclei, perinuclear spaces and cytoplasm of mesophyll cells, in sections of infected leaves. A plant infected with rhabdovirus alone was not observed in their studies, and it was not clear, whether the virus was associated with symptoms similar to those of PWV, or was symptomless in passion fruit. Virus was neither sap transmissible nor vector transmissible.

In Brazil, beside the passionfuit woodiness caused by the potyvirus and cucumber mosaic virus, several other viruses such as tymovirus (Crestani *et al.*, 1986), rhabdovirus (Kitajima and Crestani, 1985) and tobamovirus (Fribourg *et al.*, 1987) have also

been found in passion fruits. In addition to the above, a witches broom disease associated with mycoplasma-like organisms was described by Kitajima *et al.* (1981). Diseases associated with mycoplasma-like organisms were recently confirmed by Kitajima *et al.* (1986) in a systematic survey of several passion fruit production centres in Brazil.

Several other authors reported viruses affecting *Passiflora*, although the casual organisms were not usually well characterized. For example, Wilson and Satyarajan (1970) from India, described a disease of *P. foetida*, which was transmissible by grafting. The symptoms of the disease in the field consisted of yellow dots on the leaves with some vein yellowing. At times a yellow mottling of the affected leaves was also noticed.

Holmes (1961), recorded a disease of *P. foetida* from the Philippines, which was characterized by yellowing, mottling and distortion of the leaves. The authors suspected the plant was one of the alternative weed hosts of the viroid causing Cadang-cadang disease of coconut. However, Calica (1961) could not obtain transmission of the agent to healthy plants by means of cleft grafting.

Protsenko and Tamrazyan (1977) isolated a virus with particles 700 nm long from *Passiflora hybrida* with mosaic symptom in Moscow, U.S.S.R.

The above review of the worldwide literature on viruses and virus-like diseases of *Passiflora* spp. shows

that many reports have appeared since the beginning of this century. Apart from some basic electron microscopy, serology or host range studies, however, little or no detail has emerged which has thrown light on the relationship of apparently similar viruses to each other, or to other viruses. It is possible, for example, that some of the flexuous rod-shaped particles described by different authors were strains of a single virus. Furthermore, a number of authors, while describing an apparently new virus disease of *Passiflora*, failed to satisfy some or all of Koch's postulates for proving pathogenicity.

1.3 Virus diseases of *Passiflora* in Sri Lanka

Five virus-like conditions of passion fruit have been described by Seneviratne and Wickramasingha (1972, 1973 and 1974) in Sri Lanka on the basis of symptoms, although few details were given. These disorders were tentatively designated as passion mottle, passion crumple and passion blotch.

Passion decline described by Seneviratne and Wickramasingha (1972) was characterized by retardation of growth and stunting. The leaves were dark green and leathery, reduced in size, deformed, twisted and puckered, with chlorosis and vein clearing. Fruit productivity was severely affected.

The characteristic symptom of passion mottle, also described by Seneviratne and Wickramasingha (1972),

was a light and dark mottle of leaves. Mottled areas were usually sunken. Growth did not appear to be severely affected, and economic crops could be obtained despite infection, with adequate management. With the assistance of Hollings of the Glasshouse Crop Research Institute, Littlehampton (now the Institute of Horticultural Research) in England, they reported that passion mottle virus had flexuous rod similar to the virus which caused the woodiness disease of passion fruit in Australia. However, the two viruses were apparently serologically unrelated. The search for mottle tolerant clones in Sri Lanka had not yielded encouraging results and Seneviratne et al. (1983) reported that a mild strain protected clone will perform well, if grown under conditions of good management with adequate irrigation, and if the weed hosts of the virus were controlled.

Passion mosaic described by Seneviratne and Wickramasingha (1972) was characterized by a bright yellow chlorosis of leaves with prominent areas of green and yellow. Growth and productivity of vines did not appear to be severely affected by this condition. Passion decline, mottle and mosaic conditions were all graft-transmissible while passion mottle was transmissible by sap and on pruning secateurs as well.

The most prominent symptoms of the crumple condition (Seneviratne and Wickramasingha, 1973) were observed on the foliage. Leaves rolled inward from the

margin and in the younger terminal leaves, the inward rolling of leaf lobes gave a crumpled appearance. Veins were thickened and enations were produced and stems were twisted, thickened and somewhat ribbed. The virus was transmitted by chip grafting.

Passion blotch was the second sap transmissible virus detected by Seneviratne and Wickramasingha (1974). The symptoms induced in adult vines were relatively inconspicuous. Most leaves showed no symptoms but some developed yellow blotches, yellow spots and vein clearing, and also rolling and distortion. Fruit symptoms were more distinctive. There were many circular or angular depressions caused by sinking of the external layers of the pericarp. It differed from passion mottle virus in not infecting *Cassia occidentalis* and in infecting *Nicotiana tabacum* and *Capsicum annuum* with the production of systemic symptoms.

Another virus-like condition was identified by Senanayake (1972) in various locations of the western province of Sri Lanka. Foliar symptoms of the disease were chlorotic spotting, severe crinkling with curling accompanied sometimes by deformity and, in severe cases, leaves dropped prematurely. The disease did not appear to affect the size or shape of the fruit even though fruits from diseased vines showed a mottle on the pericarp. The condition was transmitted to healthy seedlings by graft inoculation with chips and terminal shoots, obtained from diseased vines at Walpita.

Since 1982, studies have been made at the Regional Agricultural Research Station, Bombuwela, on the virus-like conditions in passion fruit prevailing in the low country wet zone area. Preliminary studies have shown that passion fruit selections collected from surveying many cultivations in the region had different rates of infection ranging from 0.46 - 0.74 within one year of infection with passion fruit mottle virus (Dassanayake *et al.*, 1986). Data for assessing losses are not available, however, because yield comparisons with virus-free material have not been done.

Many descriptions of virus-like diseases in *Passiflora* species in which a virus is thought to be involved are incomplete in Sri Lanka, and in all cases a causal relationship between a virus isolate and disease has not been shown. The objectives of the present study, therefore, were to investigate the virus and virus-like diseases associated with *Passiflora* species in the low country wet zone of Sri Lanka. Viruses isolated were to be characterized at the group and, if possible, at the strain level and specific antisera prepared to determine the relationship between them. The present research programme also involved a study period in Sri Lanka in which virus epidemiology and ecology were investigated in the low country wet zone area with the objective of finding methods to rationalise the control and spread of virus diseases in passion fruit fields. Attempts were also made to demonstrate a causal relationship between

single lesion cultures of the virus isolates and disease symptoms in *Passiflora*.

CHAPTER 2 MATERIALS AND METHODS

2.1 Sources of infected *Passiflora* material*

Passion fruit virus 1 (PV1) Homagama isolates A and B from *Passiflora edulis cv. flavicarpa*.	-	Adoptive Research Unit, Homagama, Gabadawatte, Sri Lanka.
Passion fruit virus 2 (PV2) from *Passiflora caerulea*	-	In Service Training Institute, Bombuwela, Sri Lanka.
Passion fruit virus 3 (PV3) from *P. edulis cv. flavicarpa*.	-	Pahana Estate, Kalutara, Sri Lanka.

* Held under MAFF Licence in UK.

2.2 Test Plants

2.2 a) Seed Sources

Amaranthaceae:	*Gomphrena globosa* L. (Globe amaranth)	Suttons Seeds Ltd., England.
	Beta vulgaris L. cv. Globe	Suttons Seeds Ltd., England.
Chenopodiaceae:	*Chenopodium album* L.	School of Biological Science, University of Bath.

	C. amaranticolor Coste and Reyn.	School of Biological Science, University of Bath.
	C. foetidum Schrad.	School of Biological Science, University of Bath.
	C. quinoa Willd.	School of Biological Science, University of Bath.
	Spinacea oleracea L. Spinach cv. Sigma leaf	Suttons Seeds Ltd., England.
Cucurbitaceae:	*Cucumus sativus* L. Cucumber cv. Parisienne Pickling	Sharpes and Co. PLC; England.
	C. sativus L. cv. LY 58	Seed certification centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
	C. sativus L. cv. Marketer	Booker Seeds, England.
	Momordica charantia L. cv. Mc 43	Seed Certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.

	Tricosanthes anguina L. cv. LA 33	Seed Certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
Leguminosae:	*Arachis hypogaea* L. ground nut - local cultivar	Thanamalwila Sri Lanka.
	Cassia occidentalis	In-Service training Institute, Bombuwela, Sri Lanka.
	C. tora	In-Service training Institute, Bombuwela, Sri Lanka.
	Centrosema pubescens Benth.	Private Nursery Piliyandala, Sri Lanka.
	Crotalaria usuramoensis Bak.	Private Nursery, Piliyandala, Sri Lanka.
	Glycine max L. Soya bean cv. Bossier	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.

G. max L. cv. PB - 1	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
G. max L. cv. PM 78 - 13	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
Phaseolus vulgaris L. dwarf french bean cv. The Prince	Suttons Seeds Ltd., England.
P. vulgaris L. runner bean cv. Prizewinner	Suttons Seeds Ltd., England.
P. vulgaris L. cv. Canadian Wonder	Suttons Seeds Ltd., England.
P. vulgaris L. cv. K.W.G	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.

P. vulgaris L. cv. Top crop	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
Pisum sativum L. Pea cv. Meteor	Suttons Seeds Ltd., England.
Pueraria phaseoloides	Private Nursery, Pilliandala, Sri Lanka.
Vicia faba L. broad bean cv. Aquadulce	Suttons Seeds Ltd., England.
Vigna unguiculata L. Cowpea cv. MI 35	Regional Agricultural Research Station, Maha Illuppallama, Sri Lanka.
V. unguiculata L. cv. Bombay	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
V. unguiculata L. cv. Ilta	Seed certification Centre, Dept. of Agriculture, Peradeniya,

		Sri Lanka.
	V. unguiculata L. cv. Bushita Mae	Seed certification Centre, Dept. of Agriculture, Peradeniya, Sri Lanka.
Passifloracea:	*Passiflora caerulea* L.	Private Nursery Bath, England.
	P. edulis Sims.	Regional Agricultural Research Station, Bandarawela, Sri Lanka.
	P. edulis Sims. cv. flavicarpa	Regional Agricultural Research Station, Bombuwela, Sri Lanka.
	P. ligularis Juss.	Sita Eliya, Sri Lanka.
	P. mollissima Bailey.	Sita Eliya, Sri Lanka.
	P. quadrangularis L.	Central Agricultural Research Station, Peradeniya, Sri Lanka.

	P. suberosa L.	Bombuwela, Sri Lanka.
	P. van volxemii Triana and Planch	Sita Eliya, Sri Lanka.
Solanaceae:	*Datura stramonium* L. cv. Arbotea	School of Biological Science, University of Bath.
	Lycopersicon esculentum Mill. cv. Delight	Suttons Seeds Ltd., England.
	Nicotiana clevelandii Gray.	School of Biological Science, University of Bath.
	N. debneyi Domin	School of Biological Science, University of Bath.
	N. megalosiphon Huerck and Muell	School of Biological Science, University of Bath.
	N. tabacum L. tobacco cv. White Burley (Judy's Pride)	School of Biological Science, University of Bath.
	N. tabacum L. cv. Xanthi	School of Biological Science, University of Bath.

Petunia hybrida Vilm. cv. Birthday Celebration	Suttons Seeds Ltd., England.

2.2 b) Culture of test plants

Seeds were sown in seed trays (21 x 15cm) in a seedling compost (Levington Universal Compost, Fisons PLC.). *Datura* seeds were scarified with sand-paper before sowing. Seeds of *Nicotiana clevelandii* were sown in Levington potting compost and lightly sprayed with 0.29 mM gibberellic acid (Sigma Chemical Co. Ltd.) to promote even germination. Seeds of *Passiflora* spp. were soaked for 24 hr in water before sowing.

Seed trays were kept in a glasshouse at a temperature of 18 - 25°C and 16 hr day length. Test plants were pricked out into 9 cm diameter Polypropylene pots (BEF Products, Essex Ltd.) containing Levington 'C2' potting compost, as soon as they were large enough to handle. Seedlings were watered daily to minimise water stress. Unless otherwise stated all plants, before and after inoculation, were maintained in a glasshouse with a temperature range in winter of about 15 - 25°C and in summer of 15 - 35°C. During winter, supplementary lighting was provided to obtain proper plant growth.

During the summer months the glasshouse was shaded with coolhouse whitewash (PBI - Ltd.) to reduce incident sunlight and reduce temperature. Mercury vapour

lamps 3,500 - 4,000 Lux were used to supplement illumination, unless otherwise stated.

Some experiments were done in the Central Agricultural Research Station, Peradeniya, Sri Lanka. Potting mixture was prepared by mixing 1 : 2 : 1 parts of sand; cattle manure; top soil. All seedlings were potted in 15 cm or 20 cm clay pots and kept in a glasshouse at a temperature about 25 - 30°C.

2.3 General hygiene and pest and disease control

In order to prevent the contamination of the glasshouse with unwanted viruses, precautions were taken as follows:

1. To reduce accidental aphid transmission of viruses, infected plants of each culture were maintained in separate glasshouses.

2. The gravel between and beneath glasshouse benching was hand weeded regularly to remove weeds which might have harboured plant viruses and/or their vectors.

3. The glasshouse was maintained as a non-smoking area to avoid transmission of tobacco mosaic virus from cigarette tobacco.

4. Handling of infected plants was avoided to prevent the accidental mechanical transmission of plant viruses.

5. Scalpels, secateurs and other tools were immersed in a solution of Decon 75 detergent (Decon Labs. Ltd.) between uses.

6. Polypropylene pots were soaked in 5% (v/v) formaldehyde solution for several days before being washed in detergent and rinsed in tap water.

The following insecticides were sprayed as a precaution to prevent establishment of pests in the glasshouse.

Pesticide	Rate	Pest
Decis - Contains 25 g/l (2.5% w/w) deltamethrin with xylene, ethyl and propyl benzenes. (A pyrethroid chemical Hoechst product.)	7ml/l	White fly
Gammalin 20 gamma BHC Liquid (ICI product.)	10ml/l	White fly
Nicotine 40% Shreds (40% w/w nicotine.)	225g/560 cubic metres	Aphids (green and black fly)
Pynoset 30 natural pyrethrins and resmethin (products of	120ml/50l	Aphids (green and black fly)

Mitchell Cotts Chemicals.)

	175ml/50l	White fly, Mites (red spider)

Cleaning of glassware, mortars and pestles etc.

1. Mortars and pestles were washed thoroughly and autoclaved for 20 min at 121°C and 15 Psi.

2. Glassware etc. - Soaked in Decon 75 then washed thoroughly and rinsed several times in tap water and two changes of distilled water. They were dried before use.

3. Spectrophotometer - cuvettes Rinsed in tap water then 85% alcohol and finally in distilled water.

2.4 Fertilizers

Organic fertilizers

1) Fisons Univesal seed - sowing compost sufficient for 6 weeks growth

2) Levington M2 potting - compost medium structure compost with low pH, designed for short term nursery

	stock on seed beds or where good water retention is required. Suitable for Spring and Summer potting.
3) Levington C2 potting - compost	for long term potted plants, coarse structure compost with standard pH

Inorganic fertilizers

Liquid fertilizer -	Fisons Liquinure N:P:K - 20:20:20 plus trace elements

2.5 Chemicals

Unless otherwise stated laboratory chemicals were obtained from Sigma Ltd. or British Drug House (BDH) and were Analar grade.

2.6 Measurement of pH

The pH of aqueous solutions were measured electrometrically using a PW 9420 digital pH meter (Philips). Before reading pH measurement, the meter was calibrated with standards of pH 4, 7, and 9.

2.7 Mechanical inoculation of test plants

Plants to be inoculated were placed in a dark box in the glasshouse for 16 - 24 hr before inoculation, to increase their susceptibility to virus infection (Bawden and Roberts, 1948).

The virus inoculum for sap transmission was obtained from systemically infected leaves with clear symptoms. Young leaves were sometimes used as sources of inoculum, since they often contain lower levels of tannins (Fulton, 1966). The inoculum was prepared by thorough homogenization of the tissue in a chilled mortar with a pestle in the presence of cold buffer. Leaf : buffer ratio was 1:5 or 1:10 w/v. Preliminary experiments were done with 0.05 M potassium phosphate buffer pH 7.8 (Yarwood and Fulton, 1967) containing 100 g/l Polyclar AT. Unless otherwise mentioned, 0.02 M tris-HCl buffer (Tris-hydroxymethylaminomethane) pH 7.8 containing 1g/l sodium sulphite (Na_2SO_3) was used for subsequent studies. The inoculum was used directly or was filtered through a muslin cloth to remove the large cellular debris. Celite abrasive (Hopkins and Williams) was added before inoculation (Kalmus and Kassanis, 1945) or occasionally plants to be inoculated were dusted with 400 mesh carborandum.

Inoculum was applied with a finger over the whole upper surface of the test leaf. When several inocula were involved, different fingers were used for

each inoculum. Hands were thoroughly scrubbed with liquid soap (liquid) and water, and dried after each inoculum. Inoculated plants were rinsed briefly with tap water to remove inhibitory deposits (Yarwood and Fulton, 1967) and were covered with damp newspaper for 16 - 18 hr to reduce mechanical damage. Inoculated plants were then grown in a glasshouse at a temperature of about 15 - 25°C during winter and at 15 - 35°C in summer. For a few experiments growth cabinets (Fisons or Saxcils) were used.

Plants from different spp. were inoculated at the following stages:

Chenopodium	spp.	-	4 - 8 leaf stage
Cucurbitaceae	spp.	-	cotyledon stage
Leguminosae	spp.	-	first pair of primary leaf stage
Passiflora	spp.	-	4 - 6 leaf stage
Solanaceae	spp.	-	2 - 4 leaf pair stage

2.8 Quantitative experiments

When lesion counts were made, leaves of *C. amaranticolor* were inoculated in Latin square design. When whole plants were used, completely randomised design was used with the same spp. Lesion numbers (x) were transformed to value y according to Kleczkowski (1968).

$y = \log_{10}(x + c)$ for $x > 10$ and

$$y = \log_{10} 1/2(x + c / (x^2 + 2xc)) \text{ for } 1.5 < x < 10$$

Results were tested for significance using analysis of variance. Duncans's Multiple Range test (Gomez and Gomez, 1976) was used to evaluate the signicant difference between treatment means. Any two means in each column having a common letter were not significantly different at the level of probability tested. Least significant difference (L.S.D.) values were used to compare treatment means in a few experiments.

2.9 Single lesion isolates

Each virus to be studied was established as a single-lesion isolate to eliminate mixtures of viruses and/or strains (Walkey, 1985). Well separated local lesions were excised with a sterile blade and ground in a watch glass with few drops of cold buffer. A small amount of celite was added and the homogenate inoculated on to young *C. amaranticolor* plants. The procedure was repeated 3-4 times using similar lesions each time. Finally each culture was bulked up in *C. quinoa*, and transferred to *Passiflora foetida* and *P. flavicarpa*. Pure cultures from *P. foetida* were used as the source of inoculum, unless otherwise stated.

2.10 *In vitro* properties

For the determination of *in vitro* properties sap samples were prepared from systemically-infected

leaves of *P. foetida*. Sap was extracted by grinding leaves in distilled water and filtering through two layers of muslin. Samples were treated as indicated below and after adding equal weights of celite, were assayed for infectivity.

2.10 a) Longevity *in vitro* (LIV)

The storage time after which sap containing virus loses its infectivity is the longevity *in vitro*.

Infected leaves of *P. foetida* were ground in a mortar with a pestle at 1:10 dilution (w/v) with distilled water. Sap was passed through muslin cloth and divided between several sterile test tubes which were sealed with aluminium foil. Samples were stored at room temperature, and individual aliquots assayed after increasing periods of storage time. Samples were assayed on *C. amaranticolor* plants.

2.10 b) Longevity in leaf tissues

Longevity in leaf tissue was done according to Rosario *et al.* (1964).

Virus-infected leaves were cut into small pieces and mixed thoroughly. Samples were then assayed at intervals, after being allowed to dry at room temperature for several days. The dried leaves were immersed in distilled water for a few minutes, then ground in a mortar with a pestle and the sap assayed on *C. amaranticolor*.

2.10 c) Thermal inactivation point (TEP)

The temperature at which the virus is inactivated after a 10 minute exposure is referred to as the thermal inactivation point (Noordam, 1973).

Virus-infected leaves were ground in distilled water at 1:10 w/v. The homogenate was passed through muslin cloth and samples were divided between a number of Eppendorf tubes (1.5 ml), equilibrated at different temperatures in Griffin-dri-blocks. These tubes were left for 10 min after which they were rapidly cooled on ice. The content of each tube was tested on *C. amaranticolor* plants in a Latin square design.

2.10 d) Dilution end point (DEP)

This is the dilution at which sap from infected plants fails to infect test plants when mechanically inoculated. The dilution end point is usually quoted as between two dilutions, the lower one of which is still infective and the next higher one, which is not.

Dilution end-point determinations were done according to Noordam (1973). To estimate the DEP, tenfold dilutions of sap were prepared in water and each was tested on *C. amaranticolor* leaves in a Latin square design.

2.11 Storage of virus isolates

2.11 a) Over calcium chloride

Fresh virus-infected leaves were collected and cut into small pieces. Granular calcium chloride was spread over the bottom of a 4 cm sterilin Petri dish, 1.5 cm in depth. The weight of the calcium chloride used was double that of the water in the tissue . The weight of water in passion fruit leaves, was estimated on the basis of 75% of the weight of the fresh tissue. A perforated support was placed over the calcium chloride and infected diseased samples (0.2 g) were spread evenly on the top of the screen. The edges of Petri dishes were sealed with parafilm.

2.11 b) Over silica gel

Fresh virus-infected leaves were chopped into small pieces and placed on cotton wool pads over 12 g silica gel (self indicating) in 25 ml Universal bottles. The screw caps were sealed with Parafilm, and the bottles were placed in a plastic bag containing more silica gel. The bag was sealed and kept in a deep freeze at -20°C and 4°C.

2.11 c) In polythene bags

Infected leaves were cut into small pieces and stored in sealed polythene bags at -20°C and 4°C.

Infectivity tests were made after different periods of storage, by assay on *C. amaranticolor* plants in a Latin square design.

2.12 Centrifugation

Low speed centrifugation was performed at 4°C, in an MSE High Speed 18 refrigerated centrifuge (Fisons PLC.) using the 6 x 250 ml or 8 x 50 ml aluminium fixed-angle rotors. An MSE microcentrifuge was used for centrifugation of small samples (< 1.5 ml) in Eppendorf tubes.

High speed centrifugation was carried out at 4°C in an MSE Prepspin 75 ultracentrifuge using 8 x 25 ml fixed angle rotor.

All figures quoted for centrifugal force are the maximum ralative centrifugal force in g, calculated from nomograms supplied by the rotor manufacturers.

2.13 Spectrophotometry

The ultraviolet absorption spectra of partially-purified and purified virus preparations were determined using a Shimadzu U.V. - visible recording spectrophotometer. Matched-pair 3 ml and 1.5 ml semi-micro quartz cuvettes were used.

2.14 Dialysis

Samples were dialysed in 8/32 inch Visking tubing (Medical International Ltd.), which had beeen

washed before use by boiling for 10 min in each of 2 changes of 0.001 M EDTA. Dialysis was carried out at 4°C unless otherwise stated.

2.15 Density gradient centrifugation

To obtain highly purified virus for production of antisera and to study the other physical properties, partially-purified virus was centrifuged in a caesium sulphate gradient. The virus suspension was adjusted to 23 ml with BK buffer (0.1 M boric acid, 0.1 M KCl, pH 8.0), and 7.5g of caesium sulphate was then added to a median density approximately 1.27 g cm^{-3}). A density gradient was formed by centrifugation at 139,000 x g for 16 - 20 hr at 10°C. The virus band was removed manually by sterile syringe. The method is based on Hammond and Lawson (1988).

2.16 Light microscopy

An Olympus light microscope (Olympus Optical Co. Ltd., Japan) and stereo microscope (SWIFT Instrument International S.A.) were used for light microscopic investigations. Magnification of 1000 x was used to scan for inclusions.

2.16 a) Light microscopy of inclusion bodies

Tissues were stained according to Christie and Edwardson (1977), for the detection of inclusion bodies .

2.16 a) (i) Preparation of the Azure A staining solutions

The stock solution of Azure A was prepared by dissolving 0.1g of dye in 100 ml of 2 - methoxyethanol (Aldrich Ltd.) . A stock solution of 0.2 M Na_2HPO_4 (dibasic sodium phosphate) was prepared and stored at 4°C until used. The Azure A staining solution was prepared by mixing 18 drops of Azure A stock solution with 2 drops of the dibasic phosphate solution.

2.16 a) (ii) Preparation of the O - G staining solution

The stock solutions of Calcomine Orange 2RS and Luxol brilliant green BL were prepared by dissolving 0.25 g of dye in 25 ml of 2 - methoxyethanol. The solution was then stirred thoroughly and filtered through coarse filter paper. Solutions of green and orange dyes were prepared and stored separately . The staining mixture was prepared by mixing one part water, three parts orange stock solution and six parts green stock solution in a small watch glass. Water content was maintained at approximately 10% of the total volume.

2.16 b) Staining epidermal strips

Tissues were stained for about 5 - 10 min and staining solution was then removed. The tissues were then washed in several changes of 95% ethanol, with about 5 - 10 sec in each change until excess dye was removed. Ethanol was removed and a small amount of 2 - methoxyethyl acetate (Aldrich Ltd.) was then added.

After 5 min, tissues were removed and mounted in a drop of Euparal medium on a glass slide. The staining procedure was the same for both O - G and Azure A stains.

2.16 c) Thick tissue pieces

Stem pieces were cleared of chlorophyll and other pigments by soaking for approximatley 30 min in 2 - methoxyethanol, and then stained 10 - 15 min with O - G and Azure A stains.

2.17 Electron microscopy

Specimens were viewed in a JEOL 100 CX, JEOL 1200 EX II, or a 2000 FX transmission electron microscope and scanning microscope (JEOL - JSM T330). Unless otherwise mentioned, crude, partially-purified, or purified virus samples were examined after negative staining with 20 g/l phosphotungstate (PTA) acid (Taab Labs) adjusted to pH 6.5 with sodium hydroxide. Preliminary studies were done with either PTA or 20 g/l uranyl acetate.

2.17 a) Leaf squash method

Walkey and Webb (1968) introduced the 'squash homogenate' technique as a useful sources of particles. Virus infected leaves with clear symptoms were squashed on a glass slide with a drop of PTA. The crushed material was further pressed with another glass-slide, to extract the sap. A fine Pasteur pipette was used to draw up a small amount of the sap mixture. One drop of the

sap was placed on the clamped, carbon or formvar - coated copper grids of 3.05 mm diameter and 200 mesh (Agar Aids). Excess liquid was carefully removed from the grid using a small piece of filter paper. After 1 - 2 min drying, the samples were examined in the electron microscope.

2.17 b) Purified samples

Partially - purified and purified virus samples were diluted 1 : 10 or 1 : 50 v/v in negative stain. A drop of the diluted solution was then placed on either carbon or formvar coated grids and the excess fluid carefully removed using filter paper and the grid air-dried. Pre-treatment of grids with spreading agent such as 0.1 g/l bacitracin (Sigma Ltd.) did not improve the distribution of virus particles.

2.17 c) Thin sections

Small pieces of infected *P. foetida* and *P. flavicarpa* leaf tissues including one veinlet were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for 4 hr at 4°C, and post-fixed in 1% osmium tetroxide in the same buffer for 2 hr at 4°C . After dehydration with an acetone series, they were embedded in epoxy resin. Thin sections were cut with a diamond knife in a ultramicrotome (Omuz Reichert, Austria). They were double stained with 1% uranyl acetate and citrate, before observation.

2.17 d) Measurements of virus particles

The magnification of the microscope was calibrated with polystrene latex spheres . To calculate the mean value, at least 25 spheres were measured. All of 25 polystyrene latex spheres (234 nm diameter) measured were 4.7 - 5.1 mm diameter with a mean diameter of 4.9 mm ± .004.

2.18 Serology

2.18 a) Preparation of antisera

Antisera to several viruses were prepared in rabbits (Sandy half lop) with purified virus preparations. An initial injection was given into a marginal ear vein, (0.5 ml virus + 0.5 ml 85 g/l NaCl). One week later four to six intramuscular injections of an equal volume mixture of purified virus and Freund's incomplete adjuvant (Difco Labs) were given at weekly intervals. Each injection contained 0.2 - 1.0 mg virus.

Test bleeds of 5 ml were taken from a ear vein one week after the first injection. Serum was collected after the blood had been allowed to coagulate at room temperature, and then stored frozen at -20°C. Antiserum titres were determined against homologous antigen in microprecipitin tests. For some antisera, titres were monitored weekly over several weeks.

2.18 b) Double diffusion test

Double diffusion tests (Van Regenmortel, 1966) were made in 8.5 g/l Ionagar No. 2 (Oxoid Ltd.) containing 8.5 g/l sodium chloride and 1 g/l sodium azide in distilled water. The mixture was heated to melt the agar and the gels prepared on microscope slides using a Gelman immunodiffusion apparatus (Gelman Instrument Company U.S.A.). Glass slides were sealed using a small quantity of molten agar and agar allowed to solidify at room temperature. An appropriate amount (12 - 15 ml) of agar was then pipetted on to glass slides. Agar was allowed to solidify for 45 min and wells were cut in the gel according to a desired pattern. Gel plugs were removed and antiserum and antigens were added to the wells using glass micropipettes and the slides incubated in a moist chamber at room temperature for 48hr, and then at 4°C.

Crude sap antigens from infected plants and healthy plants were prepared by grinding leaves with a mortar and pestle with a drop of 0.2 M tris-HCl pH 7.8 buffer. Homogenate was centrifuged for 15 min at about 12,000 g in a microcentrifuge. Supernatants were used for immunodiffusion tests. Test antigens were placed in the outside wells and antiserum was placed in the central well. Partially purified virus was also used as antigen source. Plates were observed for precipitates daily and kept at 4°C for up to 1 week.

2.18 c) Microprecipitin test

As the virus isolates from *Passiflora* did not readily diffuse through agar gels, microprecipitin tests (Van Slogteren, 1955) were used to determine antiserum titres.

Using a micropipette, 1 µl of purified samples were titrated with antiserum on a premarked grid pattern drawn on graph papers, and mixed thoroughly using a cocktail stick. Then liquid paraffin was slowly poured into the Petri dish to cover the drops. Purified preparations from healthy plants and normal serum were used as controls. The dish was incubated at room temperature for 4 hr and examined for precipitation reactions using a stereo microscope with dark ground illumination. Further reading was taken after incubating the dish overnight at 4°C.

2.18 d) Enzyme - linked immunosorbed assay (ELISA)

Double antibody sandwich, enzyme-linked immunosorbent assay (DAS - ELISA) was done as described by Clark and Adams (1977), and indirect ELISA according to Mowat and Dawson (1987).

The following buffers were used

Phosphate - buffered saline (PBS) - 0.01 M phosphate (Na_2HPO_4 / KH_2PO_4) containing 0.15 M sodium chloride and 0.0003 M potassium chloride, pH 7.4; PBS with 0.5 ml/l Tween 20 (Sigma Ltd.); coating buffer - 0.05 M sodium

carbonate pH 9.6; conjugate buffer - PBS - Tween containing 20 g/l PVP and 2 g/l ovalbumin (Sigma Grade V); substrate buffer - 97 ml/l diethanolamine adjusted to pH 9.8 with M hydrochloric acid. All buffers contained 0.2 g/l sodium azide as preservative.

Polystyrene microtitre plates were obtained from Alpha Laboratories (Type 2). The enzyme was alkaline phosphatase (Type VII Sigma) and the substrate was p-nitrophenyl phosphate (Sigma) used at 1 mg/ml in substrate buffer. Hydrolysed enzyme substrate was determined by measuring the extinction at 410 nm using a Titertek Multiscan Photometer (Flow Laboratories Ltd.).

Gamma - globulin was prepared from 2ml of antiserum diluted to 8ml with distilled water and 8ml of saturated ammonium sulphate. After standing 1 hr at room temperature the precipitate was collected by centrifugation and dissolved in 10 ml of distilled water. Eight ml of saturated ammonium sulphate was added again, and the precipitate collected as before. The precipitate was dissolved in 1/2 strength PBS and then dialysed against half strength PBS, at least 3 times. The gamma - globulin was further purified by passage through a column of DE 22 cellulose (Whatman Ltd.) and the unabsorbed fraction collected. The gamma - globulin was adjusted to approximately 1 mg/ml and stored frozen in 1 ml portions.

For the conjugation of alkaline phosphatase with gamma-globulin, 5000 units of alkaline phosphatase

were dissolved in 2 ml of gamma-globulin preparation and dialysed extensively against PBS at 4°C. Then 50 µl of 10% glutaraldehyde (Sigma high purity) was added and the mixture incubated 4 hr at room temperature. Glutaraldehyde was then removed by dialysis against several changes of PBS and the conjugate stored with approximately 10 g/l bovine serum albumin (crystallised and lyophilised, Sigma Ltd.) at -20°C.

Direct ELISA

For the direct ELISA, microplates were coated with 200 µl of gamma - globulin at a concentration of 1 µg/ml. The plates were incubated at 37°C for 3 hr in a moist chamber and then washed three times by flooding with PBS - Tween.

The antigens were prepared by grinding tissues in PBS - TPO buffer and filtering through muslin. Healthy sap was used as a control. Two hundred microlitres of virus suspension were diluted in PBS plus 20 g/l PVP (40,000 m.w.), and after adding to the plates were incubated at 4°C for 16 - 18 hr. An initial wash was done immediately, to remove suspended solids and then the plate was given three standard washes before blotting dry. Two hundred microlitres of gamma-globulin alkaline phosphatase conjugate at an appropriate concentration was added to the wells and the plates were incubated at 37°C for 2 hr. Then the plates were again washed and dried before adding 200 µl of enzyme substrate (1 mg/ml p-

nitrophenyl phosphate in 10% diethanolamine). Incubation was done at room temperature.

Indirect ELISA

For indirect ELISA the same buffers, reagents and washing procedures were used as in the direct method. In indirect ELISA on plates not precoated with antibodies the plates were incubated successively with

(i) antigen diluted in coating buffer for 18hr at 4°C

(ii) absorbed or unabsorbed antiserum appropriately diluted (depending on test) in PBS - TPO buffer 2hr at 37°C.

(iii) protein A - alkaline phosphatase conjugate (0.5 mg in 30 ml PBS - TPO / 50% glycerol) diluted 1/2500 - 1/5000 in PBS - TPO, for 2 hr at 37°C

(iv) Substrate (p-nitrophenyl phosphate) solution for 4 hr at room temperature or 18 hr at 4°C.

Hydrolysed enzyme substrate was determined by measuring its absorbance at 410 nm with a plate reader. Readings were taken after the plate had been blanked against either substrate or conjugate buffer-filled wells. Mean absorbance values of A 410 nm greater than twice those of healthy controls were regarded as positive

for antigen extracted from crude sap (Sutula *et al.*, 1986). On the other hand, using antigens obtained from purified preparations, values of $\hat{A}$ 410 nm greater than 0.1 were regarded as positive (after Koenig, 1981).

2.18 e) Immunosorbent electron microscopy

Purified virus preparations were adsorbed on to carbon-coated grids. After a few seconds, the grid held in forceps was rinsed with 20 drops of 0.1 M phosphate buffer pH 7 and drained. A diluted antiserum in phosphate buffer was added and the grid was incubated in a moist chamber for 15 min at room temperature. After incubation, the grid was rinsed with 20 drops of phosphate buffer, 30 drops of distilled water and 5 drops of sodium phosphotungstate pH 6.5 and was then drained and dried before examination in the electron microscope. Control grids adsorbed with different viruses were also prepared.

2.18 f) Sources of antisera

Bean common mosaic virus (BCMV)	Dr. A. A. Brunt, Institute of Horticultural Research, Littlehampton, England.
Passion fruit woodiness virus (PWV)	Dr. A. B. Martin, Biological and Chemical Research Institute, New South Wales, Australia.

Passion fruit woodiness strain of cucumber mosaic virus (CMV-P)	Dr. A. B. Martin, Biological and Chemical Research Institute, New South Wales, Australia.
Passion fruit ringspot virus (PRV)	Dr. J. C. Thouvenel, Institut International de Recherche Scientifique pour le Developpement a Adiopodoume Laboratoire de Phytovirologie, Adiopodoume, Côte D'Ivoire.
Potato virus Y	Dr. R. G. T. Hicks, School of Biological Science, University of Bath, England.
Watermelon mosaic virus	Dr. A. A. Brunt, Institute of Horticultural Research, Littlehampton, England.

2.19 Extraction and analysis of double-stranded RNA (dsRNA).

The isolation of double-stranded RNA (dsRNA) from virus infected plant tissues provides a new approach to virus detection and identification (Morris and Dodds, 1979) . Double-stranded RNA (>0.1 x 10^6 daltons) has been detected in large amounts only in plants infected with RNA viruses, so that the method provides a tool for the detection of a suspected but unconfirmed disease of viral origin without the need to purify virus particles.

Disease tissue was phenol extracted to isolate cellular nucleic acids, and viral dsRNA was selectively purified from other nucleic acid by binding to cellulose powder in 15 - 17% ethanol in small columns (Franklin, 1966). The product is analyzed by gel electrophoresis and dsRNA identified by RNase treatment.

The isolation and properties of virus-specific dsRNA from tissue infected with RNA viruses have been well documented. For example, Dodds and Bar-Joseph (1983) extracted dsRNA from plants infected with closteroviruses and Jordan *et al.* (1983) used double-stranded RNA as an indicator for the presence of viruses in Avocado plants.

Experiments were carried out to extract the dsRNA from virus-infected *Passiflora* spp.

Extraction and purification of double-stranded RNA was based on the method of Morris and Dodds (1979).

The following reagents were used

(i) 2 STE buffer (1 x STE = 0.1 M NaCl, 0.05 M Tris, 0.0001 M EDTA) containing 20 g/1 PVP. plus 0.1% 2-mercaptoethanol.

(ii) Water-saturated phenol containing 1 g/1 hydroxyquinoline

(iii) Chloroform : Pentanol 25:1 (v/v)

(iv) 100 g/l SDS

Young leaves with clear virus symptoms were ground to a fine powder in liquid nitrogen in a chilled mortar with a pestle. This powder was extracted with 14 ml of double strength STE buffer, 2 ml of 100 g/l sodium dodecyl sulphate, 20 ml of water - saturated phenol, 7 ml of chloroform : pentanol (25:1) and 16 mg of bentonite. The extract was shaken for 30 min at room temperature. The emulsion was broken by centrifugation (10,000 x g) for 20 - 30 min and the aqueous phase adjusted to 17% ethanol v/v. The dsRNA was purified by one cycle of CF 11 cellulose chromatography on a small column. The column was washed with 80 ml of 83% STE buffer v/v, 17% ethanol v/v and eluting the column with 15 ml of single strength STE buffer to remove the dsRNA. The final samples were adjusted to 66% ethanol v/v and stored at -20°C. Samples were centrifuged at 3,000 x g for 30 mins. and prepared for electrophoresis by resuspending pellets in 100 µl of electrophoresis buffer (0.04 M Tris-HCl, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.8) and 5µl of glycerol. Electrophoresis was on a cylindrical 5% v/v polyacrylamide gel (7.5 cm x 6 mm) in electrophoresis buffer for 16 hr at 2 mA / tube or 8 hr at 6 mA / tube.

The λDNA cut with Hind III (Boehringer Corporation Ltd.) restriction enzyme was used as a molecular weight marker. Gels were stained in 10 - 50 ng/ml of ethidium bromide for 1 hr at room temperature, and destained in several changes of distilled water for 8 - 10 hr. The nature of

the fluorescent band was identified by poststaining digestion of the gel with ribonuclease 50 µg/ml (ribonuclease A from Bovine pancreas - Sigma Ltd.) in water ('low' salt) or 0.3 M sodium chloride ('high' salt) and DNase (deoxyribonuclease, from beef pancreas (Sigma Ltd.) 10 µg/ml in the presence of 5 mM $MgCl_2$.

2.20 Estimation of capsid protein molecular weight

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) has proven to be a useful tool for the separation and identification of polypeptide chains (Maizel, 1966 ; Shapiro *et al* ., 1966; Vinuela *et al*., 1967). These authors also reported that this technique could be used for the rapid and simple estimation of molecular weights of proteins and their subunits. The polyacrylamide electrophoresis was done according to the method described by Weber and Osborn (1969).

The marker proteins and purified virus preparations were heated in a water bath at 100°C for 5 min in 0.01 M sodium phosphate buffer pH 7, 10 g/l SDS and 1% 2-mercaptoethanol.

The following marker proteins were used

Ribonuclease (m.w. 13,700 daltons), Ovalbumin (m.w. 43,000 daltons), Bovine serum albumin (m.w. 66,000 daltons) and Chymotrypsinogen (m.w. 25,000 daltons). All marker proteins were used at 0.4 mg/ml.

The gel buffer contained 7.8 g $NaH_2PO_4.H_2O$, 38.6 g of $Na_2HPO_4.7H_2O$ and 2 g/l SDS. For the 10% acrylamide solution, 22.2 g of acrylamide and 0.6 g of methylene bisacrylamide were dissolved in water to give 100 ml solution. Insoluble material was removed by filtration through Whatman No. 1 filter paper.

The glass gel tubes were 7.5 cm long, with inner diameter of 5 mm. Fifteen millilitres of gel buffer was mixed with 13.5 ml acrylamide solution. Then 1.5 ml of freshly made ammonium persulphate solution (15 mg/ml) and 45 ml of N,N,N,N, tetramethylethylenediamine were added. After mixing, each tube was filled with this solution. Before the gel hardened a few drops of water were carefully layered on top of the gel solution.

For each gel, 3 µl of tracking dye (bromophenol blue in water, 1 drop of glycerol, 30 µl of buffer (0.01 M sodium phosphate buffer pH 7, 10 g/l SDS and 1% 2-mercaptoethanol) and 30 µl of protein solution were added.

The electrophoresis apparatus was filled with electrophoresis buffer (gel buffer diluted 1:1 v/v in water) and equilibration before the application of sample was achieved by passing a current of 2 mA / tube for 30 min. Electrophoresis was stopped when the marker dye had moved three quarters down the gel. After electrophoresis, the gels were removed from the tubes and

the length of the gel and the distance moved by the dye were measured.

The staining solution was prepared by dissolving 1.25 g of Coomassie blue in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid and removing insoluble materials by filtration through Whatman No 1 filter paper. The gels were placed separately in plastic Petri dishes with staining solution for about 8 hr.

The gels were removed from the staining solution, rinsed with distilled water, and placed in destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of water) for 12 hr. The length of the gels after destaining and the positions of the blue protein zones were recorded.

The mobility of protein was calulated according to the following formula

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye}}$$

The mobilities were plotted against migration of the known molecular weight markers expressed on a semi- logarithmic scale. Molecular weight estimations were repeated at least three times.

2.21 *In vitro* culture of *Passiflora* tissues

Virus infected passion fruit stems, cut into segments (2 cm), were surface sterilized in 5% sodium hypochlorite (20 g/l w/v available chlorine; Fisons PLC.) containing a few drops of Tween 20 for 15 min. Stem pieces were rinsed 3 times in sterile distilled water, 10 min for each rinse, and transferred to sterile medium using flamed forceps. Preparations and subsequent transfers were conducted in a laminar flow cabinet (Centronic Europe Ltd.).

The pre-mixed medium (Flow Laboratories Ltd.) consisted of Murashige and Skoog (1962) salts, minor elements and organic constituents except for sucrose and growth regulators (see Appendix 1). To this, 20 g/l sucrose and 0.05 µM 6 benzyl amino-purine (BAP, Sigma Ltd.) were added and the pH was adjusted to 6 with M potassium hydroxide. Agar (Lab m M. C. 2) was added to 6 g/l and the volume made up to 1 litre. The medium was heated to dissolve the agar, and screw top jars were filled with (30 ml / jar) culture medium. These jars were autoclaved at 121°C for 15 min.

The lids of the jars were removed and the mouth of tube, flamed by holding over a burner flame for a few seconds. The stem pieces with axillary buds were placed on the surface of the culture media, and the mouth of the jar was once again flamed. Immediately the jar was sealed with a lid. The cultures were grown at 25°C in a

Baird and Tatlock cooled incubator, illuminated with 20 w fluorescent tubes, giving a flux density of 30 $\mu E/m^2.s$ and 16 hr photoperiod.

2.22 Epidemiological studies

2.22 a) Trapping methods for aphids

Yellow water bowl traps (Moericke, 1951)

The yellow round traps were 30 cm in diameter and 15 cm in depth. A circle is the most logical shape to use because it represents the same shape and size in all directions. A gauze covered outlet 1.5 cm in diameter was made in the side of the bowl to prevent aphids being washed out by heavy rains. Traps were half-filled with clean water and a small amount of Cu_2SO_4 was added to each basin to prevent the fungal decay of the insects. In addition, a small amount of detergent was also added to the basin to improve trapping of aphids.

Sticky traps

Two types of sticky traps were used.

(i) Vertical cylindrical traps (Broadbent *et al.*, 1948)

Yellow colour painted, thick 'Eslone pipe' (Anton, PVC Ltd.) 30 cm length and 10 cm in diameter.

ii) Horizontal sticky traps (Heathcote, 1955 ; Broadbent, 1957)

These were 30 x 30 cm galvanised plates painted in yellow colour.

Both types of sticky traps had the same trapping area. The trapping surfaces of these were covered with cellophane sheets coated with transparent grease.

2.22 b) Aphid identification

Aphid maceration was done according to Blackman and Eastop (1984) before identification.

Maceration to remove the soft tissues of the specimens prior to mounting was done with the specimen tubes in a water bath kept near boiling point. The following stages were involved.

Specimens were gently boiled in 95% alcohol for 2 min. Then the alcohol was pipetted off and boiled with 100 g/l potassium hydroxide for 3-5 min. The KOH was pipetted off and the specimens were washed with distilled water three times at least 10 min for each wash. After this, distilled water was removed and glacial acetic acid was filled up to 1 cm depth and left for 3 min. This step was repeated 3 times with fresh glacial acetic acid. The specimens were then cleared using clove oil and left for 20 min. One to two aphids were transferred to a drop of

D.P.X. mountant on a clean microscope-slide and the appendages rapidly re-arranged. Finally specimens were carefully covered with a coverslip and dried at room temperature before examination under the microscope.

The following keys were used to identify the aphids (Blackman and Eastop 1984):

1. Key to polyphagous Aphis species (apterous viviparae only)

(a) dorsal abdomen with an extensive black patch centred on tergiles 4 - 5. Cauda black, tongue shaped, rather pointed and with 4 -7 hairs.

(*Aphis craccivora*)

(b) dorsal abdomen with preserved specimens unpigmented or with scattered black markings. Cauda pale or dark, but if black it usually bears more than 7 hairs.

Cauda pale, sometimes dusky in large specimens but then clearly much paler than siphunculi

siphuncule uniform dark. Hairs on hind femur all shorter than diameter of femur at it's base

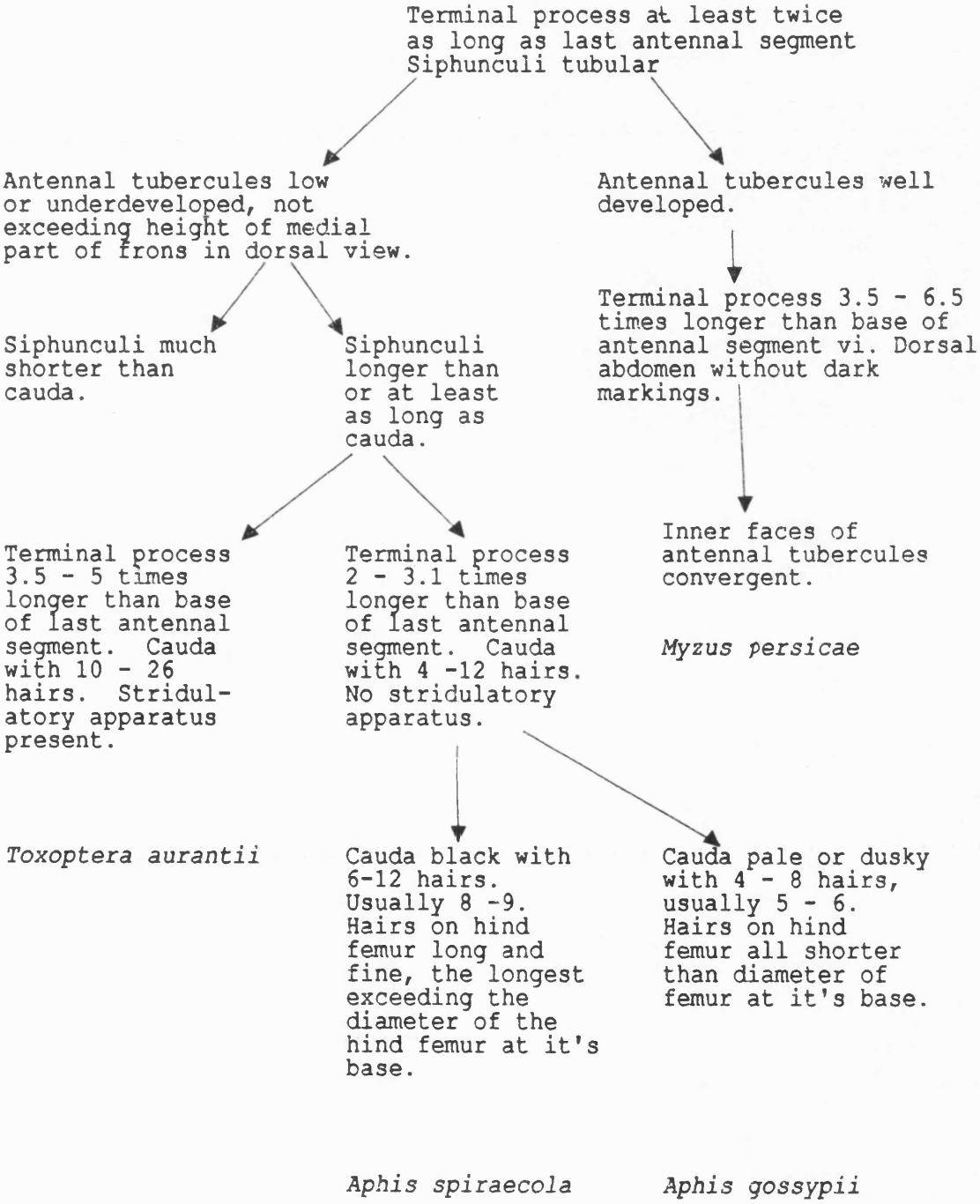
(*Aphis gossypii*)

Cauda dark like siphunculi

dorsal abdomen without any dark markings

(*Aphis spiraecola*)

2. Key to aphids polyphagous on tropical trees and shrubs (apterous vivaparae only).



2.22 c) Aphid transmission test

Transmission experiments were based on Noordam (1973).

Aphids were collected from different plant spp. They were reared on their natural host or chinese cabbage (*Myzus persicae*) under laboratory conditions and the first generation off-spring, presumed to be virus free (Sylvester, 1969; Miyamoto and Miyamoto, 1966) were used for transmission studies.

Aphids starved for 1 - 2 hr were allowed to feed on portions of infected leaves for a period of 5 min and 30 min. Fifteen to twenty aphids were immediately transferred and allowed to feed on each healthy passion fruit seedlings for another 5 or 30 min. Plants were assayed for infection by back inoculation of sap extracts to *C. amaranticolor* and observing plants for symptom development.

2.22 d) Graft transmission

To see whether the viruses from *Passiflora* could be transmitted by grafting, wedge grafting was done using 2 - 3 month old passion fruit seedlings as rootstocks. Scions for grafting were collected from diseased *P. flavicarpa* seedlings. The rootstocks were cut when the girth of the stock was about the same as that of the scion. A 1 - 1.5 cm split was made through the wood on the rootstock. Scions of 6 - 8 cm long were selected

from diseased plants and their basal ends were shaped in the form of a wedge. The cut end of the scion was then inserted through the split made in the rootstock. The union was wrapped well with parafilm and the grafted plants covered with an opaque polythene bag. The polythene bag around the grafted parts was removed 2 weeks after grafting.

CHAPTER 3 *PASSIFLORA* VIRUSES

3.1 Isolation and identification of passion fruit virus 1 (PV1)

3.1.1 Field symptoms

The foliar symptoms of the original passion fruit sources of PV1 type isolates were yellow and green mottle, leaf crinkling and slight leaf deformation. Initially young leaves showed chlorotic spotting but leaf mottling was the most reliable symptom, being present all the year round in infected plants of *P. edulis cv. flavicarpa* (Plate 2.). Growth and vigour was affected and fruit production also reduced, compared to apparently healthy plants. Diseased fruits remained small in size with or without the green and yellow mottle like symptoms on the rind. The plants from Homagama-A showed milder symptoms in the field than Homagama-B. In this location, infected plants were about two years old.

3.1.2 Isolation from *Passiflora edulis cv. flavicarpa*

Virus isolates were transmitted from young leaves with symptoms of *P. edulis cv. flavicarpa*, ground in 1:10 (w/v) 0.05 M phosphate buffer pH 7.8 containing 100 g/l Polyclar AT (insoluble PVP), to a range of test plants including *Chenopodium* spp., legume spp. and *Passiflora* spp. *Chenopodium amaranticolor* induced discrete local lesions, 10 - 15 days after inoculation

Plate 2: Foliar symptoms on *Passiflora edulis cv.flavicarpa* naturally infected with PV1 (a) vine in the field (b) mature leaves.

(a)

(b)

and used as a single local lesion host to establish pure cultures of PV1 and for infectivity assays. Pure cultures of PV1 Homagama-A from *P. foetida* were used as the source of inoculum for experiments, unless otherwise stated.

3.1.2 a) Comparison between different sources of inoculum

An experiment was done to determine whether young leaves (Fulton, 1966), mature expanded leaves or flower petals (Milbrath and McWhorter, 1953; Fulton, 1966), were the best source of inoculum.

Samples of each tissues were taken from virus-infected *P. foetida*. Inoculum was extracted from 0.05 M phosphate buffer pH 7.8 plus 100 g/l Polyclar AT. Two dilutions, 1:5 and 1:10 w/v were made. Treatments were inoculated according to a 6 x 6 Latin square design using *C. amaranticolor*. Young leaves and mature expanded leaves produced means of 57 and 139 lesions / leaf respectively, at 1:10 dilution. At 1:5 dilution also, mature expanded leaves induced more lesions (102 lesions / leaf) than young leaves (23 lesions / leaf). Extracts from petals did not induce any lesions. The results indicated that fully expanded mature leaves were a significantly better source of inoculum than young leaves or petals at two dilutions ($P < 0.05$). The results also indicated that passion fruit sap contained an inhibitor of infection as lesion numbers increased slightly with dilution from 1:5 to 1:10 (w/v).

3.1.2 b) Comparison between different extraction media

It is advantageous to add buffer to extracted plant sap, or else to grind the plant tissue in a buffer. Both the virus and the test plants are influenced to a large extent by the buffer. This is partly due to the effect of the pH and partly due to the ionic strength of the buffer. Choice of buffer, its hydrogen ion concentration and its molarity may be important factors for transmission of viruses (Kado, 1972).

(i) Influence of different buffers on isolation of PV1

Infected leaves were chopped into small pieces and mixed well. The sample was divided into four portions of equal weight and ground with different buffers at 1:10 w/v dilution. *C. amaranticolor* plants of the same size and age were used as an assay host by inoculating 6 leaves at similar positions.

The results indicated (Table - 1) that tris-HCl was significantly better than other buffers tested ($P < 0.05$).

(ii) Influence of hydrogen ion concentration in extraction buffer for isolation of PV1 virus from *P. foetida* to *C. amaranticolor*.

Infected leaves from *P. foetida* were chopped into small pieces, mixed thoroughly and divided into five

Table 1: Effect of different buffers on the transmission of PV1

Buffers (0.05 M, pH 7.8)	Mean no of lesions / plant
Sodium borate	733 b
Sodium citrate	499 bc
Tris-HCl	1272 a
Phosphate	454 bc
cv%	16.9

equal weight portions. Each portion was ground in 1:10 (w/v) in 0.05 M tris-HCl buffer at five different 'H' ion concentrations. Treatments were applied to *C. amaranticolor* leaves according to a 5 x 5 Latin square design. Results are summarised in Table 2.

Table 2: Influence of 'H' ion concentration of Tris-HCl buffer on infectivity of PV1

'H' ion concentration	Mean no. of lesions / leaf
6.0	29
7.0	35
7.8	42
9.0	43
10.0	39
cv%	13.8

More lesions per leaf were produced with tris-HCl buffer at pH 7 - 9. Treatment differences were not statistically significant, however, although slightly fewer lesions were probably produced at pH 6 or pH 10; from the observations made, pH values in the 7 - 8 region were routinely used for sap transmission. Gibbs and Harrison, (1976) reported that plant phenolase activity is reduced at relatively high pH values.

(iii) Influence of molarity of tris-HCl buffer on infectivity of PV1

Infected leaves of *P. foetida* were divided into small pieces and were mixed thoroughly. Five equal weight portions were used and each portion was ground in 1:10 dilution (w/v) of tris-HCl buffer pH 7.8 at different ionic strengths. Each inoculum was assayed on *C. amaranticolor* plants according to a 5 x 5 Latin square design. Results are summarised in Table 3.

Table 3: Influence of molarity of tris-HCl buffer on infectivity of PV1

Molarity	Mean no. of lesions/leaf
0.01	6bcd
0.02	57a
0.05	7bc
0.10	12b
0.20	2bcd
cv%	20.3

The results show that tris-HCl buffer at 0.02 M gave the highest lesion number (significant at $P < 0.01$). None of the other molarities differed significantly from each other ($P > 0.05$).

(iv) Effect of additives on isolation of PV1

Proteins, polysaccharides, enzymes, polyphenols and tannins are the most common inhibitors in plants (Fulton, 1966). Inhibitors may interfere with the infection process or inactivate virus in the inoculum. Chemicals are often added to extraction buffer to overcome the effects of inhibitors. For example, the action of polyphenoloxidases may be prevented by incorporation of reducing agent such as sodium sulphite (Bald and Samuel, 1934) and sodium thioglycollate (Hopkins and Williams; after Fulton, 1966). Alternatively, chelating agents such as sodium ethylenediaminetetra acetate (EDTA) were also used since they sequester the copper ions necessary for phenol oxidase activity (Fulton, 1966). Chemicals such as polyvinylpyrrolidone (Kosuge, 1965) may compete with virus particles for phenols in the extraction media (Matthews, 1981). Polythylene glycol (Ramaswamy and Posnette, 1971) may also reduce the combination between virus particles and phenolic compounds. The action of other inhibitors may be overcome by the addition of bentonite (Yarwood, 1966) to the grinding buffer.

Effect of additives on isolation of PV1

Infected *P. foetida* leaves were cut into small pieces and mixed thoroughly. The sample was divided into six equal weight portions. Each portion was then ground in 1:10 dilution (w/v) of 0.02 M tris-HCl buffer pH 7.8 containing different additives. Treatment differences was assayed by inoculating *C. amaranticolor* plants according to a 6 x 6 Latin square design. Results are given in Table 4.

Table 4: Effect of additives to 0.02 M tris-HCl, pH 7.8 buffer on the infectivity of PV1

Additives	Mean no. of lesions / leaf
Bentonite 10 g/l	28 e
EDTA 0.01 M	89 abcd
Sodium sulphite 1 g/l	116 ab
Sodium thioglycollate 1 g/l	175 a
PVP (m.w. 43,000) 10 g/l	104 abc
PEG 1 g/l	79 abcd

Bentonite produced significantly ($P < 0.05$) fewer lesions than other treatments. Sodium sulphite and sodium thioglycolate induced the highest lesion numbers although treatment differences were not significant among the other five additives tested. The enhancing activity of sodium sulphite was thus shown to be similar to that of sodium thioglycollate, and as this chemical has been

used before for one of the *Passiflora* viruses (e.g. De Wijs, 1974a), sodium sulphite was used routinely in experiments with Tris buffer.

3.1.2 c) Factors affecting mechanical transmission

Host range experiments showed *C. amaranticolor* to be a useful local lesion assay host for PV1. A number of experiments were done, therefore, to optimise conditions for assay.

(i) Influence of pre-inoculation dark treatment on susceptibility of *C. amaranticolor* to PV1

Chenopodium amaranticor plants were grown in a glasshouse at a temperature of 22-25°C with a photoperiod of 16 hr and a light intensity of 3,500-4,000 Lux. After dark treatment of 48, 24 and 0 hr, inoculum was applied to these plants. Inoculum was prepared by grinding infected leaves, in 0.02 M tris-HCl buffer pH 7.8 plus 1 g/l sodium sulphite. Seven *C. amaranticolor* plants were used for each treatment and four leaf pairs from base to top were rubbed with inoculum. After inoculation, the plants were covered with damp newspaper for 16 hr and were grown at 16-19°C in a glasshouse with 16 hr photoperiod and a light intensity of 3,500-4,000 Lux. The number of lesions / plant formed on inoculated leaves was used as a measure of their susceptibility. Results are given in Table 5a.

Table 5a: Effect of pre-inoculation dark period on the susceptibility of *C. amaranticolor* plants to PV1

Pre-inoculation dark period (in hours)	Mean number of lesions / plant
48	649
24	845
0	569
cv%	4.4

The 24 hour dark period resulted in the highest number of lesions, while those plants without any dark treatments produced the fewest lesions. However, the treatment differences were not statistically significant ($P > 0.05$) even though cv% was low. Pre-inoculation darkening was, however, used routinely, as it may have some effect, as other workers have found (Bawden and Roberts, 1947, 1948). In many laboratories, it is now a common practice to give pre-inoculation dark treatment to increase the susceptibility of plants to infection (Yarwood 1957; Siegel and Zaitlin, 1964).

Further investigations were made to study the effect of dark treatment under different temperature regimes. *Chenopodium amaranticolor* plants were kept at mean temperatures of 19°, 25° and 34° C. in growth cabinets, and inoculated after 72, 48, 24 or 0 hours in darkness. The dark treatment was given by covering the

leaves with 9 x 5 cm black polythene bags and the experiment was designed as a 4 x 4 Latin square design. The inoculum was prepared as above and the results are given in Table 5b.

Table 5b: Effect of dark treatment under different temperature conditions on lesion development in *C. amaranticolor*

Dark treatment in days	Mean no of lesions/leaf at different temperature		
	19° C	25°C	34°C
3	328	281 ab	217 ab
2	347	379 ab	226 ab
1	356	454 a	240 a
0	183	139 c	79 c
cv%	11.9	5.9	5.7

The data indicated that all plants with a dark treatment, produced more lesions than those without a dark treatment. However, these treatment differences were not significant ($P > 0.05$) at 19°C. They were significant ($P < 0.05$) at 25°C and 34°C. It seems that dark treatment is more effective when plants are grown at higher than at lower temperatures.

(ii) Effect of post-inoculation washing on transmission of PV1 visus

Inoculum for this experiment was prepared from infected *P. foetida* leaves, by grinding them in 0.05 M

phosphate buffer pH 7.8 containing 1 g/l sodium thioglycolate. Celite (2 mg/ml) was added and the inoculum applied with a finger on the upper surface of the leaves. Washing was done by holding the inoculated plants under a tap with a slow flow of water for about 2 seconds, immediately after inoculation. Control treatment was left without washing. Lesions were counted 25 days after inoculation. Seven *C. amaranticolor* plants were used for each treatment.

The washed treatment showed higher numbers of lesions per plant (32) compared to unwashed control (25). However, it was not statistically significant ($P>0.05$). Yarwood (1972) reported that washing increased the infection when the inoculum contained caffeine and / or a high concentration of celite, than when these chemicals were in low concentration.

A study on the effect of washing when celite is used as an abrasive (Kalmus and Kassanis, 1945) was made by using three concentrations of celite with or without washing.

Treatments were distributed in an 8 x 8 Latin square design. The inoculum was prepared by grinding infected *P. foetida* leaves in 0.02 M tris-HCl pH 7.8 buffer containing 1 g/l Na_2SO_3. Dilution was 1:10 (w/v). Results obtained are given in Table 6.

Table 6: Effect of washing on infection of PV1 with celite abrasive

Treatments	Mean no of lesions / leaf
Celite 8 mg/ml, washed	22 c
Celite 8 mg/ml, unwashed	4 d
Celite 2 mg/ml, washed	97 a
Celite 2 mg/ml, unwashed	100 a
Celite 1 mg/ml, washed	31 b
Celite 1 mg/ml, unwashed	30 b
No Celite, washed	1 e
No Celite, washed	1 e
cv%	12.9

Washing had no effect with (1 mg/ml) and (2 mg/ml) treatments. Both washed and unwashed treatments Celite at 2 mg/ml gave the highest lesion number, which was highly significant ($P < 0.01$). A significant difference was found between washed and unwashed treatments with 8 mg/ml Celite. Treatments without Celite gave significantly ($P < 0.05$) lower values. Results suggest that washing treatment was effective when a higher concentration of celite was used. However, to standardise infection 2 mg/ml was used routinely; washing was also continued as this made it easier to count lesions by removing residual inoculum.

(iii) Effect of post-inoculation covering on the transmission of PV1

The effect of post-inoculation covering was studied using 14 *C. amaranticolor* plants of uniform size. Three leaf pairs from base to top were inoculated with extracts prepared by infected *P. foetida* leaves, grinding in 1:10 (w/v) dilution 0.02 M tris-HCl pH 7-8 buffer containing 1 g/l Na_2SO_3. After inoculation, seven plants were covered with damp newspaper overnight. The remaining plants were left uncovered. Lesion counts were taken to assess treatment differences. Post-inoculation covering resulted in a significantly ($P < 0.01$) higher lesion development (458 lesions / plant) than the treatment with no covering (285 lesions / plant). Covering may reduce inoculation damage by helping to maintain turgidity of wounded leaf tissues.

3.1.2 d) Factors influencing symptom expression

(i) Influence of growing temperature, before inoculation on symptom development

A small scale experiment was designed to study the influence of growing temperature on the susceptibility of *C. amaranticolor* plants to infection with PV1. This experiment was done during, winter in a glasshouse with different temperature ranges. Light intensity and day length were 4,500 - 5,000 Lux and 16 hr respectively for each treatment.

Inoculum was prepared by grinding infected leaves in 0.02 M tris-HCl pH 7-8 buffer containing 1 g/l Na_2SO_3 at a dilution of 1:10 w/v. Celite(2 mg/ml)was used as an abrasive. Each treatment was given to eight *C. amaranticolor* plants, after about 4 weeks at each temperature. Inoculum was rubbed on leaf pairs from base upwards, on each plant, and after inoculation, plants were placed at 19-22°C. Table 7 shows the results of comparing the relative susceptibility of *C. amaranticolor* plants to the virus at different temperatures.

Table 7: Relative susceptibity of *C. amaranticolor* plants to virus infection by *Passiflora* virus 1

Average temperature (°C)	Mean no of lesions / plant
15	101
18	253
27	3
cv%	0.1

Plants grown at a temperature of 18°C showed a significantly ($P<0.01$) higher number of lesions than those at other temperatures. Those grown at 27°C produced the lowest lesion numbers. The results showed, therefore, that high growing temperature reduced the number of lesions on *C. amaranticolor*. Plants grown at 27°C had dark green, tough leaves, which were bigger than those in other plants. The results suggest that

temperature before inoculation was one of the important factors affecting the susceptibility to virus infection. The affect may be indirect through temperature influencing plant development.

(ii) Influence of post- inoculation temperature on symptom development

A study on the effect of the post-inoculation temperature on the susceptibility of *C. amaranticolor* plants to PV1, was done during winter. Before inoculation, indicator plants were grown at 22°C temperature with 16 hr day length and a light intensity of 3,000-4,000 Lux. Inoculum was prepared by extraction of virus-infected leaves in 0.02 M tris-HCl pH 7.8 buffer plus 1 g/l Na_2SO_3. Eight plants of *C. amaranticolor* were used for each treatment. Immediately after inoculation, they were kept at an average temperature of 15°, 18°, and 22°C in seperate glasshouse for symptom development. The post-inoculation light regimes were similar at each treatment. The results are shown in Table 8.

Table 8: Influence of post inoculation temperature on lesion development in *C. amaranticolor*

Average temperature (°C)	Mean no of lesions / plant	Time taken for lesion development
15	40 b	12
18	812 a	6
22	751 a	6
cv%	0.52	

Results indicated that plants grown in a glasshouse at 18°C or 22°C after inoculation induced significantly ($P<0.05$) more lesions, than those grown in a glasshouse at 15°C. It also appeared that the inoculation period for lesion development was shorter at 18°C and 22°C than at 15°C. The highest number of lesions were produced when grown at 18°C after inoculation, although results were not significant ($P > 0.05$) from those at 22°C. Lesions produced by plants grown at 18°C and 22°C were more distinct than those grown at 15°C. Jensen, (1973) reported that increasing temperature up to a certain point, increased the rate of systemic movement and decreased the time before the first appearance of systemic symptoms.

(iii) Effect of post-inoculation illumination on lesion development

In this experiment the effect of a short period of illumination of 6 hr, immediately after inoculation was studied in relation to production of lesions in *C.amaranticolor* by PV1. These plants were grown in a greenhouse at 22°C with 16 hr day length and illumination of 3,000-4,000 Lux by warm white fluorescent lamps plus natural light.

All the test plants were kept in the dark for 24 hr before inoculation. The inoculum was prepared as in Chapter 2. Three leaf pairs from the base upwards were inoculated on each plant. All the plants were

rinsed in tap water after inoculation. Inoculated plants were placed under different light intensities for 6 hr at 22°C and were then transferred to normal growing conditions, when they were covered with damp newspaper for 17 hr and left for observation. The following results were obtained:

Table 9: Effect of light intensity on lesion development in *C. amaranticolor* inoculated with PV1

Source of illumination	Light intensity (Lux)	Mean no of lesions/plant
Dark box	0	267
Day light	1,000	308
Mercury lamp	5,500	363
Sodium light	8,000	344
cv%		4.5

The results suggested that higher artificially produced light intensities might be slightly better for the development of lesions. Plants kept in darkness or under natural light produced fewer lesions than those kept under the supplementary illumination. However, treatment differences were not statistically significant ($P > 0.05$). Huguelet and Hooker (1966) pointed out a period of darkness immediately after inoculation delayed the appearance of lesions by about the same time as the dark period and the number of lesions appearing was reduced.

(iv) Combined effect of growing temperature and illumination before inoculation on lesion development

A study of the combined effect was made using *C. amaranticolor* plants exposed to different conditions for nearly 45 days before inoculation. Eight *C. amaranticolor* plants were used for each treatment. Inoculum was extracted from *P. foetida* in 0.02 M tris-HCl pH 7.8 buffer plus 1 g/l Na_2SO_3. For each treatment, three leaf pairs from base to top were inoculated. Plants were covered with damp newspapers after rinsing and placed under normal growing conditions for lesion development. Results are given in Table 10:

Table 10: Combined effects of growth temperature and illumination before inoculation on lesion development in *C. amaranticolor* plants inoculated with PVl

Average temperature °C	Light intensity in Lux	Mean no of lesions / plant
33 - 34	6500	157 b
20	3900	201 ab
18	4760	328 a
cv%		7.2

The highest temperature and light intensity combination significantly ($P < 0.05$) reduced lesion development compared to lesion formation at 18°C and

4,760 Lux. The 20°C and 3,900 Lux treatment was not significantly different from the other two treatments ($P > 0.5$).

For subsequent assay work, *C. amaranticolor* test plants were grown at about 18°C, kept dark for 24 hr before inoculation, briefly rinsed and covered after inoculation, and maintained at about 18-22°C with 4,500-5,000 Lux illumination.

3.1.3 Host plant response

Host range tests were done by mechanical inoculation to 37 plant species belonging to five families. The inoculum was prepared from PV1 infected *P.foetida* leaves. Infected leaves were extracted with 0.02 M tris-HCl pH 7.8 plus 1 g/l Na_2SO_3 or 0.05 M phosphate buffer pH 7.8 with 100 g/l Polyclar AT. Celite abrasive (2 mg/ml) was added to the inoculum before inoculation. Experimental plants were examined periodically for symptoms, up to a period of four months. The infection of symptomless plants and infected plants was confirmed by back inoculation to *C. amaranticolor* and *P. foetida* using sap extracted from inoculated leaves 21 days after inoculation and from newly emerged leaves about 60 days after inoculation. All plant species were tested at least 3 times in different seasons. Consistent host reactions are given in Table 11.

Host reactions of selected hosts:

Passiflora edulis cv. flavicarpa

The first reliable symptom was turning downwards of the affected leaves 10-14 days after inoculation. These were also slightly crinkled, and vein yellowing was apparent on some leaves. A definite green and yellow mottle was prominent within about 3 weeks after inoculation. The tissues of light green areas may be raised or sunken thus giving the leaf a puckered or a crinkled appearance. In addition, numerous chlorotic spots (1-1.5 mm diameter) were occasionally observed. A significant leaf-area reduction was not observed when compared to healthy plants.

Passiflora quadrangularis

The first symptom of infection was a vein clearing of young, non-inoculated leaves. These appeared 14-20 days after inoculation. Inoculated leaves showed yellow mottle symptoms. Systemically-infected leaves developed chlorotic mottle and flecks 3 to 4 weeks after inoculation. Infected leaves were slightly crinkled but plant growth was not severely affected.

Passiflora foetida

This herbaceous species produced systemic symptoms. The first reliable symptom of the disease appeared 7 - 10 days after inoculation, on young non-inoculated leaves. A definite light yellowish-green/dark-green mottle appeared, 14-18 days after

inoculation. In mature leaves, numerous raised irregularly shaped yellowish spots scattered over the leaf surface (Plate 3) 3 - 4 weeks after inoculation. Inoculated leaves showed chlorotic mosaic and mottling symptoms.

Passiflora mollisima

Inoculated leaves produced a green and yellow mottle, three weeks after inoculation. Plants produced systemic symptoms with yellow spots, mottle and mosaic afterwards with slightly deformed leaves. Areas of intense pigmentation were observed for infected flowers (Plate 4).

Chenopodium amaranticolor

The first symptoms of infection were small circular, yellowish non-necrotic spots measuring about 1-1.5 mm in diameter on inoculated leaves (Plate 5). These spots appeared 10-15 days after inoculation. These spots enlarged to about 2 mm size in diameter and had a tiny white centre about 3 weeks after inoculation. Several days after, these lesions became enlarged to about 3 mm in diameter and the yellowish spots became reddish pink in colour. Finally, lesions became brown. Virus was not systemic in this host.

Plate 3: Chlorotic spots on old leaf of PV1 infected *Passiflora foetida*.

Plate 4: Chlorotic mosaic and mottle symptoms on *Passiflora mollissima* infected with PV1 (a), flowers of same plant showing pigment intensification (b).

(a)

(b)

Plate 5: Chlorotic local lesions on innoculated leaf of Chenopodium amaranticolor infected with PV1.

Chenopodium quinoa

Yellow mottle symptoms were frequently observed on inoculated leaves, about 3 weeks after inoculation, but there was no systemic infection.

Phaseolus vulgaris cv. The Prince

Infected primary leaves produced tiny purple coloured lesions 3-4 weeks after inoculation. These lesions enlarged to 3-4 mm diameter about 5-6 weeks after inoculation, and their shape was often indistinct. Infection did not spread systemically.

Cassia occidentalis

Young systemically-infected leaves produced a slight leaf curl with some vein yellowing, 2-3 weeks after inoculation. Mature leaves produced a chlorotic mottle, and inoculated leaves showed diffuse chlorotic mottle.

Table 11: Host plant reaction of PV1

Plant Species	Local Reaction	Systemic Reaction
Amaranthaceae		
Gomphrena globosa (Globe amaranth)	NL	-
Chenopodiaceae		
Chenopodium album	CS	-
C. amaranticolor	CS	-

C. foetidum	CS	-
C. murale	CM	-
C. quinoa	CM	-
Leguminosae		
Cassia occidentalis	CMo	VY, Lc, CMo
C.tora	NL	-
Crotalaria usaramoensis	CM	CM
Phaseolus vulgaris	NL	-
(cv. Prince)		
P.vulgaris	NL	-
(cv. Canadian Wonder)		
P.vulgaris	NL	-
(cv. K. W. G.)		
P.vulgaris	NL	-
(cv. Top crop)		
Vigna unguiculata	NL	-
(cv. Bushita mae)		
Passifloraceae		
Passiflora edulis	CM, CMo	CMo, MLD
P. edulis cv. flavicarpa	CM, CMo	CMo, MLD
P. foetida	CM, CMo	VY, CMo, CS
P. ligularis	CMo	CMo
P. mollissima	CMo	CMo, CF, CM
P. quadrangularis	CMo	VY, CMo, CF
P. van volxemii	CM, CMo	CS, CMo
Solanaceae		
Nicotiana clevelandii	CS	CS

Petunia hybrida	SL	-

Abbreviations

CM = Chlorotic mosaic	NL = Necrotic lesions
CMo = Chlorotic mottle	MLD = Mild leaf distortion
CF = Chlorotic flecking	VY = Vein yellowing
CS = Chlorotic spots	SL = Symptomless local

\- = No infection

Species not infected

Arachis hypogaea, *Beta vulgaris* cv. Globe, *Centrosema pubescens*, *Cucumis sativus* cv. LY 58 and Parisienn Pickling, *Datura stramonium* cv. Arbotea, *Glycine max* cvs PB1, Bossier and PM 78 - 35, *Lycopersicon esculentum* cv. Delight, *Nicotiana debneyi*, *N. megalosiphon*, *N. tabacum* cvs White Burley and Xanthi, *Passiflora suberosa*, *Pisum sativum* (Pea) cv. Meteor, *Pueraria phaseoloides*, *Spinacia oleracea* cv. Sigma leaf, *Tricosanthes anguina* cv. LA 33, *Vicia faba* cv. Aquadulce and *Vigna unguiculata* cvs IIta, PB1, MI35 and Bombay.

3.1.4 *In vitro* properties

In vitro properties were repeated at least twice in different seasons.

3.1.4 a) (i) Longevity *in vitro* (LIV)

Results indicated that after 6 days at room temperature (20 - 23°C), the virus was still infectious

in sap extracts of *P. foetida* when assayed on *C. amaranticolor*, but was not infectious after 7 days.

3.1.4 a)(ii) Longevity in leaf tissue

Virus was not inactivated after drying the infected leaves of *P. foetida* at room temperature (20 - 23°C) for 7 days, which was the last interval examined.

3.1.4 b) Thermal inactivation point (TEP)

Samples heated above 75°C were not infective but chlorotic local lesions were induced on *C. amaranticolor* when samples were heated to 30 - 70°C for 10 min. There was a sharp drop in infectivity of PV1 between 50 - 70°C and the virus was inactivated in *P. foetida* sap between 70 - 75°C which is the thermal inactivation point (Fig. 1).

3.1.4 c) Dilution end point (DEP)

The results (Fig. 2) indicated a DEP between 10^{-5} and 10^{-6} for PV1.

Data from the graph (Fig. 2) suggest that PV1 reaches quite high concentrations *in vivo*. The slight increase in infectivity noted with low dilutions also confirmed the presence of an inhibitor in *P. foetida* sap.

3.1.4 d)(i) Storage of PV1

Three different storage methods (Chapter 2.11) at two different temperatures were examined, at different

Fig.1 Thermal inactivation of PV1 in Passiflora foetida sap.

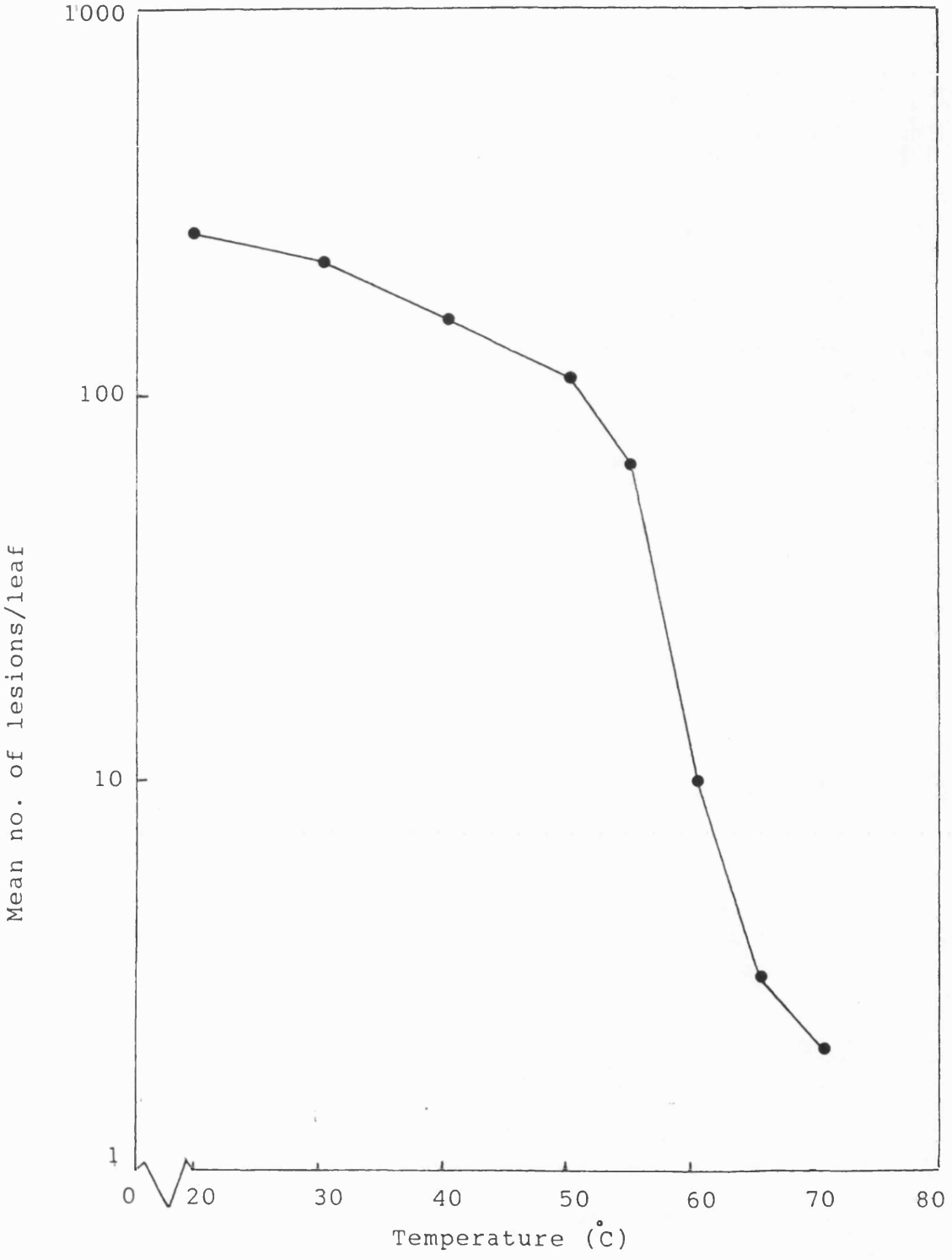
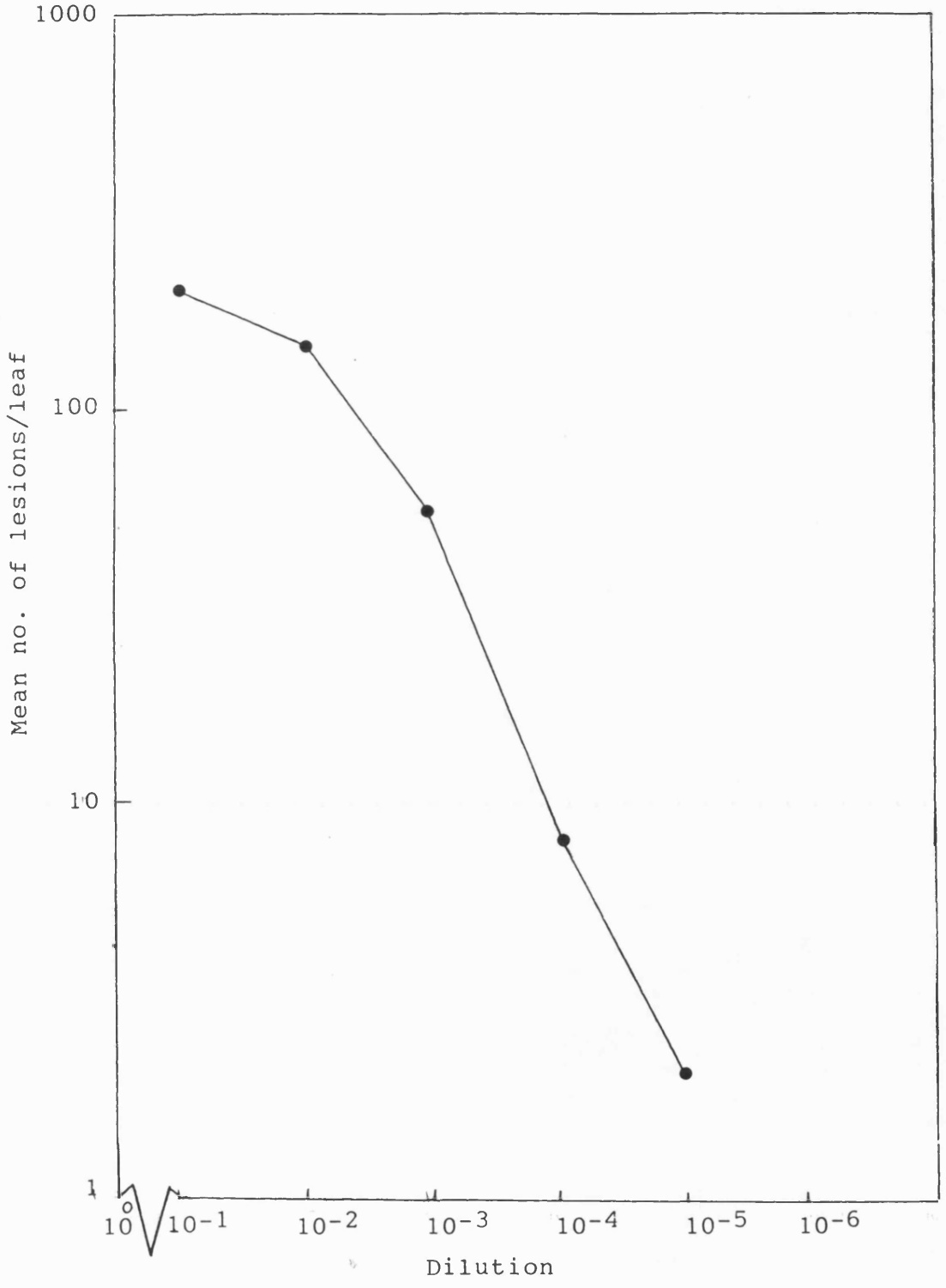


Fig.2 Dilution curve of PV1 in Passiflora foetida sap.



intervals, for the retention of infectivity (Table 12a), assayed on *C. amaranticolor*.

Table 12a: Comparison of different methods of storage for Passion fruit virus 1 in *P.foetida* leaves

Storage Method	Infectivity Reaction days after storage			
	14	30	60	360
Silica gel, -20°C	+++	+++	+++	+++
$CaCl_2$, -20°C	++++	++++	+++	+++
Polythene bag, -20°C	+++	+++	+++	+
Silica gel, 4°C	+++	+++	+++	+
$CaCl_2$, 4°C	+++	+++	+++	+
Polythene bag, 4°C	++++	-	-	-

Abbreviations

++++ Strong reaction ++ Weak reaction

+++ Intermediate reaction + Very weak reaction

\- No reaction

Results have indicated that drying over $CaCl_2$ or silica gel at -20°C were the best storage methods for PV1. Sealed polythene bags at 4°C were suitable only for short term storage. The virus survived drying over silica gel or $CaCl_2$ at 4°C but infectivity was very low after 360 days.

3.1.4 d) (ii) Liquid nitrogen

Preliminary experiments were done to study the effect of low temperatures on the stability of PV1. This method employed ultra low temperatures to inhibit the growth of microorganisms. It involved pre-cooling (Planer mini-freezer unit, type R202) 1 ml aliquots of PV1 virus prepared by grinding *P. foetida* leaves in 0.02 M tris-HCl pH 7.8 buffer (1:10 dilution w/v) in 10% (v/v) glycerol, sealed in 1.5 ml polypropylene ampoules. The samples were frozen for one minute at -20°, -37° and -54°C and the samples stored in liquid nitrogen.

One sample was stored without pre-cooling in liquid nitrogen, and another stored at 4°C was taken as control. All frozen samples were stored for 24 hr and assayed for infectivity. Treatment differences were studied according to a 5 x 5 Latin square design. The results are given in Table 12b.

Table 12b: Effect of storage of PV1 in liquid nitrogen for 24 hours

Storage Treatment	Mean no. of lesions / leaf
1 min at -20°C, stored in liquid N	32
1 min at -37°C, stored in liquid N	36
1 min at -54°C, stored in liquid N	43
directly in liquid N	36
stored at 4°C	21

The cooling treatment has not shown any marked deleterious effect on PV1 and all samples stored in liquid nitrogen, induced lesions slightly higher than the control at 4°C. De Wijs and Suda Bachmann (1979) found that deep freezing infectious sap was satisfactory for storing some viruses, but deep freezing was unsatisfactory for some others (Marcinka and Musil, 1977). Walkey (1985) also recommended storing virus in sap under liquid N.

3.1.5 Purification

Martini (1962) reported, a simple method to purify the virus isolated from *Passiflora edulis* in Western Nigeria. In this method virus was purified and concentrated by heat clarification and high speed centrifugation using systemically-infected leaves of *P. edulis*. In the present study, virus was initially purified from *P. foetida* using this protocol.

One part of infected leaf tissues of *P. foetida* (15 g) was ground in two parts of distilled water (w/v) and strained through muslin. The extract was then heated to 45°C for 5 min, and clarified at 3,000 x g for 10 min. The supernatant fluid was centrifuged at 75,000 x g for 100 min and the pellets were stored under 5 ml of water at 4°C for 2 hr. Pellets were resuspended in water and clarified at 10,000 x g for 5 min. The supernatant fluid was centrifuged at 57,000 x g for 75 min and the pellet resuspended in 2 ml of water. Infectivity of purified

samples when assayed on five *C. amaranticolor* plants was low and produced means of 20 lesions per plant. The method of clarification was also unsatisfactory as the supernatant remained a dark green colour. More systematic attempts were made therefore, to develop a satisfactory clarification method.

3.1.5 a) (i) Determination of initial buffer for purification

A comparison was made of the infectivity of extracts from infected *P.foetida* leaves when homogenised in various buffers including phosphate, borate, and tris-HCl.

Twelve grams of diced infected leaves were mixed thoroughly and divided into 3 equal weight samples. Each sample was ground (1:2 w/v) in a chilled mortar with a pestle and mixed with one of the above buffers to give three treatments. Inoculum was applied to *C. amaranticolor* plants in a 6 x 6 Latin square design. Transmissions were done immediately and also after allowing the extracts to stand at 4°C for 5 hr. Results are shown in Table 13.

Borate buffer induced more lesions when assayed immediately, than phosphate and tris-HCl buffers. Results obtained in this experiment in which tris-HCl gave the fewest lesions were in contrast with the earlier experiment (Chapter 3.1.2) in which higher lesion numbers

were found with this buffer. However, retention of infectivity after 5 hr was highest in tris-HCl buffer.

Table 13: Retention of infectivity of PV1 in different buffers

Buffer	Mean no. of lesions / leaf		Percentage of infectivity retained after 5 hr
(0.05M,pH7.8)	0 hr	5hr	
Phosphate	89	68	77
Tris-HCl	63	61	95
Borate	130	83	71

From earlier virus transmission studies, and the results of this experiment, tris-HCl was routinely used for virus purification, although the differences between the buffers were probably not marked, especially after 5 hr at 4°C.

(ii) Determination of optimum hydrogen ion concentration in extraction buffer

A systemically-infected *P.foetida* leaf sample was chopped and divided into 3 equal weight samples. Each portion was ground in a series of 0.05 M tris-HCl buffers of varying pH values. The inoculum was diluted (1:10 w/v) and assayed immediately after extraction and also after 5 hr at 4°C. Treatments were distributed according to a 6 x 6 Latin square design and results are presented in Table 14.

Table 14: Effect of hydrogen ion concentration on susceptibility of *C.amaranticolor* plants to PV1

pH	Mean no. of lesions / leaf		Percentage of infectivity retained after 5 hr
	0 hr	5hr	
7.0	82	34	41
7.8	67	38	57
9.0	12	1	7

Results indicated that infectivity was moderately well preserved at pH 7 and 7.8 but not at pH 9. Retention of infectivity after 5 hr at 4°C was better with tris-HCl buffer at pH 7.8 than at pH 7.0. Subsequently pH 7.8 buffer was used for routine virus extraction and purification. Neutral or slightly alkaline buffers are normally used for extraction of virus (Brakke, 1967). However, at pH 9 virus was probably becoming damaged (Matthews, 1981) although such values can also help reduce aggregation for some viruses (Hull, 1985).

(iii) Determination of optimum ionic strength in extraction buffer

Infected *P. foetida* leaf samples were chopped and mixed thoroughly and divided into three equal weight portions. Each portion was ground in tris-HCl pH 7.8 buffer of varying molarities. Infectivity was assayed

immediately and after standing for 5 hr at 4°C in a 6 x 6 Latin square design using *C. amaranticolor* (Table 15)

Table 15: Effect of different ionic strengths of tris-HCl buffer on the infectivity of PV1

Ionic strength	Mean no. of lesions / leaf		Percentage of infectivity retained after 5 hr
	0 hr	5hr	
0.01 M	100	30	30
0.02 M	150	60	40
0.05 M	148	137	93

The results indicated that stabilization was better in 0.05 M buffer than in 0.01 M or 0.02 M. Ionic strength of 0.05 M was, therefore, selected for routine virus extraction from *P.foetida*.

(iv) The effect of sap dilution on infectivity of PV1

The effects of sap dilution on the infectivity of PV1 was studied using 0.05 M tris-HCl pH 7.8 buffer as the diluent. Infected *P. foetida* leaves were chopped and ground in this buffer to get the four dilutions of 1:1, 1:2, 1:5 and 1:10 w/v. Inoculum was assayed immediately and five hours after extraction at 4°C. Treatments were distributed using an 8 x 8 Latin square design.

Table 16: Effect of dilution of sap on infectivity of PV1

Dilution	Mean no. of lesions / leaf		Percentage of infectivity retained after 5 hr
	0 hr	5hr	
1:1	66	64	98
1:2	79	69	87
1:5	60	51	84
1:10	56	29	51

Infectivity was well stabilized by 1:1 or 1:2 dilution. Extracts were probably slightly less infective at 1:5 and 1:10 dilutions, particularly after 5 hr, than 1:1 and 1:2 dilutions. Therefore, 1:2 dilution (w/v) was routinely used for virus extraction.

(v) Effect of chemical additives to extraction buffer on the retention of infectivity

It is often important to include a reducing agent, chelating agent or phenol antagonist in the buffer used during maceration of tissues to prevent the loss of virus through oxidation and enzymatic activity, or by precipitation, and to prevent adsorption of coloured components to the virus.

Suitable additives for stabilization of some viruses during purification were found experimentally and included sodium sulphite (Sun and Hebert, 1972), sodium thioglycollate (De Sequira and Lister, 1969; Brunt, 1971 ;

and Bos *et al.*, 1972) and polyvinylpyrrolidone (Kosuge, 1965). These chemicals were tested in the present experiment.

Infected leaves of *P. foetida* were diced and divided into equal weight portions and each portion ground in 0.05 M tris-HCl pH 7.8 buffer 1:2 (w/v) with various additives. Inoculum was rubbed on *C. amaranticolor* leaves immediately after extraction and after incubating for 5 hr at 4°C. Treatments were assayed according to an 8 x 8 Latin square design and results are given in Table 17.

Table 17: Effect of buffer additives for stabilization of PV1 during purification

Additives to buffer (0.05 M, tris-HCl, pH 7.8)	Mean no. of lesions per leaf		Percentage of infectivity retained
	0 hr	5hr	after 5 hr
Ex. 1,			
No additives	76	64	85
Na_2SO_3 1 g/l	82	69	84
Sodium thioglycollate 1 g/l	60	51	84
PVP 1 g/l	56	29	51
Ex. 2,			
Na_2SO_3 1 g/l	173	120	69
Sodium thioglycollate 1 g/l	42	27	63
PVP 1 g/l	88	57	64

In the first experiment the virus was stabilized well in buffer only and in buffer with Na_2SO_3. PV1 was poorly stabilized in PVP.

In the second experiment, sodium sulphite was superior to the other chemicals and was, therefore, routinely used for purification.

3.1.5 b) Determination of suitable clarification method

On obtaining an extract from an infected tissue, it is desirable to remove as much plant material as possible before the virus is concentrated. The ideal clarification procedure is one in which no virus is lost. In practice a compromise is usually reached between the amount of virus retained and the amount of contaminating materials removed.

(i) Comparison of different clarification methods

Twelve grams of infected leaves of *P. foetida* were chopped, mixed well and divided into five equal weight samples. Four portions were homogenized (1:2 w/v) in cold 0.05 M tris-HCl pH 7.8 buffer containing Na_2SO_3 (1 g/l). One portion was homogenized with distilled water. Extracts were filtered through double layers of muslin before clarification. Treatments were then assayed on *C. amaranticolor* in a 5 x 5 Latin square design.

Chloroform method (Francki, 1972)

Ten millilitres of chloroform were added dropwise to 10 ml of extract. Then, the mixture was stirred gently at 4°C for 45 min. After clarification, the mixture was centrifuged at 10,000 x g for 20 min. The aqueous phase was retained for assay.

Butanol method (Brunt, 1971)

N-butanol was added dropwise to 10 ml of extracts to give 8.5% butanol (v/v) in the mixture. The mixture was shaken gently at 4°C for 45 min, and subjected to 10,000 x g centrifugation for 20 min. The supernatant was retained for assay.

Triton X-100 method (Van Oosten, 1972)

One percent Triton X-100 (v/v) was added dropwise to 10 ml of extraction while stirring gently. After stirring the mixture for 45 min at 4°C it was centrifuged at 10,000 x g for 20 min. The supernatant was retained for assay.

Control

Extracts were stirred for 45 min at 4°C and centrifuged at 10,000 x g for 20 min. The supernatant was retained for assay.

The efficiency of different methods of clarification could be seen from the colour of the supernatants, and infectivity assay (Table 18).

Table 18: Efficiency of different clarification methods

Clarification method	Colour of supernatant*	Mean no. of lesions / leaf
Chloroform	light greenish brown	248
N-butanol	light brown	209
Triton X-100	green	138
Water (control)	dark green	124
Buffer (control)	dark green	131
cv%		16.2

* adjusted to same volume before assay

The results indicated that clarified extracts induced more lesions than those in water or buffer only. Chloroform treatment produced the highest number of lesions but n-butanol was also satisfactory. Infectivity was retained with both butanol and chloroform treatment but was diminished by Triton X-100. -butanol removed more pigmented host material than chloroform. Butanol was therefore used in the standard purification procedure. The lesion counts between chloroform and butanol were not statistically significant ($P > 0.05$), but these were both different from the other treatments.

3.1.5 c) Concentration of virus

Virus was concentrated by the polyethylene glycol (PEG) single phase system (Hebert, 1963) in the presence of 0.03 M NaCl. Virus infected *P. foetida* leaves were extracted in a chilled mortar with 0.05 M tris-HCl buffer

containing 1 g/l Na_2SO_3. Sap was passed through double layers of muslin and divided into four equal volumes. These were clarified in different ways and then concentrated by PEG.

After clarification by n-butanol, chloroform and Triton X-100 as described in Chapter 3.1.5b., samples were then concentrated by 60 g/l PEG 6,000. The treatments and the control were stirred with PEG and 0.03 M NaCl for 2 hr at 4°C and then centrifuged at 10,000 x g for 20 min. The pellets were resuspended in 2 ml tris-HCl buffer and left overnight. Then after low speed centrifugation (3,750 x g for 10 min) the supernatant was assayed immediately and retained for 5 days at 4°C and assayed again. A Latin square design (4 x 4) was used and the results of the experiment are given in table 19.

Results indicated that n-butanol plus PEG treatment, was better than either chloroform plus PEG or Triton X-100 plus PEG. In this experiment chloroform-clarified preparation induced the lowest lesion count although in a previous experiment it gave satisfactory results. Triton X-100 clarified preparations induced higher lesion numbers after five days at 4°C than immediately after purification. This may have been due to an experimental error or a delayed effect of the Triton X-100 in reversing aggregation of PVl. n-butanol 8.5% plus 60 g/l PEG combination was used as the standard purification method.

Table 19: Effect of different clarification methods and PEG concentration on infectivity of PV1

Clarification Method	Colour of partially purified preparation	260/280 (uncorr-ected)	Mean no. of lesions per leaf (immediately)	(after 5 days)
N-butanol (8.5% v/v)	light brown, glassy	1.5	91a	13
Chloroform (1% v/v)	light brown, glassy	1.5	1bc	1
Triton X-100 (1% v/v)	dark green	1.4	5ab	17
Control	light green	1.5	4bc	1
cv%			16.9	23.6

3.1.5 d) Comparison of n-butanol and Triton X-100 clarification method

According to preliminary electron microscope observations of PV1 (described in Chapter 3.1.7), the presence of pinwheel inclusions and flexuous rod-shaped particles about 840 nm long suggested that PV1 might be a potyvirus. Accordingly, it was decided to compare the n-butanol / PEG purification method with a recent potyvirus purification method described by Hammond and Lawson (1988).

Fifty grams of infected *P. foetida* leaves were harvested, two months after inoculation. Leaves were chopped, mixed well and divided into 25g portions. One portion was extracted (1:2 w/v) with 0.05M tris-HCl pH 7.8 containing 1 g/l Na_2SO_3 using a blender. The homogenate was then clarified by 8.5% (v/v) n-butanol and precipated with 60 g/l PEG and 0.03 M NaCl. The virus pellet was resuspended overnight at 4°C in 2 ml of extraction buffer and after brief centrifugation (3,750 x g for 10 min) the supernatant collected. Virus was pelleted from the supernatant by centrifugation at 60,000 x g for 90 min. Pellets were resuspended with 500 µl of extraction buffer overnight, and retained for assay purpose.

A second portion was homogenized in 2 volumes of chilled 0.5 M K_2HPO_4/KH_2PO_4 buffer pH 8.4 containing 5 g/l Na_2SO_3 in a blender. The homogenate was then filtered through muslin and the filtrate centrifuged at 10,000 x g for 20 min, the supernatant was collected and the preparation clarified with Triton X-100 added to 2% (v/v) and the liquid stirred for 15 min prior to the addition of 0.1M NaCl and 40 g/l PEG (m.w. 8,000). After stirring for 1.5 hr the PEG precipitate was collected by centrification 10,000 x g for 30 min, the supernatant being discarded. The precipitate was resuspended in 20 ml of 0.1 M boric acid and 0.1 M KCl adjusted to pH 8 with NaOH (BK buffer). The solution was then transferred to a small beaker and stirred for 1.5 hr. After

centrifugation (3,000 x g for 5 - 10 min) the supernatant was collected and carefully underlain with 5 ml of 300 g/l sucrose in BK buffer. After centrifugation at 60,000 x g for 90 min, the pellets were resuspended in 500 µl of BK buffer and the pellet homogenized.

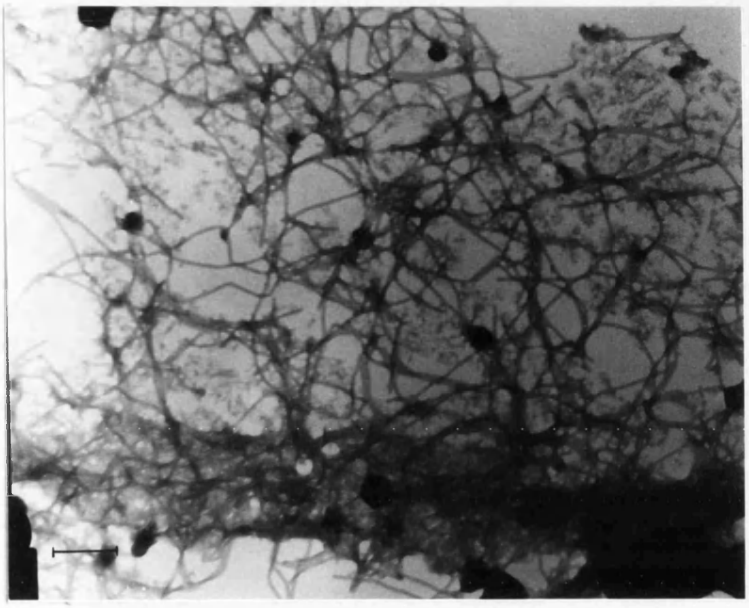
Partially-purified virus preparations were then assayed on five *Phaseolus vulgaris* (cv. The Prince) plants using the half-leaf method.

Table 20: Comparison of n-butanol and Triton X-100 purification methods

Method of Purification	No of lesions per half leaf	260/280 ratio uncorrected	Colour of the purified preparation
Butanol / PEG	14	1.29	light yellow
Triton X-100 / PEG	150	1.33	colourless

Results suggested that the Triton X-100 / PEG method was more effective than n-butanol method. The clarification was better than the n-butanol method and more lesion numbers were produced. Furthermore, many more flexuous rod shaped particles were seen under the electron microscope in Triton X-100 clarified preparations (Plate 6) than in the n-butanol / PEG method. Therefore, subsequent purification was routinely done according to the Hammond and Lawson, (1988) method.

Plate 6: Electron micrograph of partially purified PV1 from Passiflora foetida stained in 20 g/l PTA, pH 6.5; note aggregated particles. Bar represents 200 nm.



3.1.5 e) Density gradient centrifugation

In this method, most of the host contaminants including ribosomes can usually be separated from the main virus band. The virus-containing band 4.3 - 4.4 cm below the meniscus after caesium sulphate gradient (see Chapter 2.15) was opalesent and clearly visible by shining a torch through the side of tube. The presence of virus in this band was confirmed by infectivity tests and electron microscope observation (Plate 7). Virus peak fractions were collected by hand, and dialyzed against three changes of 1 litre of 0.5 x BK buffer and stored at -20°C for future use.

Ultraviolet light absorption

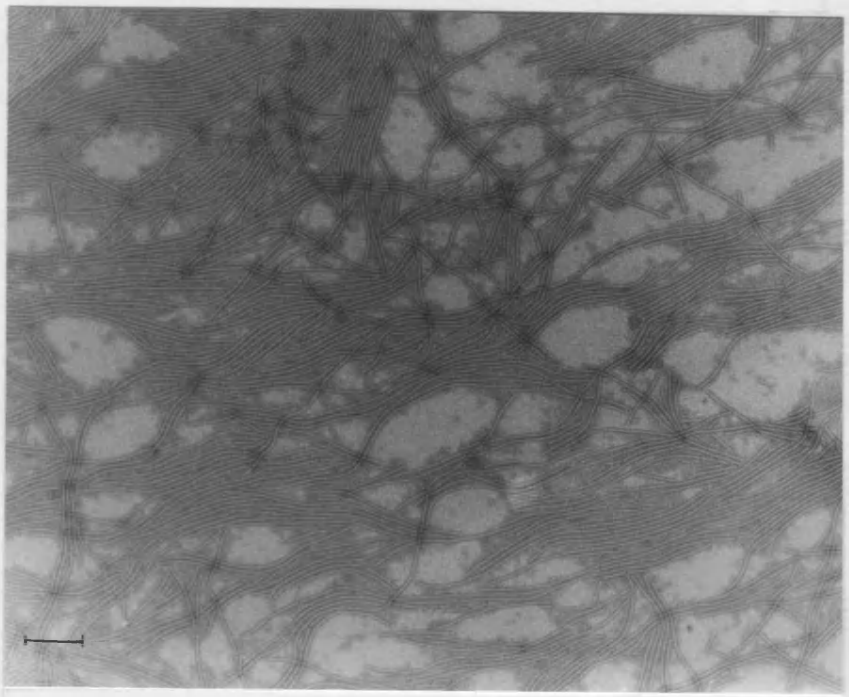
The spectrum of the dialyzed PV1 virus in BK buffer pH 8 had a minimum at 250 nm and a maximum at 260 nm and slight shoulder at 290 nm possibly due to a high tryptophan content of the protein (Fig. 3). The 260/280 ratio was 1.30 - 1.33 before correction for light scattering. Virus yield were calculated on the basis $E_{1cm}\ 0.1\%_{260nm} = 2.5$ (Hammond and Lawson, 1988). Average yields obtained for *P. flavicarpa* and *P. foetida* were 2-5 mg and 10-15 mg per 100 gm of tissue, respectively.

3.1.6 Light microscopy

Cylindrical inclusions in the epidermal cells were stained with the O-G combination (Plate 8) which were not seen in healthy tissues.

Plate 7: (a) Purified virus particles of PV1 from Passiflora foetida after caesium sulphate density gradient centrifugation stained in 20 g/ l PTA pH 6.5.
(b) Higher magnification of the same.
The bars represent 200 nm.

(a)



(b)

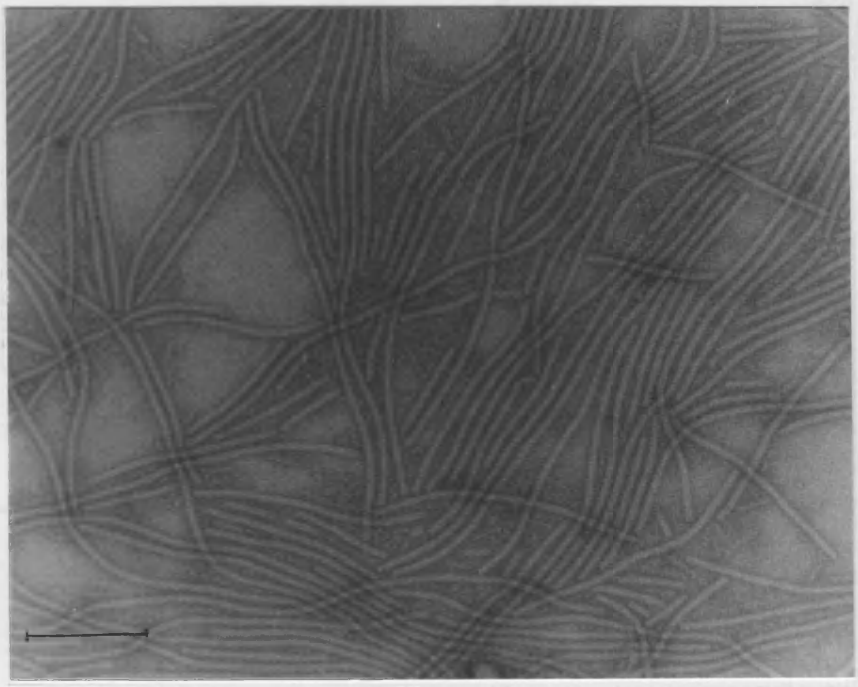


Fig.3 Absorption spectrum of partially purified preparation of PV1 from Passiflora foetida (diluted to 1/100).

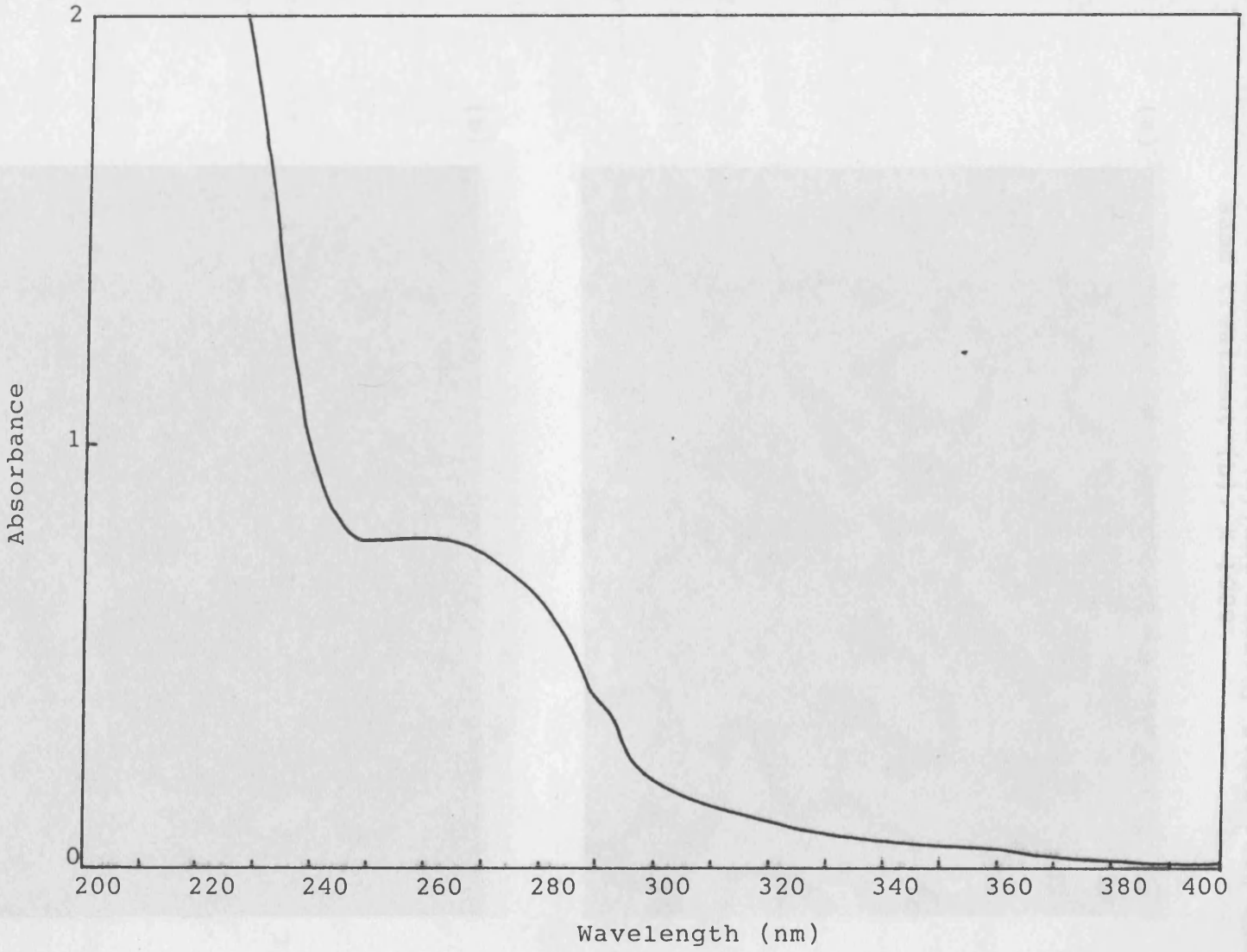
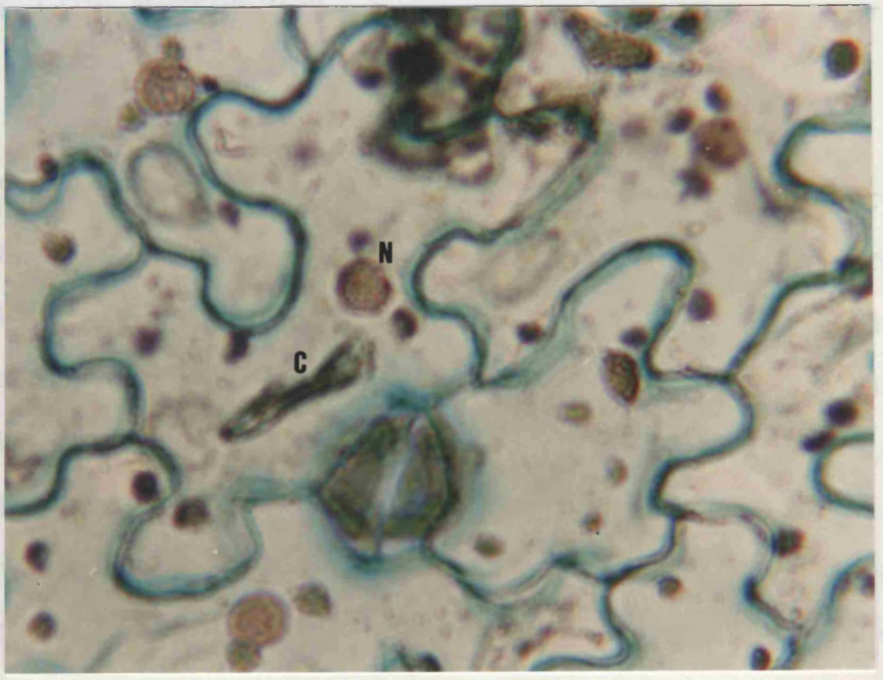
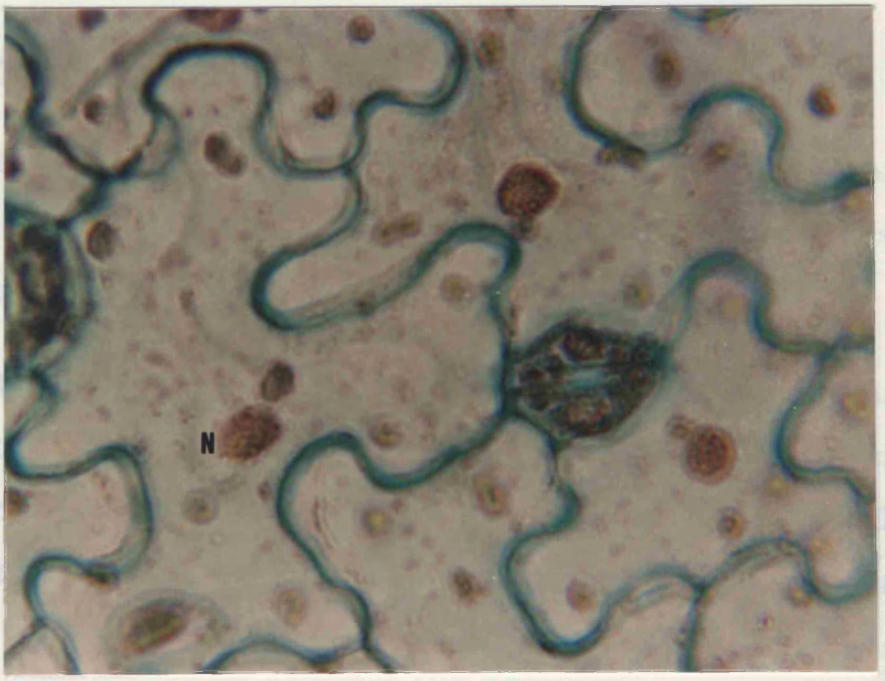


Plate 8: Passiflora foetida epidermal cells infected with PV1, containing large group of cylindrical inclusions (C) and nucleus (N) stained with the O-G combination (a),and healthy cells with the same treatment (b). X 1000

(a)



(b)



Sections through infected stems of *P. flavicarpa* showed brown probably necrotic cells in vascular bundles. There were no other abnormalities in cell organs when compared to the healthy preparations.

3.1.7 Electron microscopy

3.1.7 a) Particle morphology

To measure the particle length of PV1, electron micrographs were taken of leaf squash preparations from *P. foetida* (Plate 9). At least 100 particles were measured to calculate normal length and 50 particles were measured to calculate width. Of the 100 particles examined, 81% measured were between 600-900 nm and 4% below 600 nm and 15% over 900 nm. Particle lengths which ranged from 760-900 nm (the main peak) were used to calculate the normal length of PV1. This was found to be 841 nm $\pm$ 4.6 long (Fig 4) and 13.1 $\pm$ 0.4 wide.

3.1.7 b) Thin sections

No virus particles were found in ultrathin sections of PV1 infected tissues of either *P. foetida* or *P. flavicarpa*.

No cytoplasmic inclusions were observed in thin sections of PV1-infected *P.foetida*. However, in thin sections of diseased *P. flavicarpa*, inclusions in the form of tubular and laminated aggregates were seen. Pinwheels inclusions, typical of potyviruses were also observed (Plate 10).

Plate 9: Electron micrograph of leaf squash preparation of PV1 from Passiflora foetida stained in 20 g/l PTA, pH 6.5. Bar represents 100 nm.

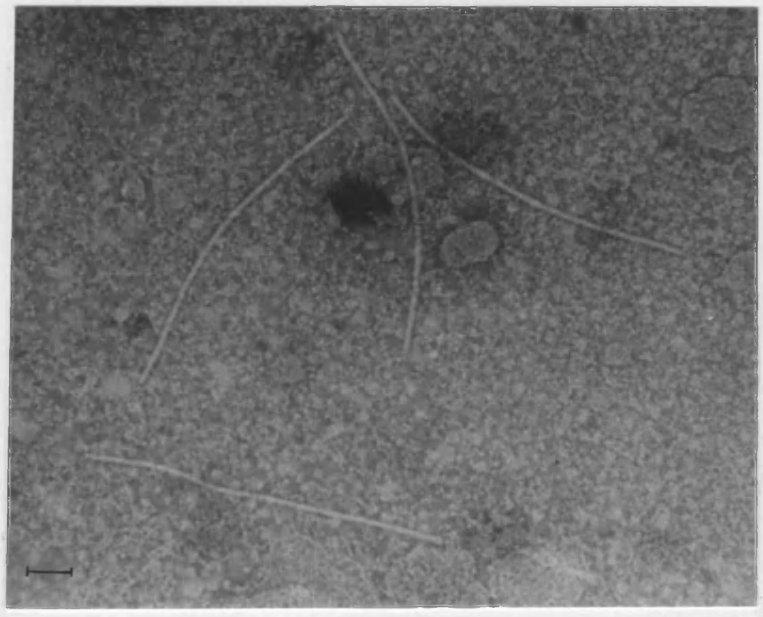


Fig.4 Distribution of particle length for PV1 in a leaf squash homogenate.
(class interval 1:33.3 nm)

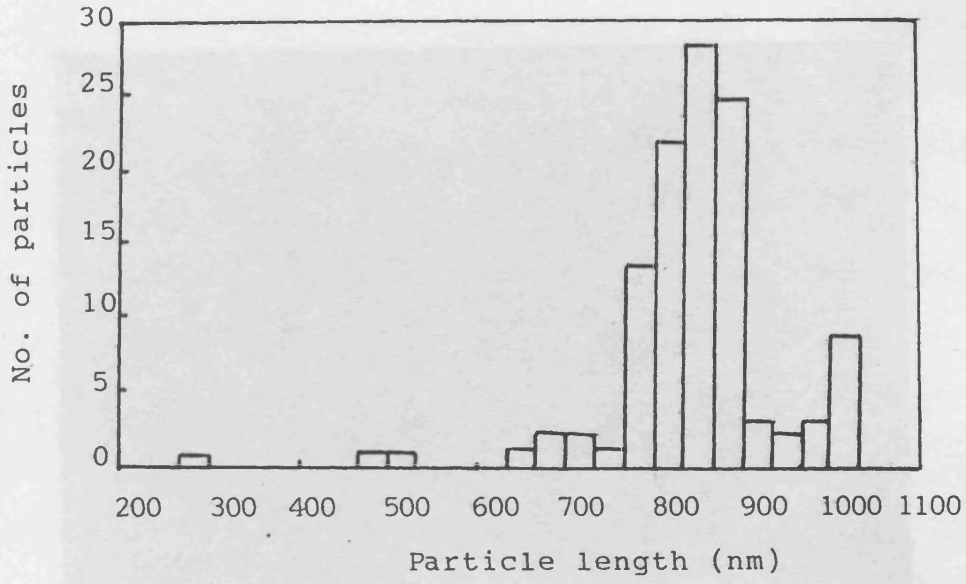
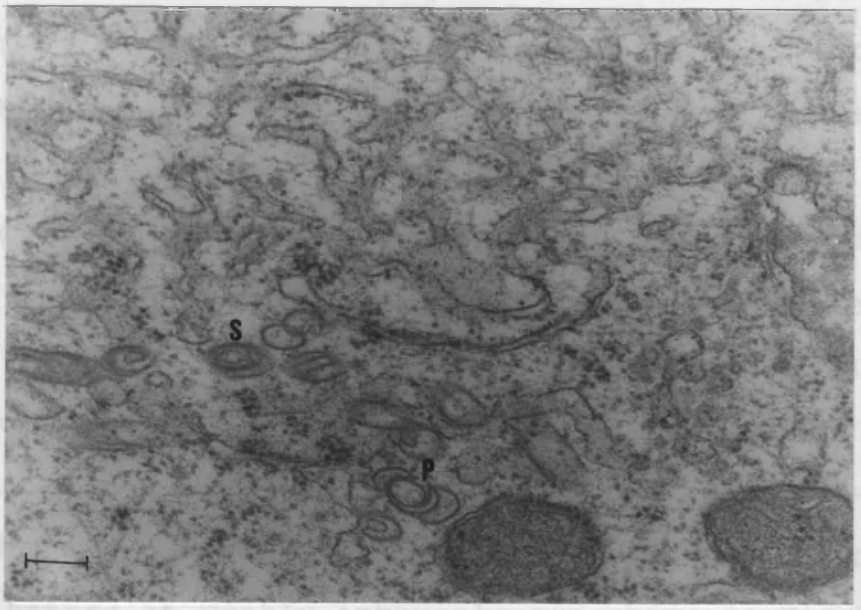


Plate 10: Electron micrograph of inclusion bodies; pinwheel (P) scrolls (S) in ultrathin section of Passiflora edulis cv.flavicarpa leaf cell infected with PV1. Bar represents 200 nm.



3.1.8 Serology

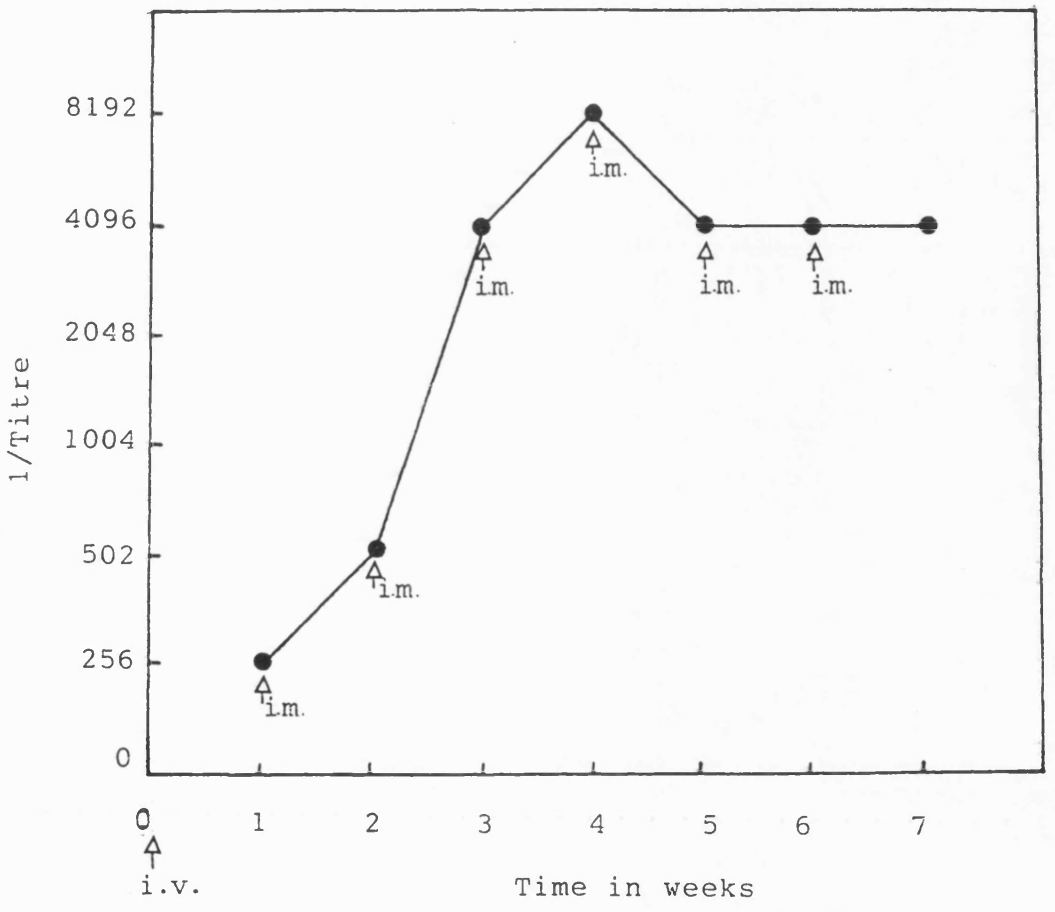
P. flavicarpa was used a source material for virus purification. Virus to be used as an immunogen was collected from the caesium sulphate gradient and dialyzed against 0.5 x BK buffer overnight before use. A single intravenous ear injection was given initially, followed by another six intramuscular injections at weekly intervals. Intravenous injections were prepared by mixing the virus (1 mg) in 8.5 g/l NaCl to give final volume of 1 ml. Intramuscular injections were prepared by mixing 1 mg virus with an equal volume of Freunds incomplete adjuvant (Difco Ltd.). Homologous reaction in microprecipitin test (Plate 11) at weekly intervals were determined with 1 mg/ml purified virus preparations. Antiserum titres at weekly intervals are shown in Fig 5.

3.1.8 a) Agar gel double-diffusion

Wetter and Milne (1981) reported that particles longer than 500 nm diffuse rather poorly in agar gels.

In this study, precipitation lines were not detected in 5 - 7.5 g/l agar gels using antiserum to PV1 and antigen prepared from PV1-infected *P. foetida* leaves or purified preparations. This was presumably due to the large particle size of PV1. In an attempt to detect virus in gel diffusion tests, antigens (crude sap and purified preparations) were treated in various ways before testing, to produce smaller length fragments,

Fig.5 Increase in antibody production of rabbit injected with PV1, measured as antiserum titre in microprecipitin tests.



⇧ Arrows - Time of injection
i.v. - Intravenous injection
i.m. - Intramuscular injection

which might diffuse more readily, through the agar. The following treatments were tested.

(i) Infected sap was sonicated 10 mins (Moghal and Francki 1976, Tomlinson and Walkey, 1967)

(ii) Infected sap was mixed 1:1 (v/v) with 3` g/l SDS and 5 g/l SDS was incorporated in the unbuffered gel diffusion medium (Purcifull and Batchelor, 1977).

(iii) Antigens were mixed with an equal volume of 5% v/v pyrrolidine solution (Shepard *et al.*, 1974; El-Nil *et al.*, 1977).

(iv) 5 g/l 3,5 diodosalicylic acid (lithium salt) was incorporated in the gel diffusion medium (Rajeshwari *et al., 1981)*.

All methods tested were unsatisfactory. Thus, pyrrolidine treated antigen had a homologous titre of 1:2 in gel double-diffusion tests, but 1:4016 in microprecipitin tests, PV1 antiserum also had 1:1 titre with healthy preparations in gel diffusion tests. Several serologically active components were also detected when antigens were treated with pyrrolidine. The serological heterogenity of the degradation products of PV1 virus with pyrrolidine may complicate the interpretation of agar double-diffusion tests. Double diffusion reaction in agar of PV1 with its antiserum is

shown in Plate 12. Unsatisfactory results in agar double-diffusion test with treated antigen may be due to the antisera to intact virus reacting poorly against the disrupted virions (Van Regenmortel, 1982). It was apparently not due to low virus concentration as up to 10 mg/ml of purified preparations were used.

3.1.8 b) Immunosorbent electron miscroscopy

Antiserum to PV1 heavily decorated homologous antigens in purified preparations, in immunologically specific electron microscopy (Plate 13). However, decoration was not observed with tobacco mosaic particles in the same preparation.

3.1.8 c) Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique for the detection of PV1 virus infection in herbaceous and woody hosts was done by using direct and indirect forms of ELISA. Antiserum with a homologous titre of 1:8,092 was adopted for the indirect version of ELISA and gamma-gobulin was prepared from antiserum collected 7 weeks after immunization, with a homologous titre of 1:4,096.

(i) Detection of PV1 infection in *P. foetida* or *P. flavicarpa* by indirect ELISA

Investigations were done to see if PV1 could be detected in *P. edulis cv. flavicarpa* and *P. foetida* leaves by indirect ELISA (Mowat and Dawson, 1987).

Plate 11:Micro-precipitin reaction of PV1 with its antiserum. Flocculation observed 5 min after mixing. Test with drops in a Petri dish,under paraffin oil.

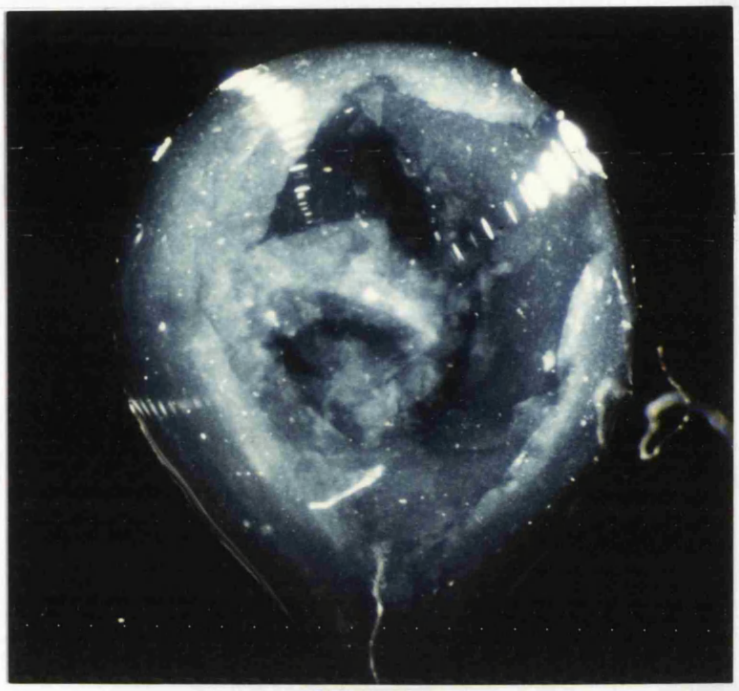


Plate 12:Double diffusion reaction in agar of PV1; purifed preparation from Passiflora foetida (P) and crude sap (C), (dissociated with pyrrolidine) with its antiserum (A). Healthy crude sap with the same treatment (H).

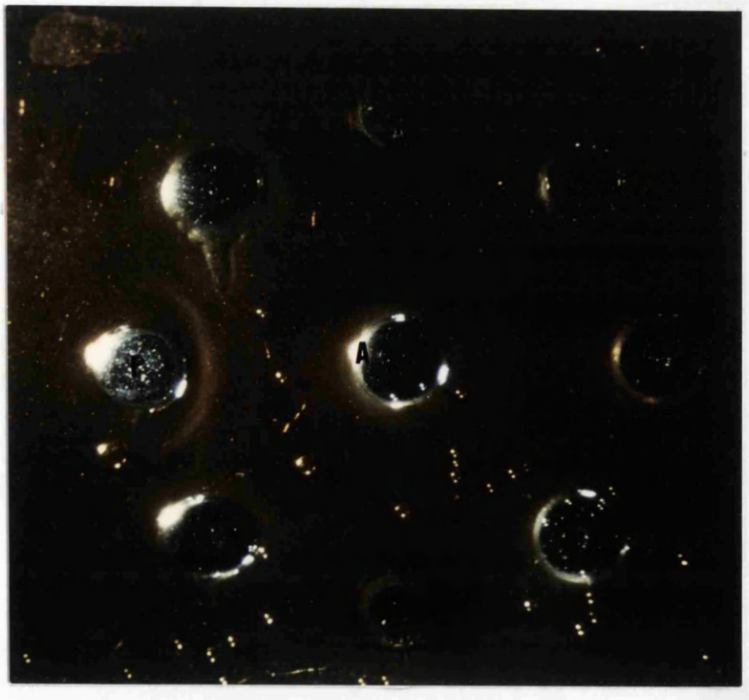
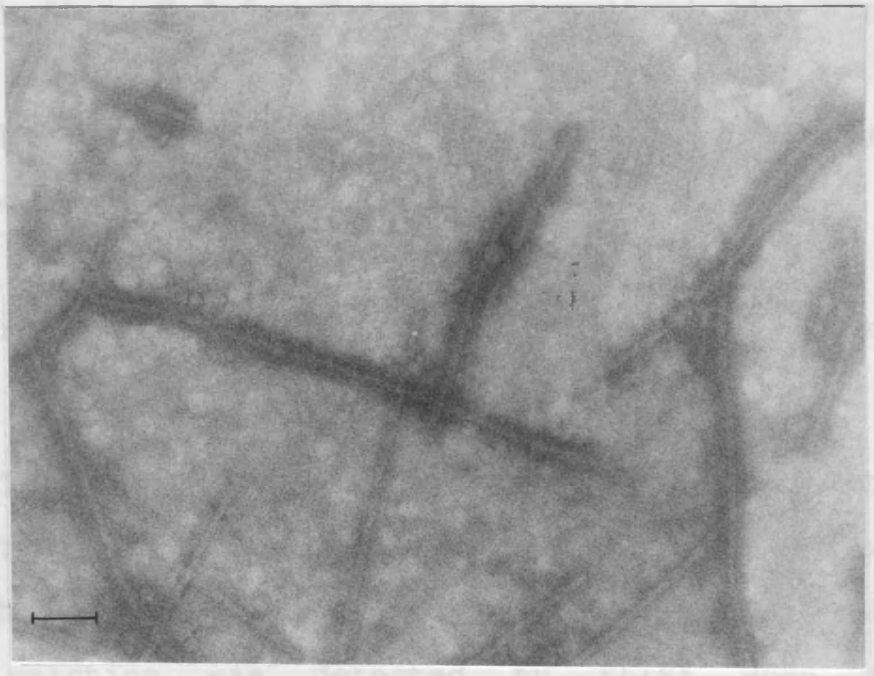


Plate 13: Reaction of PV1 to homologous antibody, immunosorbent electron microscopy. Bar represents 100 nm.

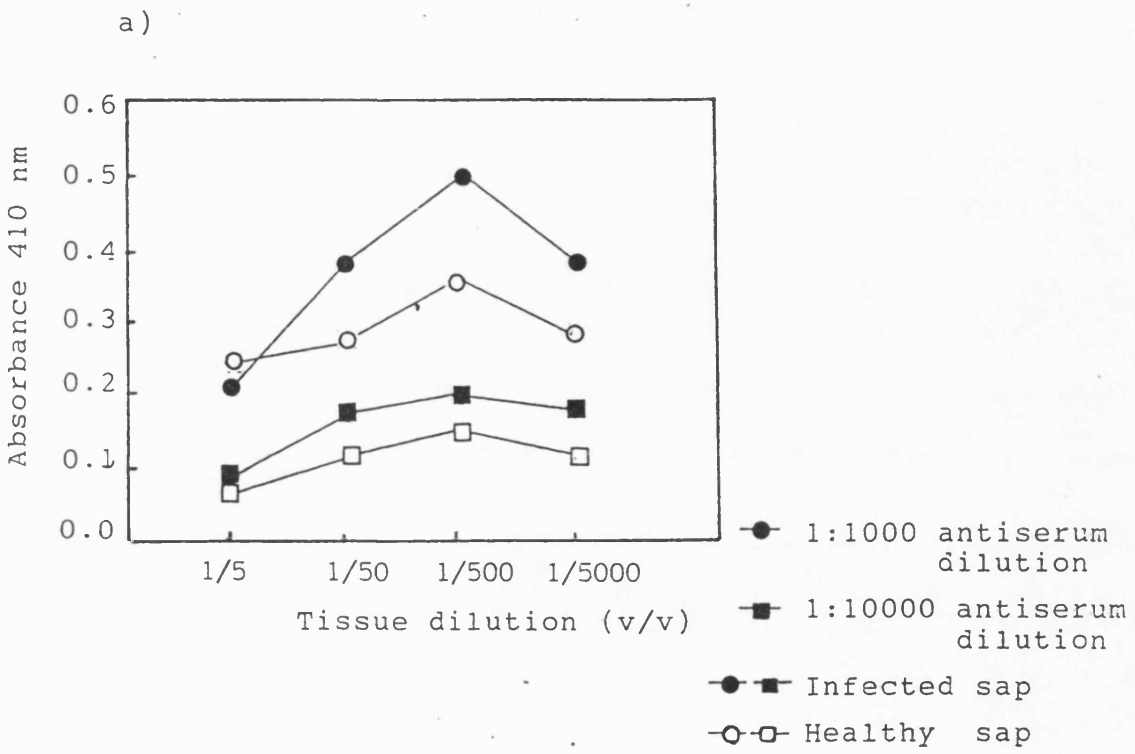


Mature, fully expanded leaves were collected from virus-infected and apparently healthy plants of *P.edulis cv. flavicarpa* and *P. foetida*. Tissues were ground in a mortar with a pestle in the presence of sodium carbonate buffer pH 9.6. Extracts were strained through muslin and $^1/_5$, $^1/_{50}$, $^1/_{500}$, and $^1/_{5000}$ dilutions made in the same buffer. Two antiserum dilutions 1:1,000 and 1:10,000 were compared in this study. A protein A conjugate dilution (stock) of 1:2,500 was found to be satisfactory. Absorbance (A 410 nm) curves are presented in Fig. 6.

There were no positive reactions with the extracts from virus-infected leaves of *P. flavicarpa* after 16 hr substrate incubation (Fig. 6a). However, a positive reaction was detected by ELISA when sap was extracted from *P. foetida* even after only one hr substrate incubation (Fig. 6b). An antiserum dilution of 1:1,000 was not suitable for ELISA due to high background values. Antigen dilutions of $^1/_{500}$ produced the highest A 410 nm value. The phenomenon has been explained by a competition between virus and host proteins during adsorption onto the uncoated plate (Lommel *et al.*, 1982).

This experiment was repeated using leaves of *P. foetida* and results are presented in Fig. 7. Antiserum dilutions of 1:20,000 (Fig. 7b) produced a better separation between diseased and healthy treatments. However, background reaction was high even at 1:10,000 (Fig. 7a) dilution of the antiserum. This could be due to

Fig.6 Absorbance curves obtained by indirect ELISA for PV1 in Passiflora edulis cv.flavicarpa leaves (a) and Passiflora foetida leaves (b).



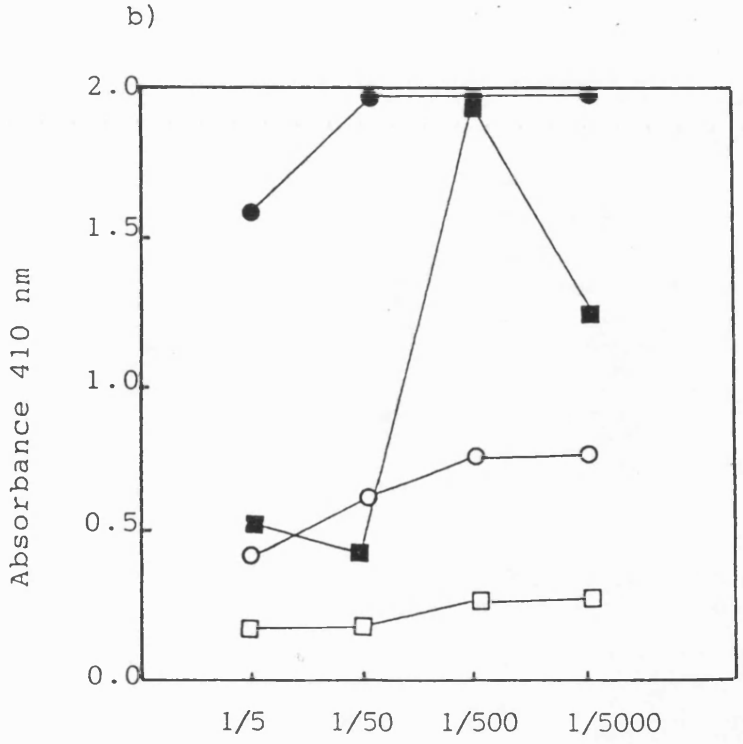
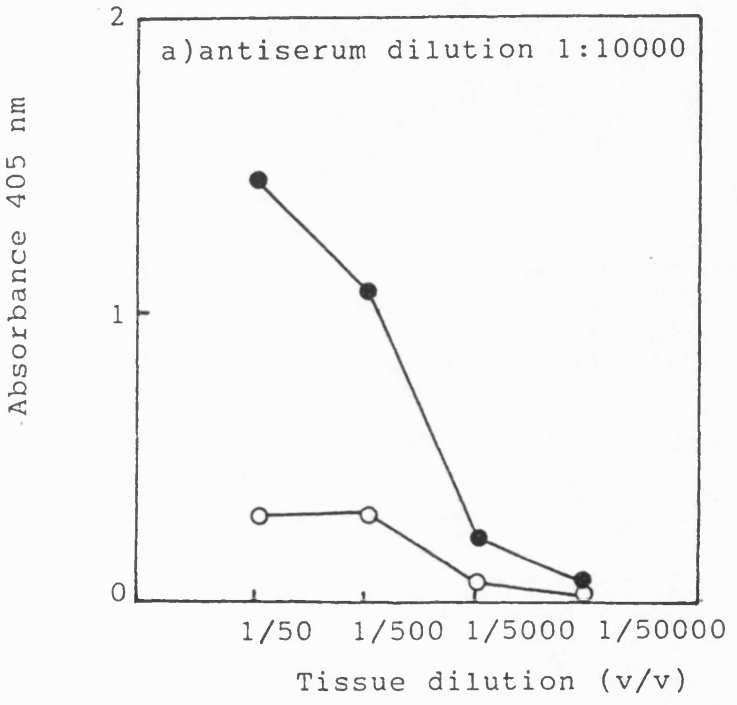
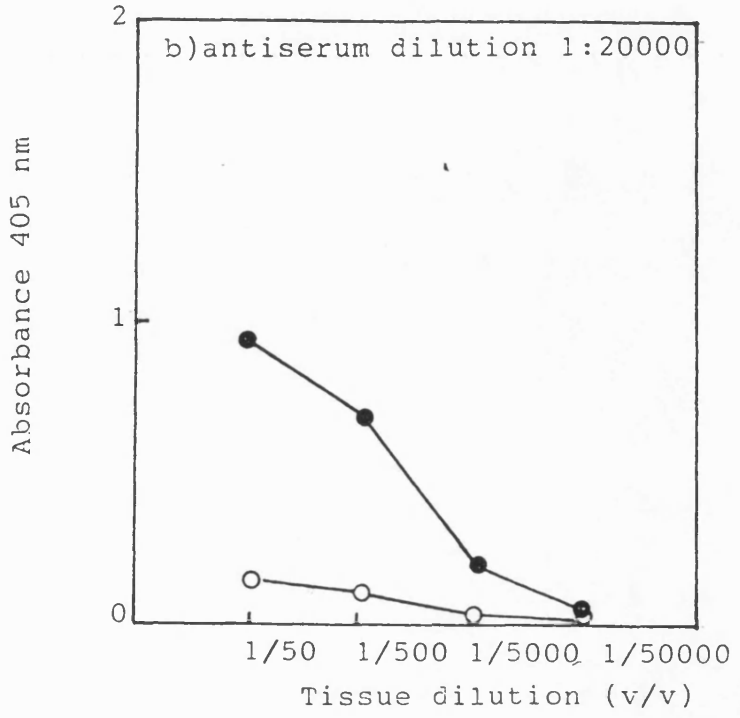


Fig.7 Absorbance data obtained with different dilutions of antiserum.
(18 hours substrate incubation at 4 °C)

● infected sap
○ healthy sap





antibodies to healthy antigens present in the antiserum used for this study.

(ii) Comparision between absorbed and nonabsorbed antisera to improve sensitivity of ELISA

Antiserum produced for PV1 was absorbed with an equal volume of a partially purified *P. edulis cv. flavicarpa* preparation from the Triton X-100 method and incubated for 2 hr at 37°C. The mixture was incubated for a further 16 hr at 4°C and the supernatant collected after centrifugation at 6,000 x g for 30 min (Chester, 1936). Viral antigens were prepared from *P. foetida* and *P. flavicapa* leaves and $^{1}/_{500}$ (v/v) and $^{1}/_{5000}$ (v/v) dilutions were made in carbonate buffer. Absorbed and unabsorbed antisera were compared at 1:20,000 dilution. The dilution of protein A - conjugate was 1:2,500. Results are given in Table 21.

Table 21: Absorbance data obtained with absorbed and unabsorbed antisera

Antigen	absorbed serum				unabsorbed serum			
Dilutions	PfoD	PfoH	PflD	PflH	PfoD	PfoH	PflD	PflH
1:500	1.368	0.038	0.238	0.085	2.000	0.113	0.952	0.462
1:5,000	1.000	0.043	0.214	0.082	1.646	0.123	0.469	0.349

Abbreviation:

Pfl - *P. edulis cv. flavicarpa*

Pfo - *P. foetida*

H - healthy

D - diseased

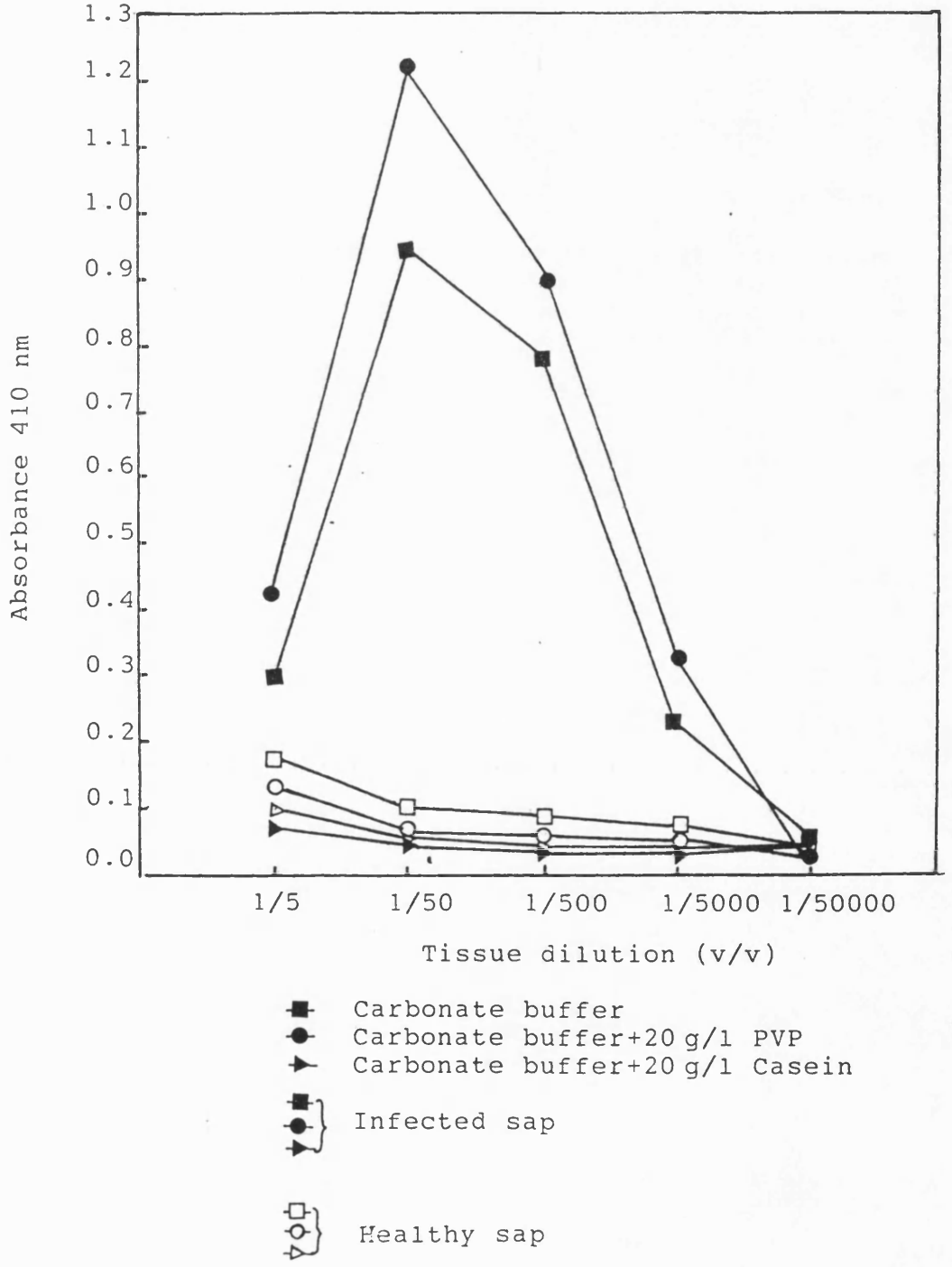
Both *P. foetida* and *P. flavicarpa* gave positive reaction with absorbed antiserum at 1/500 and 1/5000 dilutions. On the other hand, unabsorbed antiserum failed to detect the presence of virus at 1/5000 dilution of sap extracted from *P. flavicarpa*. Background values were lower in absorbed serum than unabsorbed serum, although positive values were correspondingly reduced. Subsequent studies by ELISA, however, used absorbed serum at 1:20,000 dilution, on the assumption that it is generally better to have weak negatives rather than strong positives (Clark *et al.*, 1986).

(iii) Effect of extraction medium on the detection of PV1 by indirect ELISA

Carbonate buffer pH 9.6 (Koenig, 1981), carbonate buffer plus 20 g/l soluble PVP (Clark and Adams, 1977) and carbonate buffer plus 20 g/l casein (Kenna *et al.*, 1985) were compared for detection of virus in leaf tissues of PV1 infected *P. flavicarpa*. The antigen dilutions of 1/5 to 1/50,000 were made in each buffer. The dilution of protein A-conjugate was 1:2,500 and the results are presented in Fig. 8.

At all dilutions, carbonate buffer plus 20 g/l PVP gave a positive reaction. Extracts made in coating buffer plus casein failed to give a positive reading. This may be due to less efficient binding of antigens to the ELISA plate in the presence of casein as backgrounds were not noticeably higher than the others. Accordingly,

Fig.8 Effect of extraction medium on the detection of PV1 in Passiflora edulis cv.flavicarpa leaves. (18 hours substrate incubation at 4 C)



carbonate buffer pH 9.6 with 20 g/l PVP was adopted as the standard procedure for indirect ELISA when *P. flavicarpa* was used as the source of test antigens.

(iv) Influence of leaf age on detection of PV1 in *P.edulis cv. flavicarpa* by indirect-ELISA

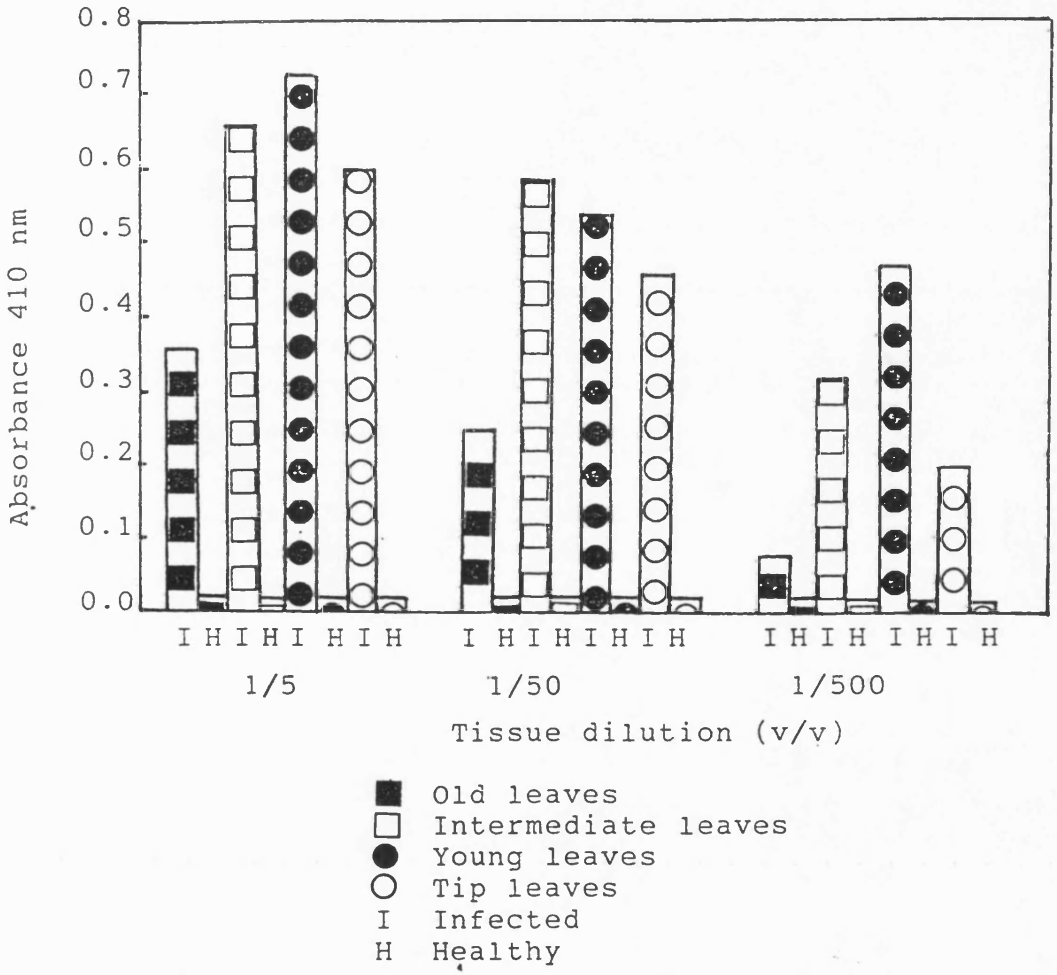
In this experiment, old, intermediate, young and tip leaves selected from the base, middle, top and tip of one shoot, were compared. Tissues were extracted using carbonate buffer with 20 g/l PVP, and dilutions of 1/50, 1/500 and 1/5,000 were used. Similar dilutions were made of apparently healthy leaf tissues as controls. Antiserum dilution was 1:20,000 and protein A-conjugate diluted to 1:2,500 and the results are presented in Fig. 9.

The results showed that there was little difference between young or intermediate aged leaves, but both apparently contained more antigen than old leaves. Very young leaves (tip) contained slightly lower levels than young leaves. Old leaves seemed to be a poor source for ELISA. The results suggested that young or intermediate leaves of *P. flavicarpa* were the best source of antigen for ELISA.

(v) Effect of extraction buffer on detection of PV1 in *P. foetida* by indirect ELISA

The following buffer / additive combinations, were tested: sodium carbonate with 20 g/l PVP, 0.05 M

Fig.9 Mean ELISA values for assay on leaves collected from healthy and PV1-infected Passiflora edulis cv. flavicarpa leaves.
(18 hours substrate incubation at 4 C)



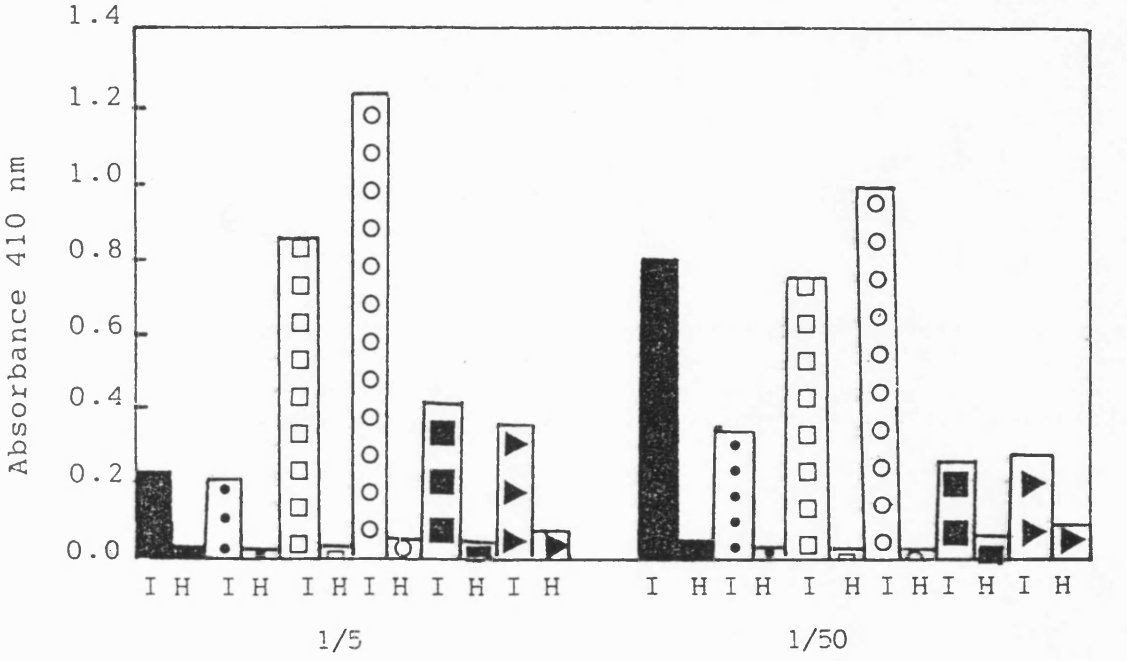
phosphate (pH 8.4), 0.5 M phosphate with 5 g/l Na_2SO_3 (pH 8.4), 0.02 M tris-HCl pH 7.8 and 0.02 M tris-HCl pH 7.8 with 1 g/l Na_2SO_3. Antiserum dilution and protein A-conjugate dilutions were 1:20,000 and 1:2,500 respectively. Results are presented in Fig. 10.

The highest A410 nm values were obtained when sap was extracted in phosphate buffer with 5 g/l Na_2SO_3 at a 1/5 dilution. Phosphate and tris buffer, with or without additives, produced higher absorbance values at 1/5 dilution than 1/50. This suggested these buffers were able to reduce the effects of inhibitors apparent when carbonate buffer was used. The addition of 20 g/l PVP (a phenol antagonist) to carbonate buffer did not increase ELISA values, with sap extracts from *P. foetida*. This contrasted with the effect of adding the anti-oxidant sodium sulphite to phosphate buffer.

(vi) Effect of tissue source on detection of PV1 by indirect ELISA

Leaves, immature stem pieces, and flower petals were collected from virus-infected and apparently healthy *P. foetida* plants, growing in a glasshouse. Equal weight portions from each tissues were ground in a mortar with a pestle, in the presence of 0.05 M carbonate pH 9.6 with 20 g/l PVP. Extracts were centrifuged at low speed (3,000 x g, 10 min) to remove large plant debris. The partially-clarified extracts were diluted in the same buffer to make 1/5 to 1/50,000 dilutions. Antiserum and

Fig. 10 Effect of extraction buffer on the detection of PV1 in Passiflora foetida leaves by indirect ELISA.
(3 hours substrate incubation)



■ Carbonate buffer
• Carbonate buffer +20 g/l PVP
□ 0.5 M Phosphate buffer pH 8.4
○ 0.5 M Phosphate buffer pH 8.4 +5 g/l Na_2SO_3
■ 0.02 M Tris-HCL pH 7.8 buffer
▶ 0.02 M Tris-HCL pH 7.8 buffer +1g/l Na_2SO_3
I Infected sap
H Healthy sap

protein A dilutions 1:20,000 and 1:2,500 respectively, were used. The following results were obtained (Fig. 11).

Sap extracted from leaves produced higher A410 nm values than extracts of stem pieces and flowers. It was difficult to detect the presence of virus in flower petals. The highest absorbance was obtained at 1/500 dilution and a strong 'prozone' effect was apparent with leaf extract. It seems that sap from immature stem pieces or flower petals is not a reliable source of virus for ELISA in this test.

3.1.8 d) Serological cross-reaction between PV1 and other potyviruses

Preliminary data (Chapter 3.1.7) suggested that PV1 belonged to the potyvirus group. Experiments were done, therefore, to determine whether PV1 would cross react with antisera to known potyviruses. This was tested using the indirect form of ELISA described by Mowat and Dawson (1987).

Antigens were prepared from PV1 infected *P.foetida*. Two dilutions of antigen; 1/500 and 1/5,000 were made but three dilutions of antiserum were prepared in PBS-TPO buffer. Protein-A dilution was 1:2,500 and the plates were incubated with substrate buffer at 24°C for 6 hr. The following results were obtained (Table 22). Reaction of PV1 to its antiserum (column one) was included as control.

Fig. 11 Effect of tissue components on the detection of PV1 by indirect ELISA.
(18 hours substrate incubation at 4 °C)

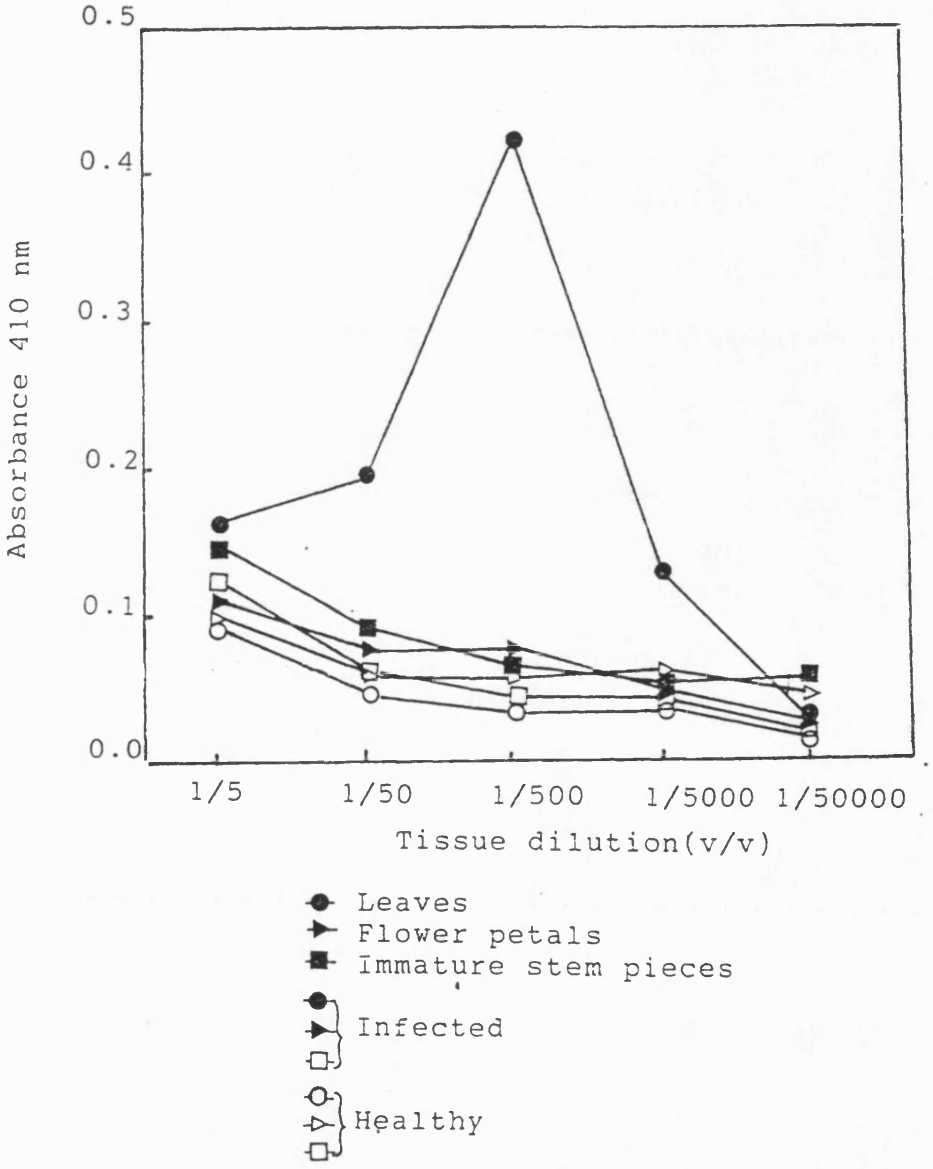


Table 22: Reaction between PV1 antigen and antisera to other potyviruses

Antigen dilution	PV1 antiserum (1:20,000)		(1:5,000)		PRV Antiserum Antiserum dilution (1:10,000)		(1:20,000)	
PV1	D	H	D	H	D	H	D	H
1:500	1.214	0.023	0.715	0.114	0.377	0.087	0.257	0.068
1:5,000	1.039	0.021	0.603	0.134	0.355	0.085	0.242	0.067
					PWV Antiserum			
1:500	1.271	0.019	1.243	0.330	0.699	0.195	0.379	0.119
1:5,000	1.126	0.023	1.248	0.334	0.768	0.209	0.420	0.128
					PVY Antiserum			
1:500	0.655	0.009	1.054	0.117	0.226	0.009	0.089	0.003
1:5,000	0.356	0.012	0.769	0.138	0.157	0.009	0.072	0.019
					WMV Antiserum			
1:500	0.945	0.019	0.282	0.160	0.160	0.098	0.101	0.075
1:5,000	0.806	0.017	0.294	0.138	0.177	0.086	0.115	0.052
					BCMV Antiserum			
1:500	0.563	0.031	0.614	0.537	0.177	0.145	0.148	0.112
1:5,000	0.209	0.017	0.671	0.561	0.188	0.158	0.160	0.113

Abbreviations

PRV - Passion fruit ringspot virus
PWV - Passion fruit woodiness virus
PVY - Potato virus Y
WMV - Watermelon mosaic virus
BCMV - Bean common mosaic virus
D - diseased
H - healthy

When antigens of PV1 were reacted against antisera to PRV (1:4,096), PWV, PVY, WMV and BCMV (1:4,096) in indirect ELISA, PRV, PWV and PVY gave positive reactions while WMV gave a borderline reaction at 1/5,000 antigen dilution. These results suggested that PV1 was related to those viruses, but without the homologous antigens it was impossible to assess this relationship further.

3.1.9 Double-stranded RNA analysis

Passion fruit leaves of *P. edulis cv. flavicarpa, P. foetida, P. quadrangularis* and *P. mollissima* were used for the extraction (4 - 30 g tissues. A single band of double stranded RNA was extracted from *P. foetida* and *P. quadrangularis*. *Passiflora mollissima* was not a good source for dsRNA extraction. The fluorescent band above the normal DNA band was identified as a stained dsRNA segment, because fluorescence was not lost after incubation at 4°C for 18 hr in DNase treatment and incubation at 25°C for 3 hr in 'high' salt RNase treatment. It was digested after incubation in 'low' salt RNase treatment within 1 hr at 25°C. The fluorescent intensity of the dsRNA band extracted from *P.quadrangularis* was higher than that for *P. foetida*, with comparable tissue weights.The average molecular weight of PV1 dsRNA found in four separate extractions was 7.0 x 10^6 daltons using λDNA as a standard.

3.1.10 Estimation of the capsid protein molecular weight

The polypeptide molecular weight of virus coat-protein is a useful criterion for the identification and classification of viruses (Hamilton *et al.*, 1981).

Viral coat proteins were analysed by gel electrophoresis in 10% polyacrylamide gels containing SDS. All preparations separated into two molecular weight components. The values estimated for the two viral coat protein forms were 33192(± 622) daltons and 25,701 (± 1.5) daltons. Some preparations had a higher molecular weight band (68,217) when electrophoresed in SDS gel. This could be possibly due to dimer formation of some viral coat protein, or inclusion protein. The molecular weight for inclusion proteins of potyviruses varied from 67,000-70,000 for different viruses (Hiebert and McDonald, 1973).

3.1.11 *In-vitro* propagation

White (1934) was the first to examine the possibilities of growing plant viruses in tissue culture. A number of different plant viruses has now been propagated in cultured tissues.

Results of the *in-vitro* propagation indicated (Table 23) that PV1 infected *P. mollissima* performed well in the MS medium containing 60 g/l agar, 20 g/l sugar and 0.05 µm BAP (Plate 14). PV1 was maintained in *P.*

Plate 14: Nodal explant of *Passiflora mollissima* infected with PVl grown *in vitro*.

mollissima in-vitro for up to 8 weeks which was the length of the experiment.

Table 23: *In-vitro* propagation of *P. mollissima* infected with PV1

Weeks after culture	* Mean number of shoots	roots	leaves	Mean height (cm)	Colour of explants
2	1.5	0	4.2	3.2	dark green
4	1.7	0.3	4.7	5.7	dark green

3.1.12 Return inoculation of PV1 to *Passiflora edulis cv. flavicarpa*

Attempts to satisfy Koch's postulates by return inoculation were successful for PV1. In the case of *P. edulis cv. flavicarpa* inoculated with a single lesion culture of PV1, symptoms similar to those observed in the field were produced. A virus with the same host range and reaction, and other properties as PV1 was isolated from diseased plants.

3.2 Isolation and identification of passion fruit virus 2 (PV2)

3.2.1 Field symptoms

Virus was isolated from two year old *P. caerulea*, an ornamental species obtained from In-service Training Institute, Bombuwela, Sri Lanka. Leaf symptoms

of this disease were conspicuous yellow spots and flecking (Plate 15). In addition to the leaves, flecking symptoms were also present on sepals and 'leaf appendages'. Leaf symptoms were present throughout the year. Vigour of the plant was apparently not markedly affected.

3.2.2 Isolation from *Passiflora caerulea*

Virus was successfully transmitted from mature leaves of *P. caerulea* ground in 1:5 (w/v) 0.05 M phosphate buffer containing 100 g/l Polyclar AT to a range of test plants including *Chenopodium* spp., legume spp. and *Passiflora* spp. *Chenopodium amaranticolor* was found as a suitable local lesion host to establish pure cultures of PV2. After three successive local lesion passages through *C. amaranticolor,* virus was multiplied in *C. quinoa* and maintained in *P. foetida* and *P. flavicarpa*. Virus-infected *P. foetida* was used for the subsequent transmission studies, unless otherwise stated.

3.2.2 a) Comparison between sources of inoculum

Virus-infected young leaves, with symptoms, intermediate leaves and flower petals of *P. caerulea* were compared as sources of inoculum. These different types of tissues were chopped, mixed and divided into two equal weight portions. Each portion was ground in either 1:5 w/v or 1:10 w/v. in 0.05 M phosphate buffer pH 7.8 with 100 g/l Polyclar AT. Treatments were assayed according to a 6 x 6 Latin square design using *C. amaranticolor* test

Plate 15: Yellow flecks on Passiflora caerulea infected with PV2 in the field.

plants. Local lesions were counted after 3 weeks and results are given in Table 24.

Table 24: Comparison of sources of inoculum for transmission of PV2 from *P. caerulea* to *C. amaranticolor*

Sources of inoculum	Mean no of lesions/leaf dilutions	
	1:5	1:10
young leaves	2 b	10 b
intermediate leaves	68 a	39 a
flower petals	0 c	0 c
cv%	21	

The above results showed that intermediate leaves at 1:5 and 1:10 dilutions provided more infectious inoculum than young leaves and the difference was significant ($P < 0.05$). Virus infection was not detected in flower petals.

A further experiment using the same buffer compared the infectivity of extracts of petals, sepals and old leaves. Infectivity was too low (mean number of lesions <1) for all treatments to draw meaningful conclusions. Some virus was, however, detected in sepal tissue though not in petals.

3.2.2 b) Effect of different buffers on the isolation of PV2

Infected *P. foetida* leaves were chopped into small pieces and mixed well. The sample was divided into four portions of equal weight and ground with different buffers in 1:10 (w/v) dilutions. *Chenopodium amaranticolor* plants were used as an assay host and treatments were distributed according to a 4 x 4 Latin square design. Lesion counts were made 3 weeks after inoculation (Table 25).

Table 25: Effect of different buffers on the isolation of passion fruit virus 2 (PV2)

Buffers	Mean no of lesions per leaf
Sodium borate	28 ab
Sodium citrate	42 ab
Phosphate	6 c
Tris-HCl	54 a
cv%	9.8

According to Table 25, extracts prepared in 0.02 M tris-HCl buffer at pH 7.8, gave highest mean lesion number although the differences between tris-HCl borate and citrate were not significant ($P > 0.05$). Inoculum extracts of phosphate buffer produced significantly fewer ($P < 0.05$) lesions than other treatments. On the basis of these results tris-HCl was

used when *P. foetida* extracts were used to inoculate *C. amaranticolor.*

3.2.2 c) Effect of buffer additives on the isolation of PV2

Infected *P. foetida* leaves were cut into small pieces and mixed thoroughly. The sample was divided into seven equal weight portions. Each portion was then ground, 1:10 (w/v) in 0.02 M tris-HCl buffer pH 7.8 with various additives. Buffer without additives was used as control. Treatments were inoculated to *C. amaranticolor* plants according to a 7 x 7 Latin square design. Lesions were counted 3 weeks after inoculation (Table 26)

Table 26: Effect of additives to 0.02 M tris-HCl, pH 7.8 buffer on infectivity of PV2

Additives	Mean no of lesions/leaf
Buffer only	16 a
Sodium sulphite 1 g/l	14 a
PVP 10 g/l	2 bd
Sodium thioglycollate 1 g/l	5 bc
EDTA 0.01 M	4 bc
Polyethylene glycol 1 g/l	2 bd
Bentonite 10 g/l	0 e
cv%	5.3

According to the above results 0.02 M tris-HCl pH 7.8 buffer with or without 1 g/l sodium sulphite produced more lesions than any other treatment ($P < 0.01$)

although buffer with sodium sulphite was not significantly ($P > 0.01$) better than buffer alone.

3.2.2 d) Effect of post-inoculation temperature on lesion development

A study of the effect of post-inoculation temperature on the susceptibility of *C. amaranticolor* to PV2 was done during winter. Indicator plants were grown at 22°C temperature with 16 hr day length and illumination of 3,500-4,000 Lux. Inoculum was prepared by maceration of *P. foetida* leaves in 0.02 M tris-HCl buffer pH 7.8 containing 1 g/l Na_2So_3. Six plants of *C. amaranticolor* was used for each treatment. Plants were kept in the dark for 24 hr before inoculation. Immediately after inoculation they were kept at average temperatures of 10, 15, 27 and 29°C in separate glasshouses. Plants were covered with damp newspaper overnight after inoculation. The following results were obtained (Table 27).

Table 27: Influence of post-inoculation temperature on lesion development in *C. amaranticolor* inoculated with PV2

Temperature	Mean no of lesions/plant
29	79 a
22	130 a
15	11 b
10	5 b
cv%	16

Results indicated that plants grown in glasshouses at higher temperatures after inoculation induced significantly ($P < 0.05$) more lesions, than those grown in glasshouses at lower temperatures. Lesions produced by plants grown at 22°C were more distinct than those grown at lower temperatures. Thereafter, *C. amaranticolor* plants were routinely kept at about 22°C after inoculation for assay purpose.

3.2.3 Host plant response

Virus-infected *P. foetida* leaves were extracted with 0.02 M tris-HCl buffer, pH 7.8 (0.10 w/v) containing 1 g/l Na_2SO_3 or 0.05 M phosphate buffer pH 7.8 with 100 g/l Polyclar AT.

Inoculated plants were examined periodically for symptoms for 2-3 months. Species with or without symptoms were inoculated to *C. amaranticolor* and *P. foetida* to confirm infection. The reactions of all species are given in Table 28. The reactions of selected hosts are further described below.

Passiflora foetida

Young leaves developed vein-yellowing and epinasty 7-10 days after inoculation. Two weeks after inoculation, terminal shoots were stunted, defoliation and stem necrosis were also observed (Plate 16). Young leaves were frequently curled, twisted and deformed. When the disease spread systemically, the plants showed

Plate 16: (a) Terminal shoot of Passiflora foetida infected with PV2, showing vein yellowing and downward leaf curl.
(b) Defoliation symptom of P.foetida seedling infected with PV2.

(a)

(b)

severe stunting. Milder symptoms were produced about 2-3 months after inoculation. An intense yellow spotting of leaves was observed in infected leaves 4 - 5 weeks after inoculation. This symptom was somewhat similar to plants infected with PV1.

Passiflora edulis cv. flavicarpa

The first reliable symptom was the turning down of infected leaves 10 - 14 days after inoculation (Plate 17a). These leaves showed vein-clearing initially. Three to four days after vein-clearing, these leaves were severely crinkled, puckered and curled. Growth was severely reduced. Necrotic patches and vein necrosis were observed on the infected leaves. Premature defoliation occurred when the virus spread systemically. Leaves showed a range of symptoms including yellow flecking, chlorotic mottle, and terminal bunchiness (Plate 17b). Symptoms on fruits were not observed due to poor fruit-set under glasshouse conditions.

Passiflora quadrangularis

Young leaves developed vein yellowing 10-15 days after inoculation. Three to four weeks after infection vein necrosis, stem necrosis, and premature defoliation were the characteristic symptoms observed (Plate 18). A few plants died due to infection, and growth was severely reduced.

Plate 17: (a) Passiflora edulis cv. flavicarpa infected with PV2 showing initial symptoms of vein necrosis and downward bending.
(b) Terminal shoot of P. edulis cv. flavicarpa infected with PV2 showing leaf puckering, crinkling, terminal bunching and tip necrosis.

(a)

(b)

Plate 18: Vein necrosis of Passiflora quadrangulari infected with PV2.

Phaseolus vulgaris cv. Prince

Infected primary leaves produced 1 mm necrotic spots 3-4 weeks after inoculation. These spots enlarged to 3-4 mm in size, two months after inoculation and occasionally spread along the veins. Virus did not spread systemically.

Table 28: Host range of PV 2

Plant Species	Local Reaction	Systemic Reaction
Amaranthaceae		
Gomphrena globosa (Globe amaranth)	NL	-
Chenopodiaceae		
Chenopodium album	CS	-
C. amaranticolor	CS	-
C. foetidum	CMo	-
C. murale	CMo	-
C. quinoa	CMo(CS)	-
Leguminosae		
Cassia occidentalis	CMo	VY,LC,CMo
C.tora	NL	-
Crotalaria usaramoensis	CM	CM
Phaseolus vulgaris (cv. The Prince)	NL(VN)	-
P.vulgaris (cv. Top Crop)	NL	-

Vigna unguiculata (cv. Bushita mae)	NL	-
Passifloraceae		
Passiflora edulis	CM,CMo	VY,LD,CF,CMo,St
P. edulis cv. flavicarpa	CM,CMo	VY,VN,LD,CF,CMo,St
P. foetida	CM,CMo	VY,VN,LD,CMo,CS,St
P. ligularis	CMo	CMo
P. mollissima	CM,CMo	VY,LD,VN,CMo,St
P. van volxemii	CM,CMo	CS,CMo,LC
Solanaceae		
Nicotiana clevelandii	CS	CS
Petunia hybrida	S	-

Abbreviations

CM = Chlorotic mosaic	NL = Necrotic lesions
CMo = Chlorotic mottle	St = Stunted growth
CF = Chlorotic flecking	VN = Vein necrosis
CS = Chlorotic spots	VY = Vein yellowing
LD = Leaf distortion	S = Symptomless
LC = Leaf curl	() = Occasional symptoms

\- = No infection

Species not infected

Arachis hypogaea, *Beta vulgaris* cv. Globe, *Centrosema pubescens*, *Cucumis sativus* cvs Ly 58, Marketer, *Datura stramonium* cv. Arbotéa, *Glycine mas* cvs PBl, Bossier, *Lycopersion esculentum* cv. Delight, *Momordica charantia* cv. Mc 43, *Nicotiana debneyi*, *N. megalosiphon*, *N. tabacum*

cvs White Burly and Xanthi, *Passifora suberosa*, *Pisum sativum* cv. Meteor, *Pueraria phaseoloides*, *Spinacia oleracea* cv. Sigma leaf, *Tricosanthes anguina*, cv. LA 33, *Vicia faba* cv. Aquadulce and *Vigna unguiculata* cvs I ta, PBl, MI 35 and Bombay.

3.2.4 *In-vitro* properties

These were determined in extracts of *P. foetida* and repeated at least twice.

3.2.4 a) Longevity *in-vitro* (LIV)

Sap was infectious up to the fifth but not 6th day, when stored at room temperature.

3.2.4 b) Thermal inactivation point (TIP)

The TIP of PV2 was between 60°C and 65°C. Most infectivity was lost, however, between 55-60°C.

3.2.4 c) Dilution end-point (DEP)

The dilution end-point of virus was found be be 10^{-4}-10^{-5} (Fig. 12).

3.2.4 d) Storage characteristics of PV2

Passion fruit virus 2 remained viable when *P. foetida* leaves were stored dehydrated either over $CaCl_2$ or silica gel at -20°C for up to two months, which was the longest storage time tested (Table 29). Storage in liquid nitrogen experiments, however, gave unsatisfactory results.

Fig.12 Dilution curve of PV2 in Passiflora foetida sap

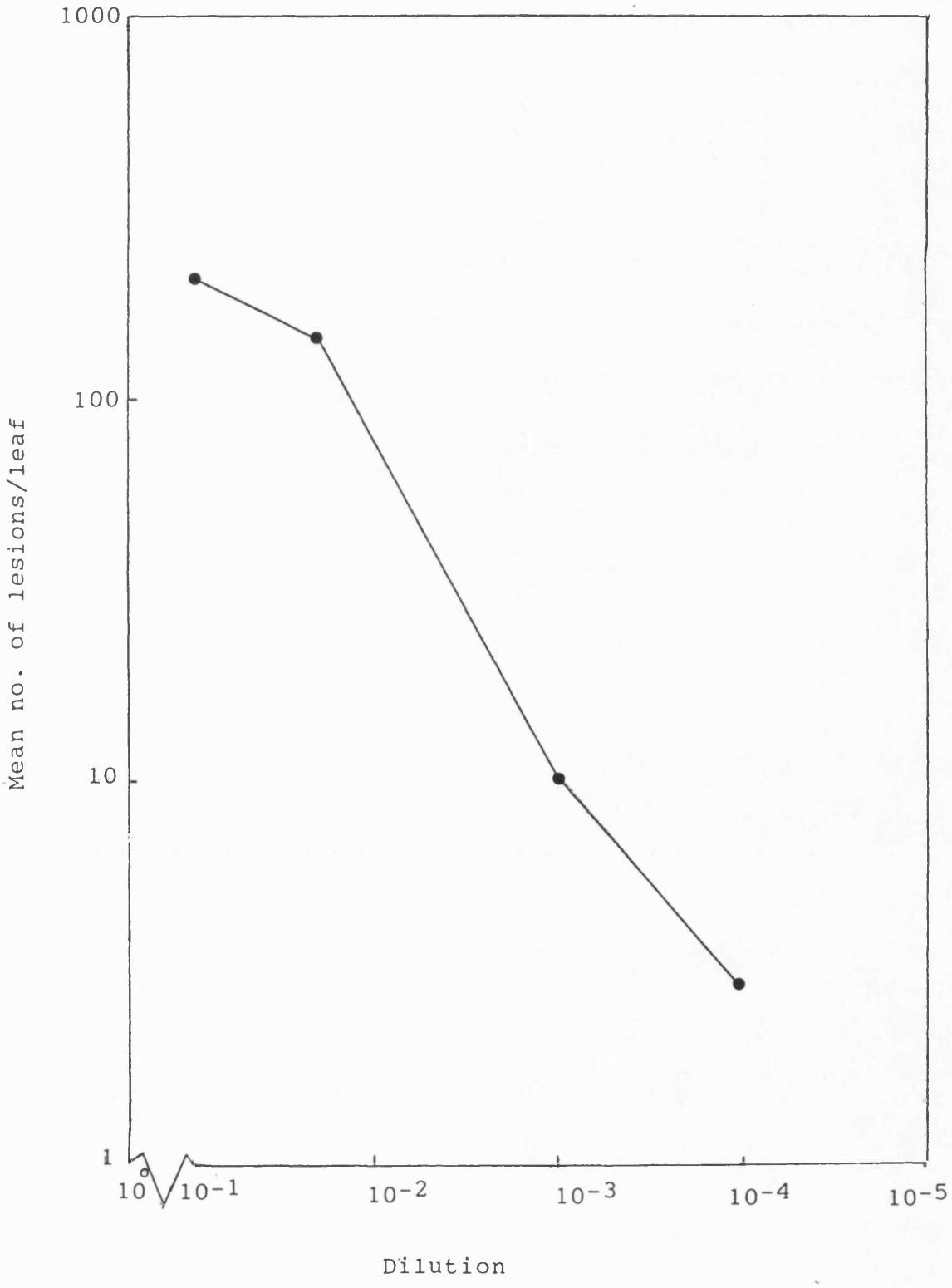


Table 29: Comparison of different methods of storage for PV2.

Storage Method	Infectivity days after storage		
	14	30	60
Silica gel, -20°C	+++	++	++
$CaCl_2$, -20°C	++	++	++
Polythene bag, -20°C	+	+	-
Silica gel, 4°C	+	+	+
$CaCl_2$, 4°C	-	-	-
Polythene bag, 4°C	-	-	-

Abbreviations

+++	moderate	+	trace
++	weak	-	no infectivity

3.2.5 a) Purification

Following the successful purification of PV1, n-butanol and Triton X-100 methods were compared for PV2.

Twenty-five grams of freshly harvested *P. foetida* was chopped into small pieces, mixed thoroughly and then divided into two portions of equal weight. One portion was subjected to 8.5% n-butanol (v/v) clarification followed by 60 g/l PEG precipitation method as described for PV1 (Chapter 3.1.5).

The second portion was purified using Triton X-100 and 40 g/l PEG, according to the method described in Chapter 3.1.5. Partially purified virus was assayed by inoculating opposite half leaves of *Phaseolus vulgaris* cv. Prince with each treatment. In addition, the colour and 260:280 nm ratio were determined. The results are presented in Table 30.

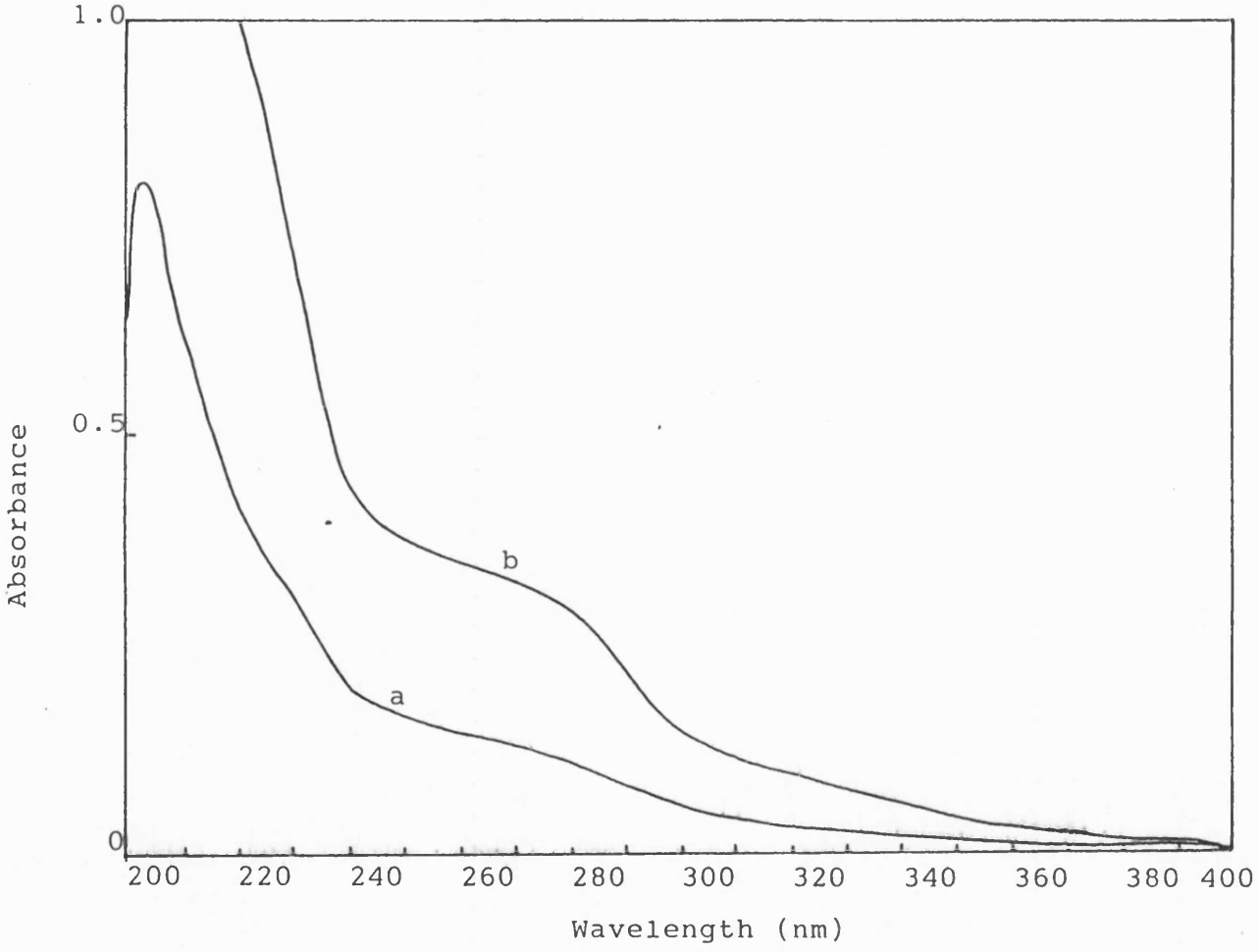
Table 30: Comparison of n-butanol and Triton X-100 method

Method of Purification	Colour of purified preparation	Mean no of lesions per half leaf *	260 / 280 ratio (uncorrected)
Butanol/PEG	light yellow	6	1.34
Triton X-100 /PEG	light yellow	171	1.26

* Mean number of 8 half-leaves

Results suggested the purification method of Hammond and Lawson, (1988) using Triton X-100 was more effective than the n-butanol method. The colour of the partially-purified preparation was satisfactory in both methods. However, the number of lesions produced by butanol clarified preparation was lower than the Triton X-100 method. The 260 / 280 ratio was typical for a rod-shaped virus with about 5% nucleic acid with either purification method (Fig. 13). Subsequent purification of PV2 was routinely done using Triton X-100 clarification.

Fig.13 Absorption spectrum of partially-purified preparations of PV2.
(a)Butanol/PEG method
(b)Triton x-100/PEG method
(samples diluted to 1:100)



3.2.5 b) Density gradient centrifugation

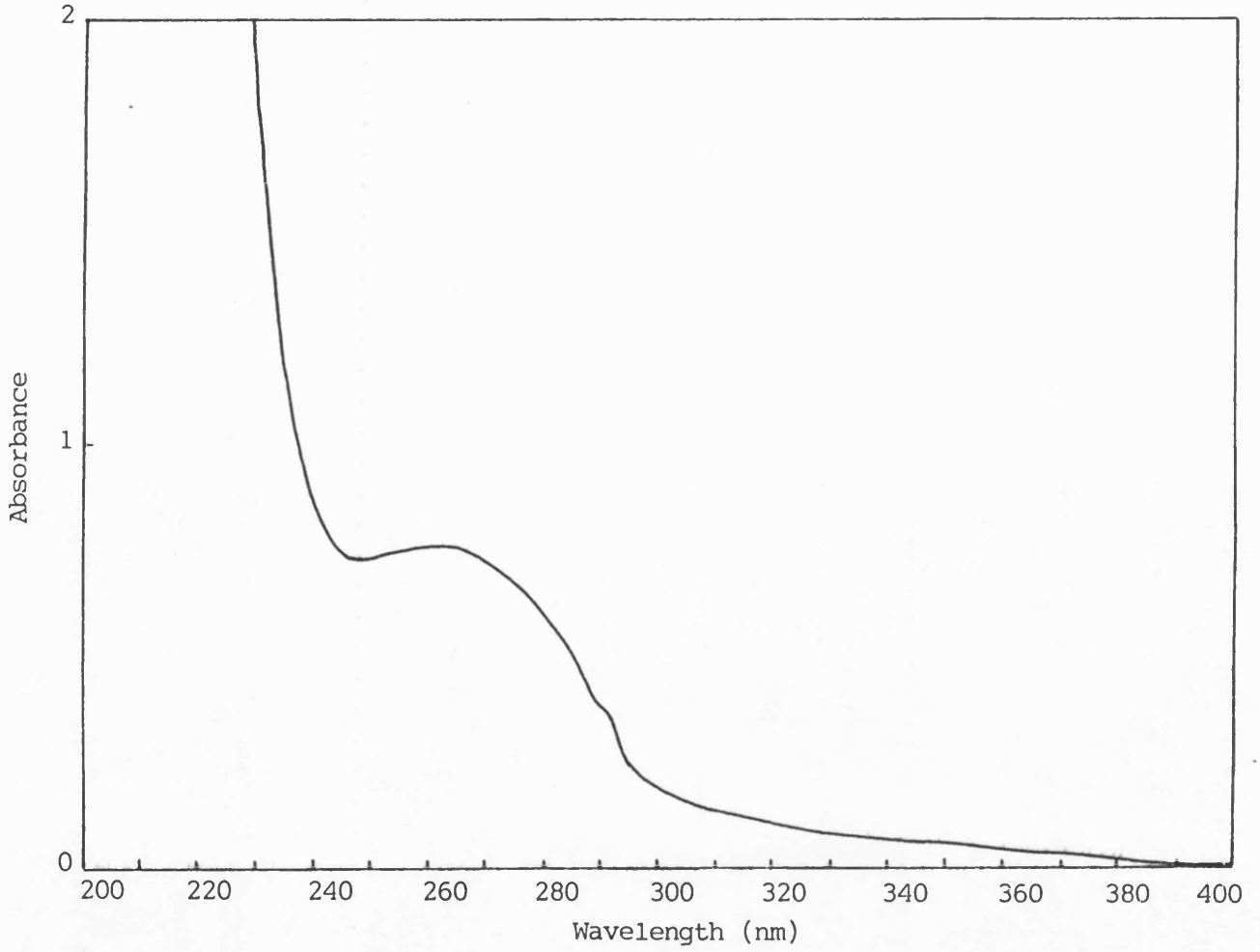
To obtain highly purified virus for the production of antisera and to study the physical / chemical properties, partially-purified virus from the Triton X-100 method was centrifuged in a caesium sulphate gradient. Density gradient centrifugation was done according to Hammond and Lawson (1988).

After 18-20 hr centrifugation in the gradient, most of the contaminated material was separated from the main virus band, and did not interfere with the collection of the virus fraction. A single virus-containing band sedimented 4.4 - 4.5 cm below the meniscus in caesium sulphate gradient was opalescent and clearly visible when viewed with a torch against a dark background. Presence of virus was confirmed by infectivity assay on *C. amaranticolor* and by electron microscopy when many flexuous rods were seen. After final purification 0.5-3mg and 10-15 mg per 100 gms were obtained for *P. flavicarpe* and *P. foetida*, respectively.

Ultraviolet light absorption

The 260/280 ratio of highly purified virus was 1.30 - 1.33 before correction for light scattering. A slight shoulder at 290 nm was a characteristic feature in the spectrum (Fig. 14) and may indicate the presence of tryptophan. This is not unusual for viruses of the potyvirus group (Hollings and Brunt 1981).

Fig.14 Absorbance spectrum of purified preparation of PV2 from Passiflora foetida (sample diluted to 1/100).



3.2.6 Light microscopy

Sections through a vascular bundle of leaves and stems of infected *P. edulis cv. flavicarpa* had an appearance similar to those through a normal leaf and stem. The most prominent feature observed in the phloem and xylem was brown coloured cells. The study revealed that the primary internal symptoms of the disease appeared in the vascular tissues, and secondarily in adjacent tissues. The necrotic cells generally stained heavily.

Stained epidermal tissues (O - G combination) of diseased *P. foetida* contained cylindrical inclusions. These were not found in healthy *P. foetida* tissues. However, these inclusions were unstained with Azure A (Plate 19).

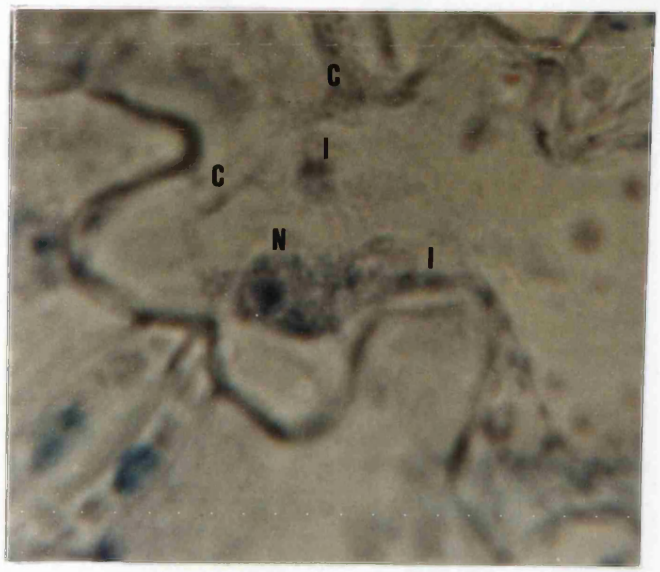
3.2.7 Electron microscopy

3.2.7 a) Leaf-squash preparations

Leaf squash preparations of infected *P. foetida and P. flavicarpa* contained flexuous rods, finely striated bundles of rods and remnants of cellular organelles. Some preparations also showed long rod-like structures, possibly from cylindrical inclusions (Plate 20).

Plate 19:(a) Passiflora foetida epidermal cell infected with PV2 containing irregular cytoplasmic inclusions (I) and nucleus (N). Possible cylindrical inclusions (C) are unstained. Stained with Azure A.X 1000.
(b)Healthy epidermal cells with the same treatment.

(a)



(b)

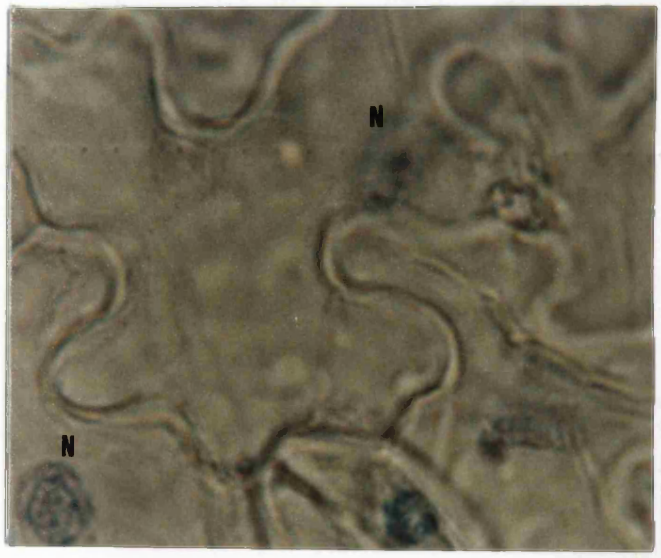
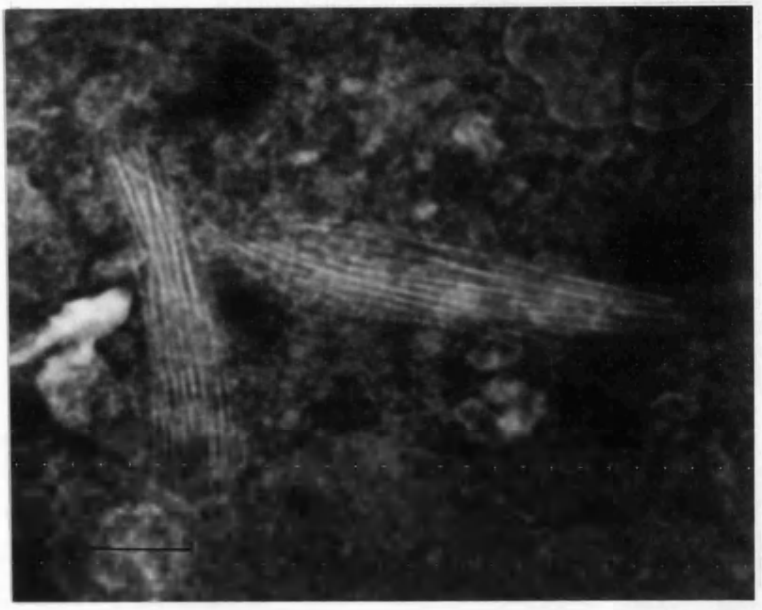
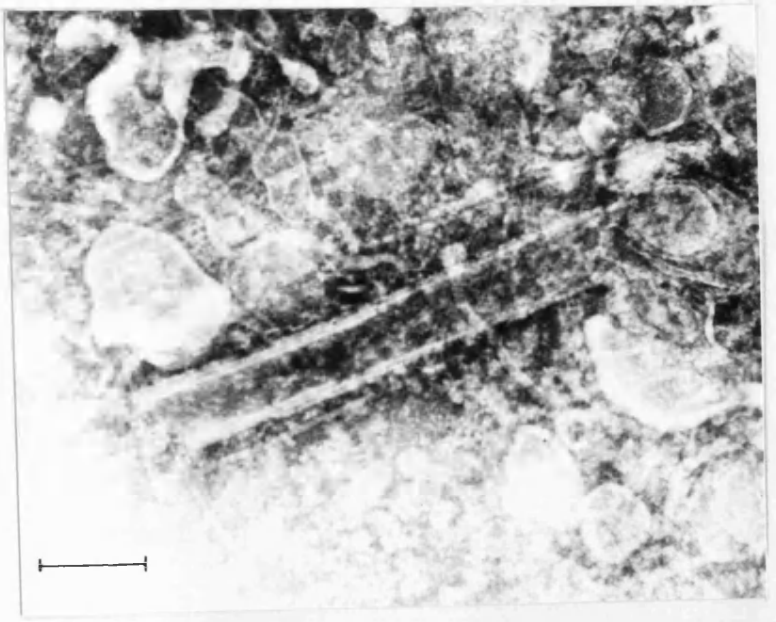


Plate 20: Leaf squash preparations of Passiflora foetida infected with PV2 showing bundles of virus particles (a), cylindrical inclusions, virus particles and cell remnants (b); stained in 20 g/l PTA pH 6.5.
The bars represent 200 nm.

(a)



(b)



3.2.7 b) Particle length of PV2

The electron micrograph negatives from leaf squash preparations were placed over a light box and measurements of length and width were made of 137 and 50 particles respectively. Of 137 particles measured, 75% were between 600-930 nm, 17% were below 600 nm and 8% above 1000 nm. It was considered that the particles below 700 nm were fragments of rods formed during the staining process while those above 1,000 nm may have been end-to-end aggregated particles. In calculating normal length, only those between the main peak (760-960 nm) were included. The normal length (mean of the main peak) of PV2 was found to be 867 nm ± 5.6 (Fig. 15) and 12 nm ± 0.34 wide.

3.2.7 c) Purified preparations

Purified virus preparations after caesium sulphate gradient were high yielding, and free of most host material (Plate 21). Virus particles were observed in both partially-purified and caesium sulphate purified preparations.

3.2.7 d) Ultrathin sections

In ultrathin sections of *P. edulis* cv. *flavicarpa* leaf tissue, similar types of inclusion bodies were found in both electron and light microscopes. No virus particles, however, were found in ultrathin sections of infected *P. foetida* or *P. flavicarpa*. In

Fig.15 Distribution of particle length for PV2 in a leaf squash homogenate. (class interval 1:33.3nm).

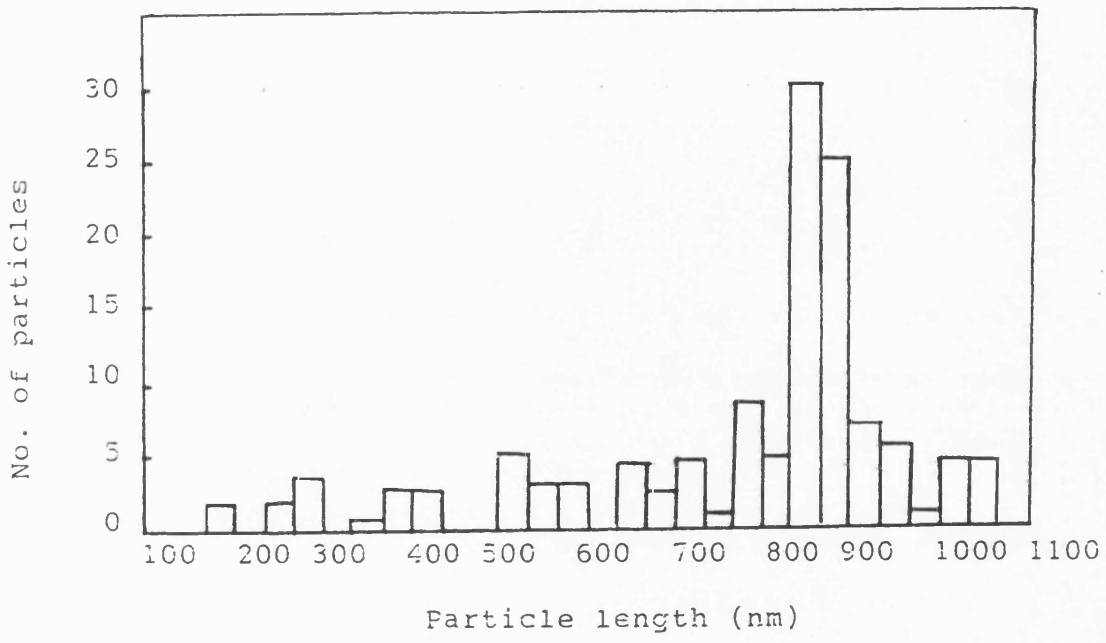
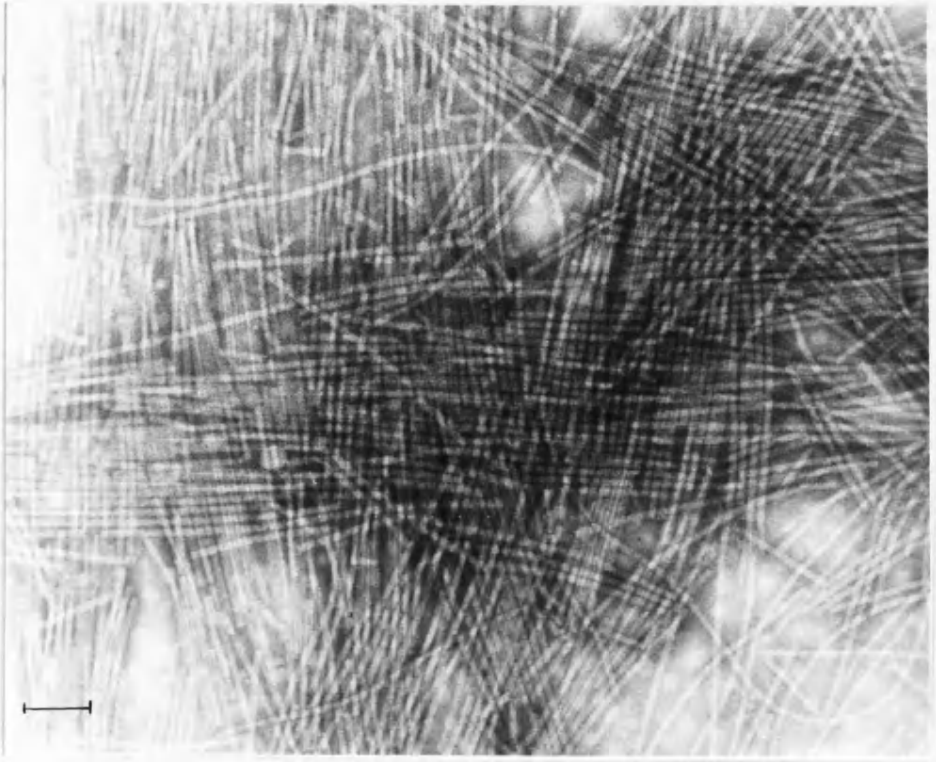
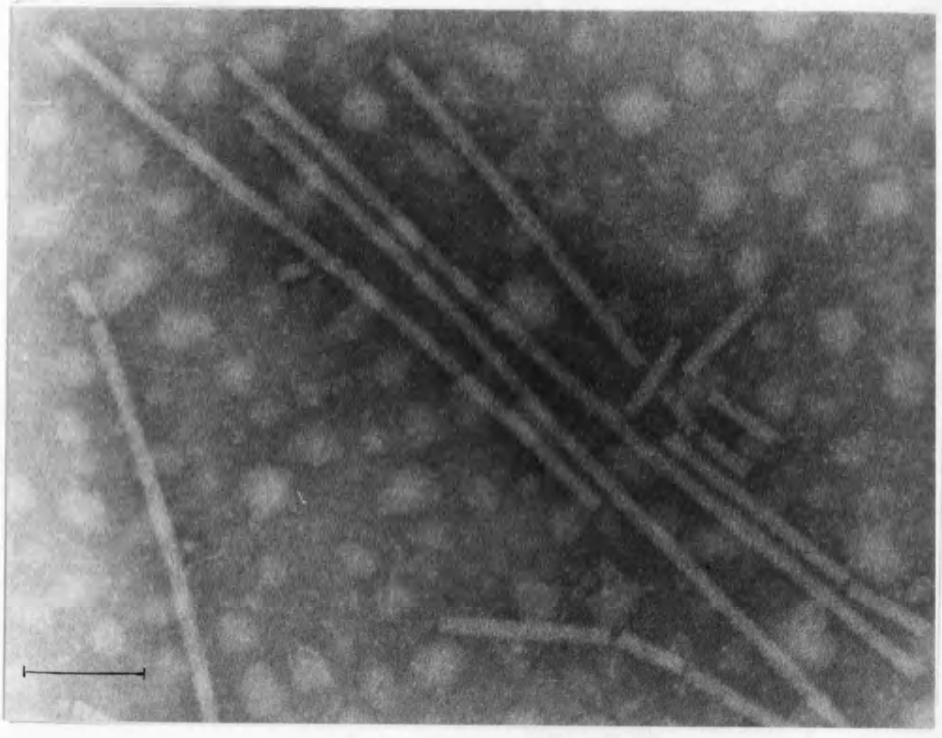


Plate 21: (a) Electron micrograp of purified PV2 from Passiflora foetida after caesium sulphate density gradient centrifugation stained in 20 g/l PTA pH 6.5.
(b) Higher magnification of (a).
The bars represent 100 nm.

(a)



(b)



thin sections of PV2 infected *P. edulis cv. flavicarpa* leaves, both tubular and laminated aggregate inclusions were found. In some areas, plates of various thickness were orientated in the form of laminated aggregates and scrolls, with the laminated aggregates predominating. In the same areas of tissue, thin curved plates converged on a central axis to form 'pinwheels' (Plate 22).

3.2.8 Serology

3.2.8 a) Preparation of antiserum to PV2

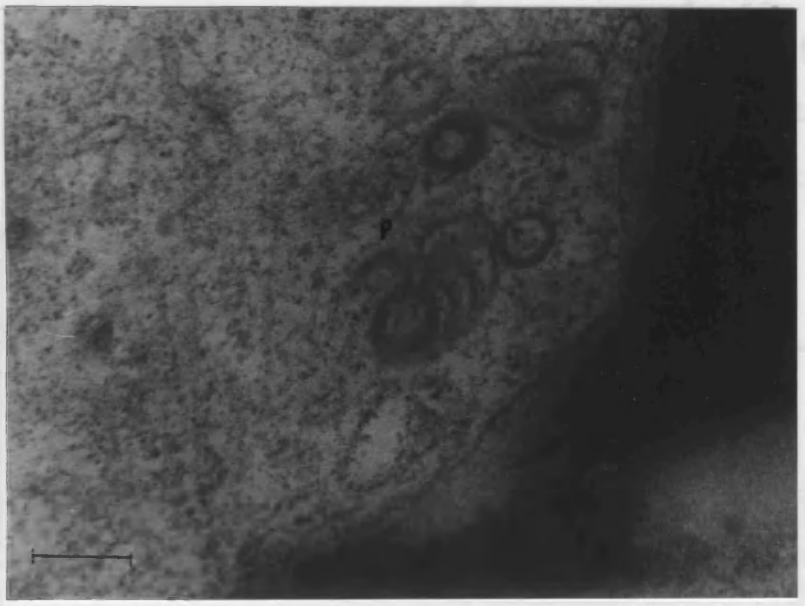
A preparation of virus obtained by Triton X-100 clarification, PEG precipitation, and caesium sulphate gradient centrifugation was adjusted to 1 mg/ml. A rabbit received a single intravenous injection and another four intramuscular injections, one week apart, containing 1 mg each of virus. Intramuscular injections were prepared by emulsifying virus with an equal volume of Freund's incomplete adjuvant. The rabbit was bled 4 weeks and 5 weeks after the first injection. Antiserum titres were determined against homologous antigen in microprecipitin tests. Antiserum titres were 1:4,096 and 1:8,192 for the 4th and 5th bleed, respectively.

3.2.8 b) Indirect ELISA

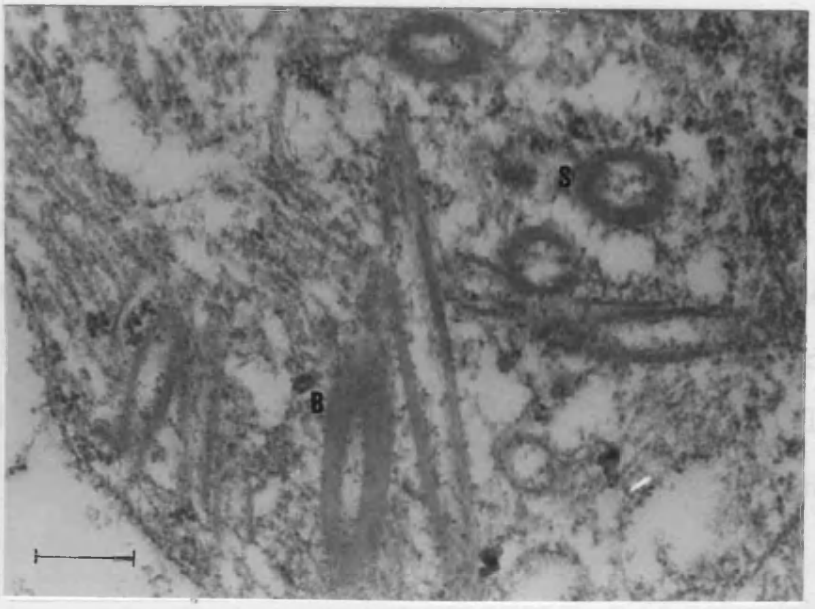
An indirect ELISA method, (plates not pre-coated with antibodies) was used to study the cross reaction between other potyviruses; - passion fruit

Plate 22: Electron micrographs of ultrathin sections of PV2 infected leaf tissue of Passiflora edulis cv. flavicarpa showing pinwheel inclusion(P), scrolls(S) and bundles (B). The bars represent 200 nm.

(a)



(b)



woodiness, passion fruit ringspot (1:4,096) Potato virus -y, water melon mosaic and bean common masaic (1:4,096).

Antigens were obtained from virus-infected *P. foetida* leaves. Two dilutions of antigens were prepared in coating buffer. Concentration of PV2 and other antisera were 1:20,000 and 1:1,000 respectively. The plate was incubated in substrate buffer at 4°C for 18 hr. The following results were obtained (Table 31).

Table 31: Cross reactions between PV2 and other potyviruses by indirect ELISA

Antigen dilutions	PV2		PWV		PRV		PVY		WMV		BCMV	
PV2	D	H	D	H	D	H	D	H	D	H	D	H
1:500 v/v	1.561	0.08	0.29	0.09	0.26	0.04	0.15	0.03	0.13	0.07	0.16	0.16
1:5,000 v/v	1.430	0.06	0.30	0.08	0.16	0.06	0.10	0.02	0.18	0.07	0.17	0.13

D = diseased

H = healthy

When the antigens of PV2 tested against antisera to PWV, PRV, PVY, WMV and BCMV in ELISA, PWV gave a clear positive reaction while PRV, PVY and WMV gave borderline positive reactions, indicating the PV2 may be related to PWV and possibly the other potyviruses, except BCMV. However, homologous antigens would be needed to confirm the degree of serological relationship, if any, and possibly higher titred antisera. The

homologous titres of most antisera were not available. Further serological studies are reported in Chapter 3.4.

When an antiserum to a passion fruit woodiness isolate of CMV(P) was tested against PV2 antigens, there was no cross reaction between PV2 and CMV(P) antiserum. This indicates that the cucumber mosaic virus seemed not to be involved in the production of severe symptoms of infected plants, as reported by Pares *et al.* (1985).

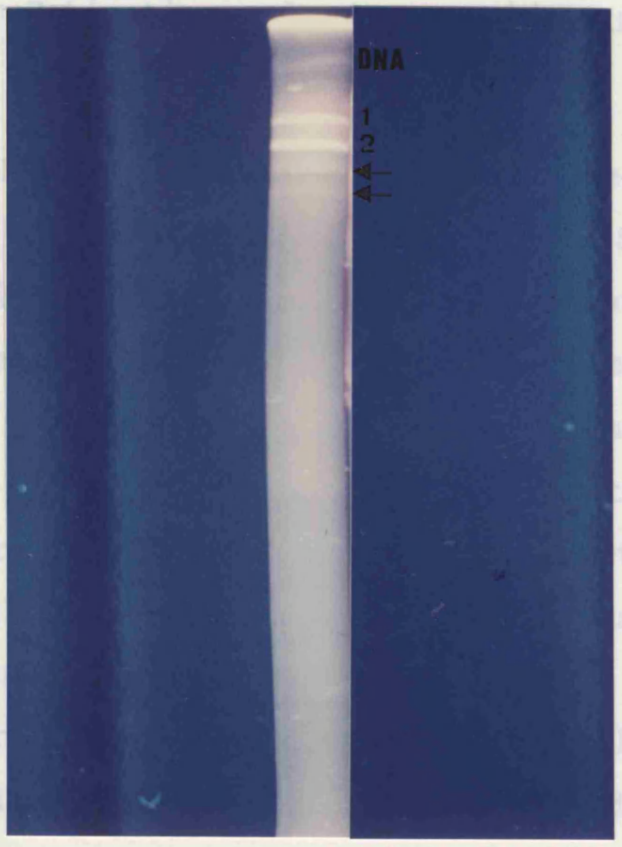
3.2.9 Double stranded RNA analysis

Double-stranded RNA was extracted from 20 g of *P. foetida* infected with PV2 (Chapter 2.19) and the sample was electrophoresed for 18 hr on 5% polycrylamide gels (1 mA/gel) and stained with ethidium bromide.

Three major bands, and two minor bands, were visible on gels after electrophoresis (Plate 23). The first band at the top of the gel, and two minor bands disappeared after 18 hr incubation at 4°C with DNase treatment (10 µg/ml). The remaining two bands were still visible after incubation with RNase (50 µg/ml) in the presence of 0.3M sodium chloride, but disappeared after incubation with RNase in water, indicating the double-stranded nature. No dsRNA was found when extracts of healthy *P. foetida* were analysed.

Using the λ Hind III digestion product of λ as standards, the molecular weights of the two dsRNA bands were approximately 7.3×10^6 and 5.0×10^6 daltons and

Plate 23: Polyacrylamide gel (5%) electrophoresis of dsRNA extracts of Passiflora foetida infected with PV2 stained in ethidium bromide. (minor bands arrowed)

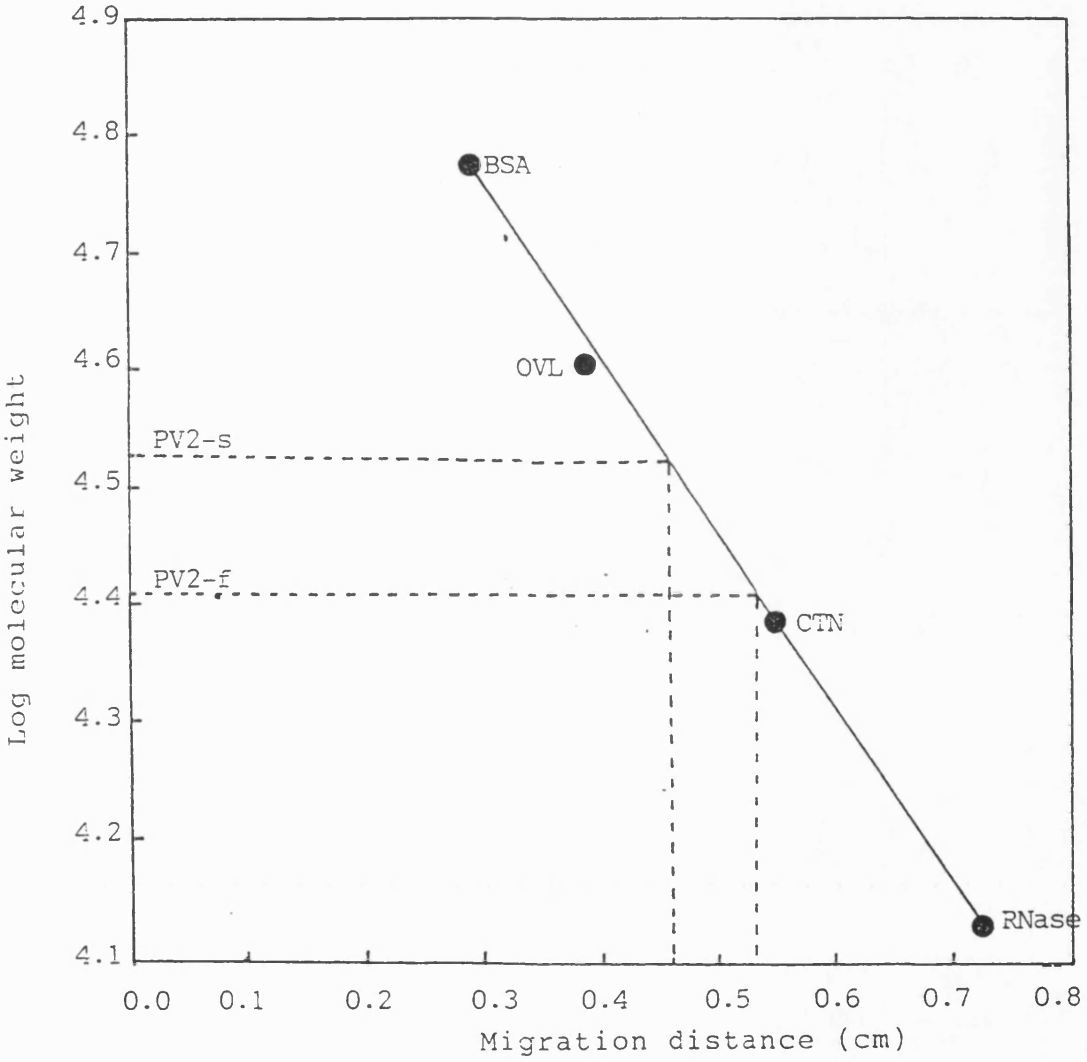


minor bands were 3.9×10^6 and 3.16×10^6. The two minor bands were probably dsRNA but were in too low concentration to restain clearly after DNase treatment. In repeated extractions it was not possible to detect dsRNA even though the sample size increased to 30 g.

3.2.10 Estimation of the capsid protein molecular weight

Most of the highly purified preparations separated into two component in 10% SDS-PAGE. The slowest migrating protein band predominated. The mean value obtained for molecular weight of the slower migrating band was 33,794 (± 1,612) daltons and for the faster migrating band was 25,654 (±1,649) daltons (Fig. 16). The faster migrating component was detected in SDS-PAGE for freshly made virus preparations as well as the preparations stored at -20°C. However, this component was present in similar proportions whether or not the sample had been stored beforehand. In addition to these two forms, a high molecular weight component (65,700 daltons) was detected in one experiment. This could be possibly due to the dimerisation of the viral coat protein or contamination with inclusion protein. The above finding of polypeptides 33,794 and 25,654 was consistent with that of other workers on potyviruses (Hollings and Brunt, 1981).

Fig.16 Determination of molecular weight of PV2 by electrophoresis on 10% polyacrylamide gels.



Abbreviations are: BSA-bovine albumin, OVL-ovalbumin
CTN-chymotrypsinogen and RNase ribonuclease with molecular weight of 66,000, 43,000, 25,000 and 13,700 respectively
PV2-s (slow) 33113
PV2-f (fast) 25,700

3.3 Isolation and identification of passion fruit virus 3 (PV3)

3.3.1 Field symptoms

The most characteristic feature of this infection was 3.5 mm sized chlorotic ringspots on intermediate and older leaves of one year old *P. edulis cv. flavicarpa*. Mottling symptoms associated with PV1 had been observed since 1982 in this field. In 1987, however, several vines started to show chlorotic ringspotting in addition to the mottling symptom in the same field (Plate 24). The symptom had been found in several other passion fruit cultivations in the Kalutara and Galle districts in Sri Lanka. Infected leaves showed chlorotic ringspots and concentric rings on leaves, especially where these were shaded. The prominent ringspotting symptom was persistent throughout the year. Mottling symptoms associated with PV1, with spotting and deformed leaves, were also observed. Infected fruits showed green and yellow mottling on the pericarp, and occasionally, green rings were also present on the skin.

Investigations were made to determine whether these new symptoms were due to environmental effects on mottle symptoms or whether it was a new virus disease or a strain of an existing virus.

Plate 24: (a) Mottling and chlorotic ringspotting on leaves of Passiflora edulis cv. flavicarpa naturally infected with PV3.
(b) Close up of ringspot symptom.

(a)

(b)

3.3.2 Isolation from *Passiflora edulis cv. flavicarpa*

Virus was isolated from young leaves of *P.edulis cv flavicarpa*. Infected leaves were macerated in 0.02 M tris-HCl pH 7.8 buffer containing 1 g/l Na_2SO_3 and inoculated to *Chenopodium* spp. legume spp. and *Passiflora* spp. *Chenopodium amaranticolor* was selected as a single lesion host as it induced well separated lesions, 10 - 14 days after inoculation. Virus was transferred through three single lesion serial passages in *C. amaranticolor* and finally in *C. quinoa*. A pure culture of PV3, maintained in *P. foetida*, was used for subsequent transmission studies.

3.3.3 Host range of (PV3)

Mechanical transmission attempts to various herbaceous and woody hosts were made by extracting infected leaf tissues in 0.02 M tris-HCl buffer containing 1 g/l Na_2SO_3 (1:10 w/v). Systemically infected *P. foetida* was used as a source of inoculum.

Plants were judged as susceptible, whenever they produced symptoms on inoculated or non-inoculated leaves within 21 days after inoculation. Inoculated plants not showing symptoms and infected plants were indexed for infection by back inoculation to *C. amaranticolor* plants. Symptoms produced on indicator plants are shown in Table 32.

Symptomatology of selected hosts are described below:

Cassia occidentalis

The first sign of infection was vein-clearing in newly developed leaves after 2-3 weeks of infection. These leaves showed slight leaf curl. When infection spread, chlorotic rings and mottling was induced in mature leaves, 4-6 weeks after inoculation (Plate 25).

Passiflora edulis cv. flavicarpa

Young developing leaves showed vein-yellowing 10-14 days after inoculation, sometimes followed by mottling and slight leaf curl. About one month after inoculation, faint chlorotic and concentric rings developed with chlorotic spotting, as well as more prominent mottling.

Passiflora foetida

Young systemically-infected leaves and some inoculated leaves developed vein yellowing, 7-10 days after inoculation. Secondary symptoms of chlorotic spots and mosaic appeared on mature fully-expanded leaves (Plate 26). This species was highly susceptible to infection.

Passiflora quadrangularis

Infected plants produced chlorotic lesions 1-2 mm diameter on inoculated leaves, 2-3 weeks after

Plate 25: Systemic ringspotting on Cassia occidentalis infected with PV3.

Plate 26: Vein yellowing, mottle and chlorotic dotting on Passiflora foetida infected with PV3.

inoculation. These spots later turned necrotic (Plate 27a) Systemically-infected young leaves showed vein-yellowing, mottling, mosaic and slight leaf deformation (Plate 27b). Vein necrosis was occasionally observed and plant growth was severely reduced.

Table 32: Symptoms induced on herbaceous and woody hosts by PV3

Plant Species	Local Reaction	Systemic Reaction
Amaranthaceae		
Gomphrena globosa (Globe amaranth)	NL	-
Chenopodiaceae		
Chenopodium album	CS	-
C. amaranticolor	CS	-
C. foetidum	CS	-
C. quinoa	CS	-
Leguminosae		
Cassia occidentalis	CMo	VY,LC,RS,CMo
C.tora	NL	-
Crotalaria usuramonensis	CM	CM
Phaseolus vulgaris (cv. Top crop)	NL	-
P. vulgaris (cv. The Prince)	NL	-
Vigna unguiculata	NL	-

(cv. Bushita mae)

Passifloraceae		
P. edulis cv. flavicarpa	CM,CMo	VY,CMo,CS,(RS)
P. foetida	CMo,CM,VY	VY,CS,CMo
P. mollissima	CMo	CS,CMo
P. quadrangularis	CL,CMo	VY,CMo,LD
Solanaceae		
Nicotiana clevelandii	SL	-
Petunia hybrida	SL	-

Abbreviations

CM = Chlorotic mosaic	RS = ringspot
CMo = Chlorotic mottle	VY = Vein yellowing
CS = Chlorotic spots	LD = Leaf distortion
NS = Necrotic spots	SL = Symptomless local
LC = Leaf curl	() = Occassional symptoms

\- = No infection

<u>The following species were not infected</u>

Arachis hypogaea Centrosema pubescens, Cucumis sativus cv. Marketer, *Datura stramonium* cv. Arbotea, *Glycine max* cv. Local, *Nicotiana megalosiphon, N. tabacum* cvs White Burley and Xanthi, *N. rustica, Passiflora suberosa, Pisum sativum* cv. Meteor, *Pueraria phaseoloides, Vigna unguiculata* local cultivar.

Plate 27: (a) Necrotic lesions on inoculated leaf of Passiflora quadrangularis infected with PV3.
(b) Vein yellowing and systemic mottling of P.quadrangularis infected with PV3.

(a)

(b)

3.3.4 *In vitro* properties

These were determined in extracts of *P. foetida* and repeated at least twice.

3.3.4 a) Longevity *in vitro*

Crude extracts of *P. foetida* kept at room temperature (20°C) lost infectivity between 4 and 5 days. Infectivity dropped gradually during ageing.

3.3.4 b) Thermal inactivation

The thermal inactivation point in crude sap of *P. foetida* was between 50°C and 60°C.

3.3.4 c) Dilution end point

The dilution end point of the virus was 10^{-3} - 10^{-4}.

3.3.5 Purification

The virus was purified from systemically infected *P. foetida* leaves, 2 months after inoculation, by the same method as PV1 (Chapter 3.1.5). After caesium sulphate gradient centrifugation, one strong light scattering band 4.5 cm below the meniscus was observed. This zone was collected manually and dialysed overnight, and virus concentrated by high speed centrifugation. Virus concentration of 0.2 - 0.5 mg/100g leaves were routinely obtained from *P. foetida*, after caesium sulphate

density gradient centrifugation. Purified virus particles are shown in Plate 28.

3.3.5 a) Ultraviolet light absorption

The uv absorption spectrum of the purified PV3 solution was typical for a virus with rod-shaped particles. The 260:280 ratio varied between 1.07 - 1.11.

3.3.6 Electron microscopy

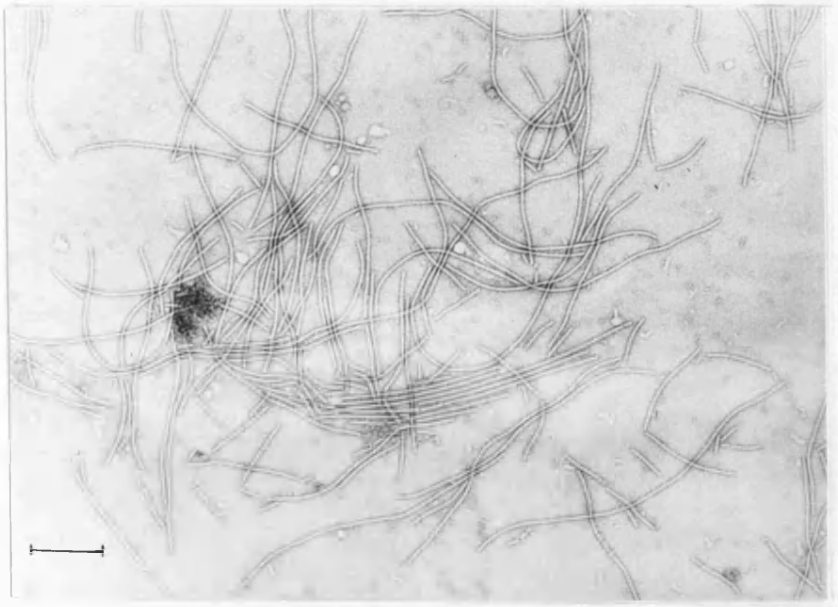
3.3.6 a) Leaf squash preparations

Several flexuous rod-shaped particles were detected in leaf squash preparations of *P. foetida* or *P. edulis cv. flavicarpa* (Plate 29).

3.3.6 b) Particle morphology

The particle morphology of PV3 was determined by electron microscopy of leaf squashes from infected *P. foetida*. Purified virus preparations were not used to determine the particle length because of extensive particle breakage or linear aggregation during purification. More than 140 particles were measured using electron micrograph negatives. A main maximum of 700 nm - 850 nm which contained over 82% of the particles was used to calculate the normal length (Fig. 17). Fifty particles were measured to calculate the width of particles. The normal particle length of PV3 as determined in leaf squashes from infected *P. foetida* was 772 nm ± 3.8, and 13 nm ± 0.17 wide.

Plate 28: Electron micrographs of purified PV3 from Passiflora foetida after caesium sulphate density gradient centrifugation, stained in 20 g/l PTA pH 6.5.
Bars represent 200 nm.



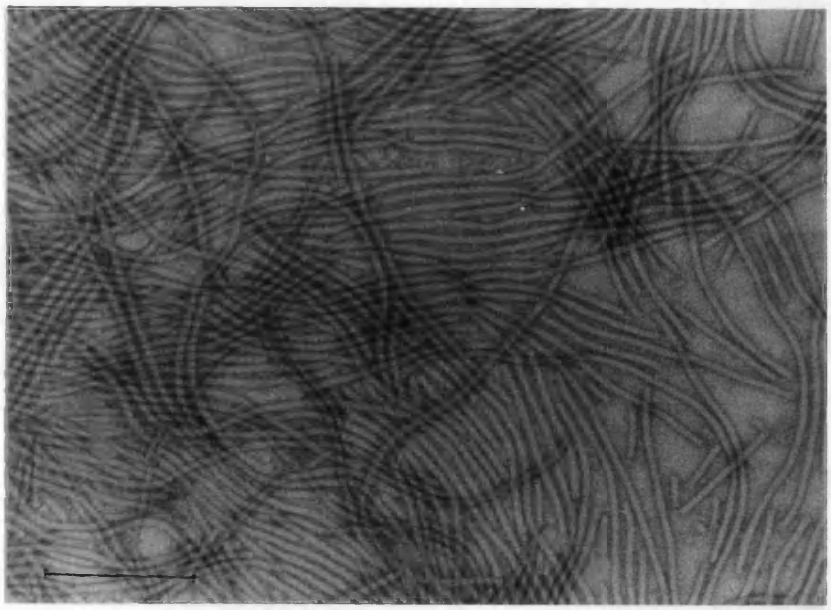


Plate 29: Electron micrograph of leaf squash preparation of PV3 from Passiflora foetida stained in 20 g/l PTA pH 6.5. Bar represents 200 nm.

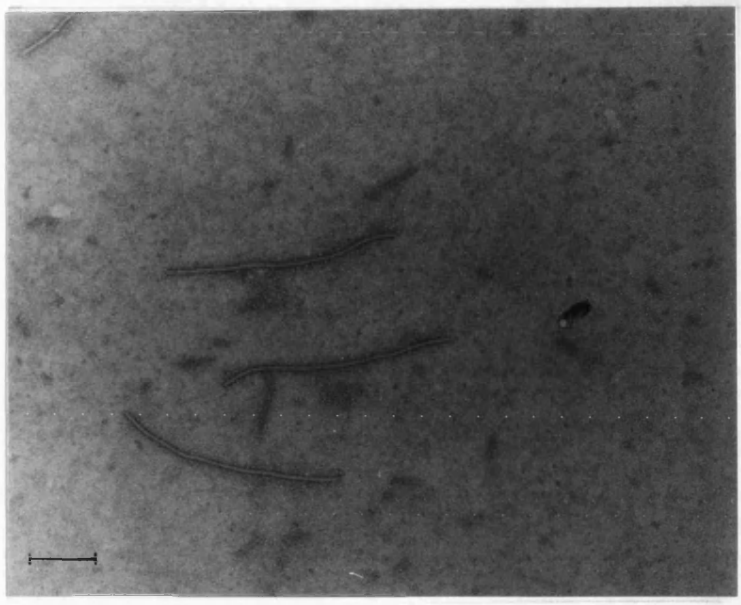
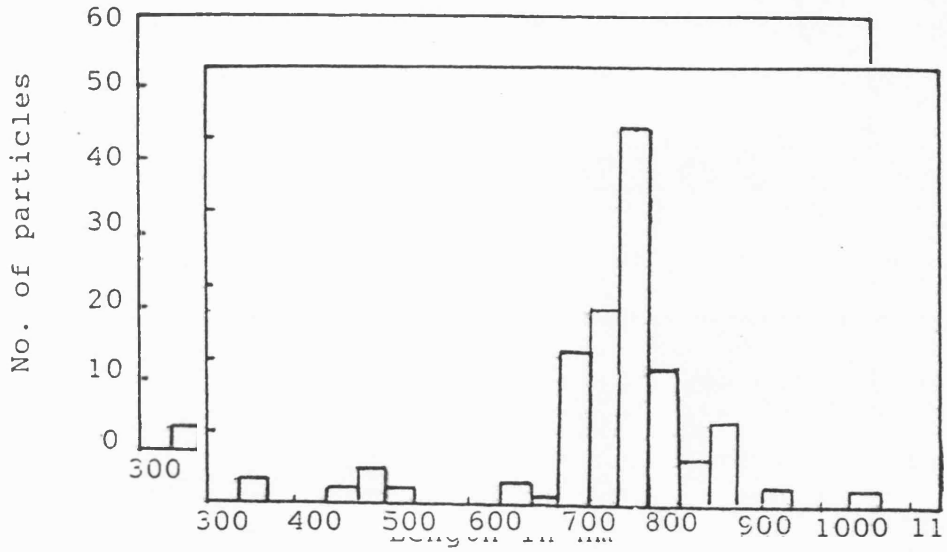


Fig.17 Histogram of the length of PV3 particles in a leaf squash homogenate. (class interval 1:33.3nm)



3.3.7 Serology

3.3.7 a) Preparation of antiserum to PV3

Antiserum was produced in Sandy half lop rabbits by first injecting 0.5 mg of purified virus intravenously followed by three intramuscular injections of 0.5 ml virus (0.2 - 0.5 mg) in 8.5 g/l NaCl mixed with equal volume of Freunds incomplete adjuvant, administered at weekly intervals. The homologous titre was determined in microprecipitin tests with purified virus at a concentration of 1 mg/ml. In these tests, 1 µl of virus antigen was titrated with an equal volume of a twofold serial dilution of antiserum in 8.5 g/l NaCl. Due to low yields of PV3 obtained from caesium sulphate gradients it was only possible to inject 0.2 - 0.5 mg of virus into a rabbit for immunization. However, an homologous titre of 1:8,192 was obtained for PV3.

3.3.7 b) Indirect ELISA

Attempts to use indirect ELISA using crude sap extracts were unsuccessful with the heterologous antisera of passion fruit woodiness virus, passion fruit ringspot virus, potato virus Y, watermelon mosaic virus and bean common mosaic virus. Further studies using purified antigens are reported in Chapter 3.4.

3.3.8 Double-stranded RNA-analysis

The detectable amount of dsRNA's were very low and were barely visible even when samples from 20 - 30 g

of *P. foetida* were loaded onto one tube gel. A single high molecular weight species dsRNA band was found in each of 2 experiments. This had an approximate molecular weight of 6.9 x 10^6 daltons.

The dsRNA nature of the band was confirmed by DNase and RNase treatments, the latter under 'low' and 'high' salt conditions. The dsRNA pattern associated with PV3 was different from that previously recorded for PV2 but was similar to that for PV1. However, the amount of dsRNA in PV3 infected tissue was very low.

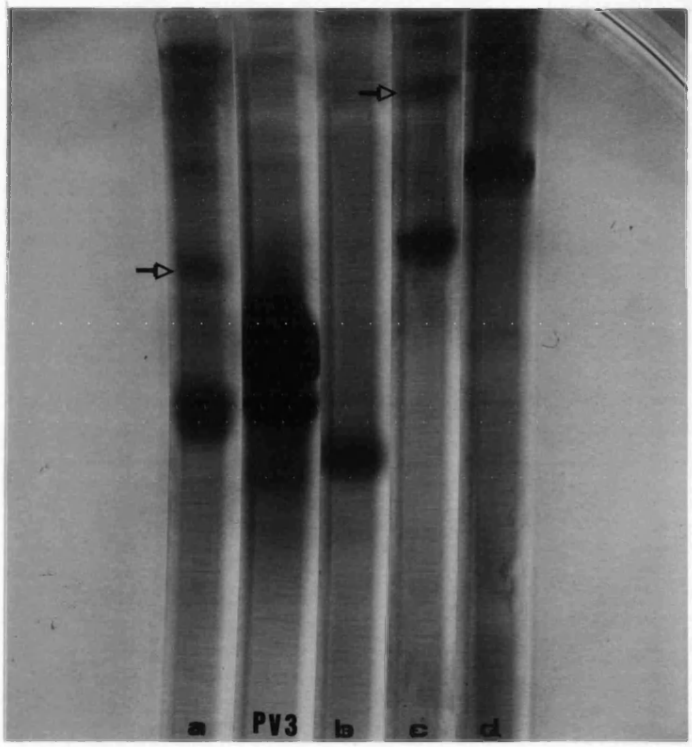
3.3.9 Estimation of the capsid protein molecular weight

The protein coat of PV3 was examined by SDS-PAGE (Weber and Osborne, 1969). Most preparations showed high and low molecular weight components (Plate 30) with mobilities indicating molecular weights of 33,681 (±519) and 25,379 (±757) daltons, respectively. Some preparations showed an additional minor component with an estimated molecular weight of 57,743 which was possibly inclusion protein of virus. Nuclear inclusion protein with a molecular weight of 54 x 10^3, for potyviruses was reported by Dougherty and Hiebert (1980 a,b).

3.3.10 Return inoculation of PV3 to *Passiflora edulis cv. flavicarpa.*

Passiflora edulis cv. flavicarpa inoculated with PV3 did not reproduce the typical chlorotic ringspot

Plate 30: Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of SDS dissociated PV3 and marker proteins. Gels from left to right with molecular weights of marker proteins indicated in parentheses: (a) chymotrypsinogen (25000), (b) ribonuclease (137000), (c) ovalbumin (43000), (d) bovine albumin(66000). Note the presence of dimers.



symptoms as observed in the field. These symptoms, however, were present shortly after infection but disappeared under the prevailing glasshouse conditions at Bath. Other symptoms produced were mottling and chlorotic spotting. Thus, although the pathogenicity of the PV3 isolate in *P. edulis cv. flavicarpa* was demonstrated, the expected ringspot symptoms were not reliably reproduced. For this particular symptom, therefore, Koch's postulates was not fulfilled.

3.4 Serological relationships between PV1, PV2 and PV3

Quantitative measurements of the degree of serological relationship between plant viruses are usually done by tube precipitin or gel double-diffusion tests. These tests have been used, for example, to provide a reliable estimate of the extent of cross-reactivity between tobamoviruses (Van Regenmortel, 1975), cucomoviruses (Devergne and Cardin, 1975), tymoviruses (Koenig, 1976) and potexviruses (Koenig and Lesemann, 1978). However, diffusion techniques are not generally suitable for detection of the longer flexuous rod-shaped viruses which diffuse poorly through agar (Gibbs and Harrison, 1976).

The degree of antigenic relatedness between two plant viruses is commonly expressed by a serological differentiation index (SDI) which corresponds to the average number of two-fold dilution steps separating homologous from heterologous precipitin titres (Van

Regenmortel and Von Wechmar, 1970). In recent years the enzyme-linked immunosorbent assay (ELISA) has replaced precipitation as the most sensitive and widely used serological test for virus detection (Clark, 1981; Van Regenmortel, 1982). The double antibody sandwich version of ELISA is most commonly used in diagnostic work, but is generally regarded as too strain specific for use in cross-reaction studies (Koenig, 1978; Koenig and Paul, 1982; Van Regenmortel, 1982).

The indirect form of ELISA, on the other hand, still uses much less antiserum than more conventional tests but can also be used without the need to prepare anti-virus conjugated gamma-globulin. This technique is especially useful for viruses that are difficult to purify or when sera are in short supply. Furthermore, indirect forms of ELISA are well suited to the study of distant relationships between viruses (Van Regenmortel and Burckhard, 1980; Devergne *et al.*, 1981; Koenig, 1981; Rybicki and Von Wechmar, 1981; Koenig and Paul, 1982). An indirect ELISA was successfully used to quantitatively measure the degree of antigenic cross-reactivity between viruses (Jaegle and Van Regenmortel, 1985).

Indirect forms of ELISA were selected to study the degree of antigenic cross-reactivity between viruses isolated from *Passiflora* species.

Antigens - Antigens used for comparative purposes were in highly purified form and stored at -20°C. Antigen for each virus was purified from *P. foetida* using caesium sulphate gradient. Jaegle and Van Regenmortel (1985) stated that purified virus preparations should be used to determine serological cross-reactivity if plates not previouly coated with antibodies were to be used in indirect ELISA tests.

Antisera - Antisera taken 28 days (PV1 and PV3) and 35 days (PV2) after the first injection were used and all had a titre of 1:8,192 for PV1, PV2 and PV3. Antisera were cross-absorbed with equal volumes of healthy purified preparations, prior to use.

Preliminary tests were done to determine the suitable antigen concentration for determining the serological cross-reactivity between the PV1, PV2 and PV3. Each serum was compared with homologous and heterologous viruses using four antigen concentrations of 2, 1, 0.1, 0.01 µg/ml.

The optimum concentrations of the detecting antigens were determined and it was found that 1 µg/ml antigen concentration was suitable to study the cross-reaction between homologous and heterologus antigens.

3.4.1 Cross-reactions between PV1, PV2 and PV3

Antisera prepared for PV1, PV2, and PV3 viruses were used to study the cross-reactions between homologous

and heterologous antigens. Antigen was used at a concentration of 1 µg/ml of purified virus. Antiserum to PV1, PV2 and PV3 were used over a range of dilution.

Serological differentiation indices (SDI) were calculated for each virus using ELISA curves obtained from plotting antiserum dilution (expressed as $\log_{-2}$) against absorbance at 410 nm (Fig. 18). Serological differentiation indices were calculated for the homologous and heterologous viruses, from the points in the curves that correspond to absorbance value of 0.5, after the method of Jaegle and Van Regenmortel, (1985). The SDI values calculated from the curves in Fig. 18 are summarised in Table 33, while SDI's calculated from microprecipitin tests are given in Table 34.

Table 33: Serological differentiation indices (SDI) for pairs of passion fruit viruses calculated from ELISA

Antiserum	Antigen	SDI from ELISA	(average SDI)
PV1	PV2	1.35	1.70
PV2	PV1	2.05	
PV1	PV3	1.1	0.78
PV3	PV1	0.45	
PV2	PV3	1.45	1.45
PV3	PV2	1.45	

Fig.18 a) Cross-reactivity between PV1,PV2 and PV3 using PV1 antiserum as measured by indirect ELISA.
(2 hours substrate incubation)

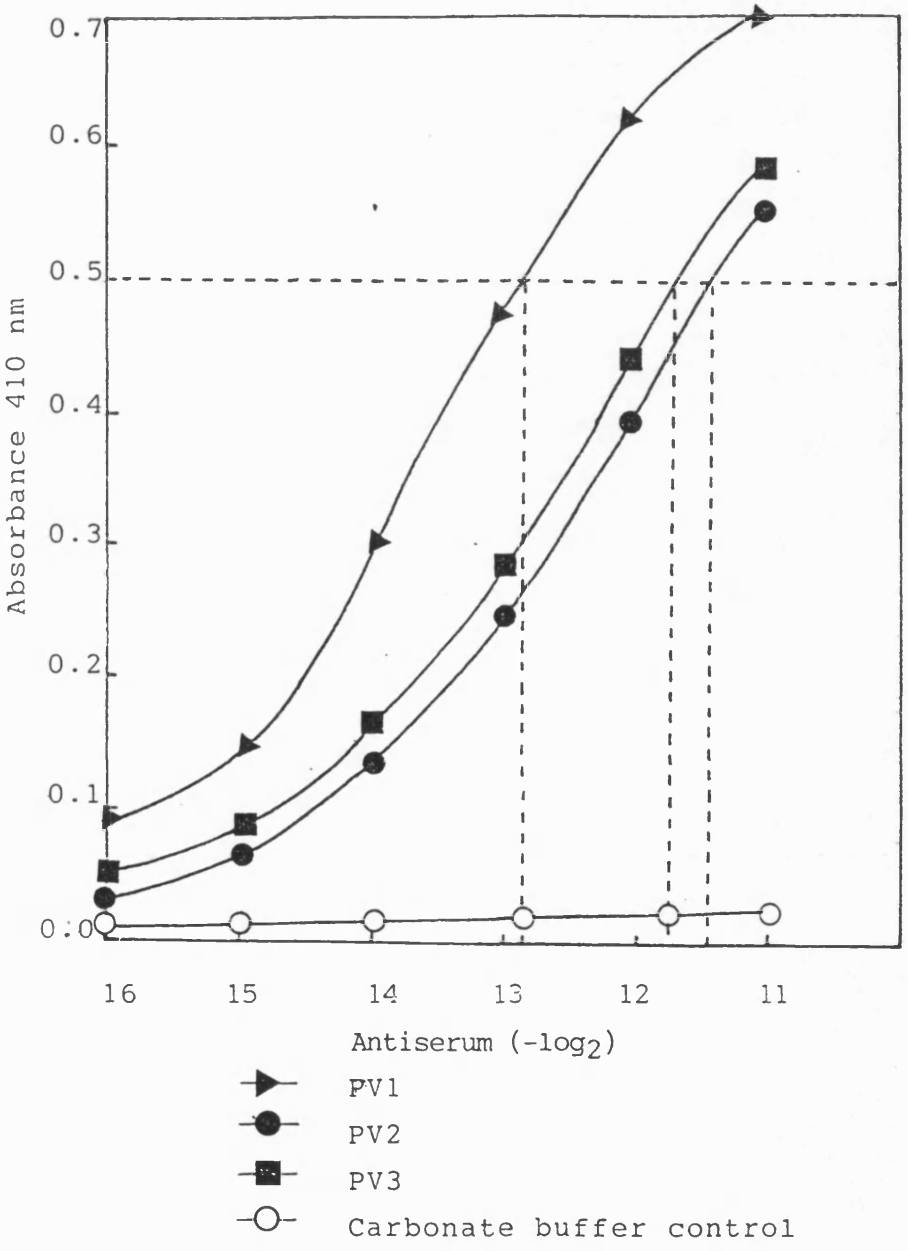


Fig.18 b) Cross - reactivity between PV1, PV2 and PV3 using PV2 antiserum as measured by indirect ELISA .
(2 hours substrate incubation)

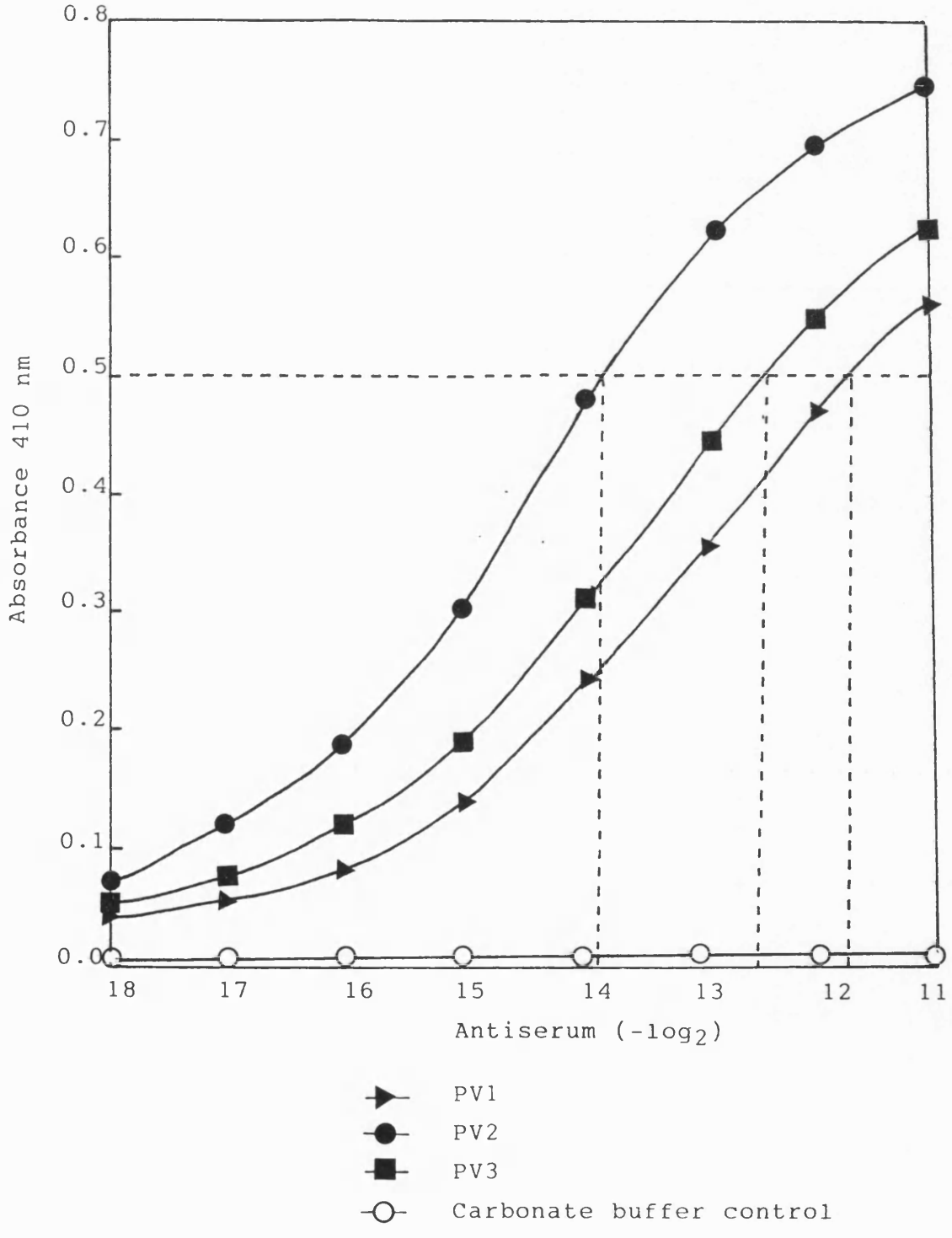


Fig.18 c) Cross reactivity between PV1,PV2 and PV3 using PV3 antiserum as measured by indirect ELISA.
(2 hours substrate incubation)

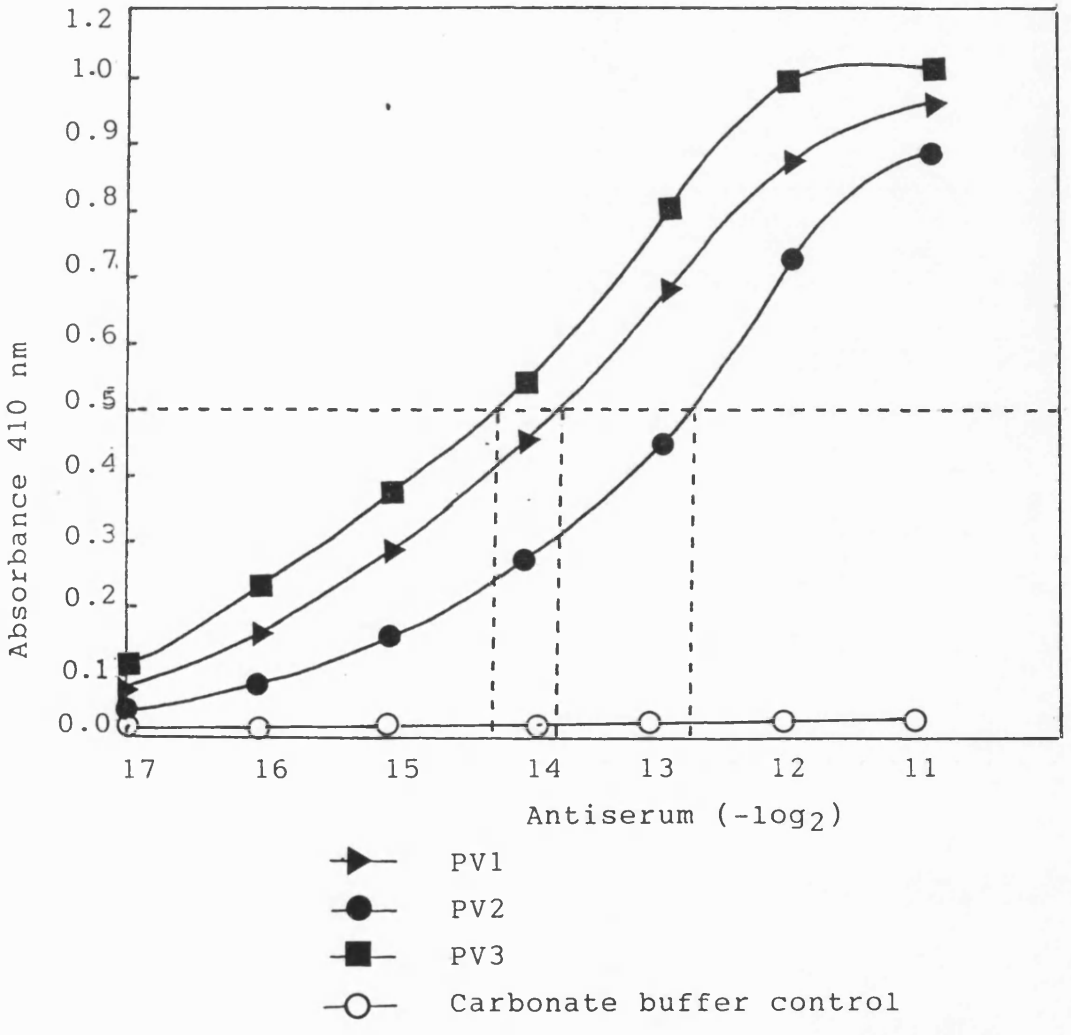


Table 34: Serological differentiation indices (SDI) for pairs of passion fruit viruses calculated from micro-precipitin test

Antiserum	Antigen	End point titre homologous / heterologous titres of unfractionated sera	Average SDI
PV1	PV2	1:8,192 / 1:1,024	3
PV2	PV1	1:8,192 / 1:1,024	
PV1	PV3	1:8,192 / 1:2,048	2
PV3	PV1	1:8,192 / 1:2,048	
PV2	PV3	1:8,192 / 1:2,048	2
PV3	PV2	1:8,192 / 1:2,048	

Results shown in Tables 33 and 34 suggested that PV1, PV2 and PV3 are probably not identical but are closely related. Passion fruit virus 1 was apparently more closely related to PV3 than PV2 while, PV2 was more closely related to PV3 than PV1. In these tests, however, only one antiserum for each antigen was used, and it is well known, however, that antisera to a given antigen may vary qualitatively depending on the rabbit used, bleed taken and immunisation schedule (Van Regenmortel, 1982). Small differences in titre (or SDI) should be treated with caution, therefore, as they may not necessarily indicate serological distinctness.

3.4.2 Cross-absorption tests

To study the serological relationships in more detail, cross-absorption tests were done using purified antigens in indirect ELISA plates not precoated with antibodies. Serological reactions between antisera and viruses were studied after absorbing the antisera with purified preparations of healthy antigens and after cross-absorption with homologous and heterologous antigens. Purified preparations of each antigen were mixed with an equal volume of antiserum and incubated for 2 hr. at 37°C. After precipitation at 37°C,the sample was further incubated at 4°C for 18 hr. Precipitates were removed by centrifugation, and the supernatant used for subsequent studies. Antigens were tested at 0.1 and 0.01 µg/ml in preliminary studies and antigen concentration of 0.01 µg/ml was selected for cross-absorption study. According to Van Regenmortel (1966) for cross-absorption studies antigen dilution should be near to virus dilution end point.

Results obtained in cross-absorption test are summarised in Table 35.

From Table 35, it can be seen that, after PV1 antiserum was cross-absorbed with PV2 and PV3 antigens separately, the antiserum was still capable of reacting with PV1 antigens. This suggested that the absorption of PV1 antiserum by excess of PV2 and PV3 antigens did not remove all of the antibodies that reacted with PV1.

Table 35: Cross-absorption test with PV1, PV2 and PV3

Antiserum	Absorbing antigens	Test antigens PV1	PV2	PV3
PV1	-	1:32,768	1:8,192	1:2,048
	PV1	1:2,048	-	-
	PV2	1:16,384	-	1:2,048
	PV3	1:8,192	-	-
PV2	-	1:8,192	1:32,768	1:4,096
	PV2	-	-	-
	PV1	1:2,048	1:16,384	1:4,096
	PV3	-	1:8,192	-
PV3	-	1:32,768	1:4,096	1:65,636
	PV3	-	-	-
	PV2	1:8,192	-	1:8,192
	PV1	-	1:2,048	1:2,048

- no reaction

Thus, PV1 apparently has some antigenic determinants not found in PV2 or PV3 antigens. When antiserum specific for PV1 was cross-absorbed with PV3 antigen, the only positive reaction was the homologous one. This indicated that PV2 had more antigenic determinants in common with PV3 than PV1.

Similarly, the test with PV2 antiserum indicated that PV2 contained antigenic determinants that were absent in PV1 and PV3 antigens. Furthermore, when

antiserum specific for PV2 was cross-absorbed with PV3 antigens, the only positive reaction was found with PV2. This may indicate that PV3 had more antigenic determinants in common with PV1 than PV2.

When antiserum specific for PV3 was cross-absorbed with PV2 and PV1 antigens, this did not remove all of the antibodies raised against PV3. This indicated that PV3 contained antigenic determinants not present in PV2 and PV1 antigens.

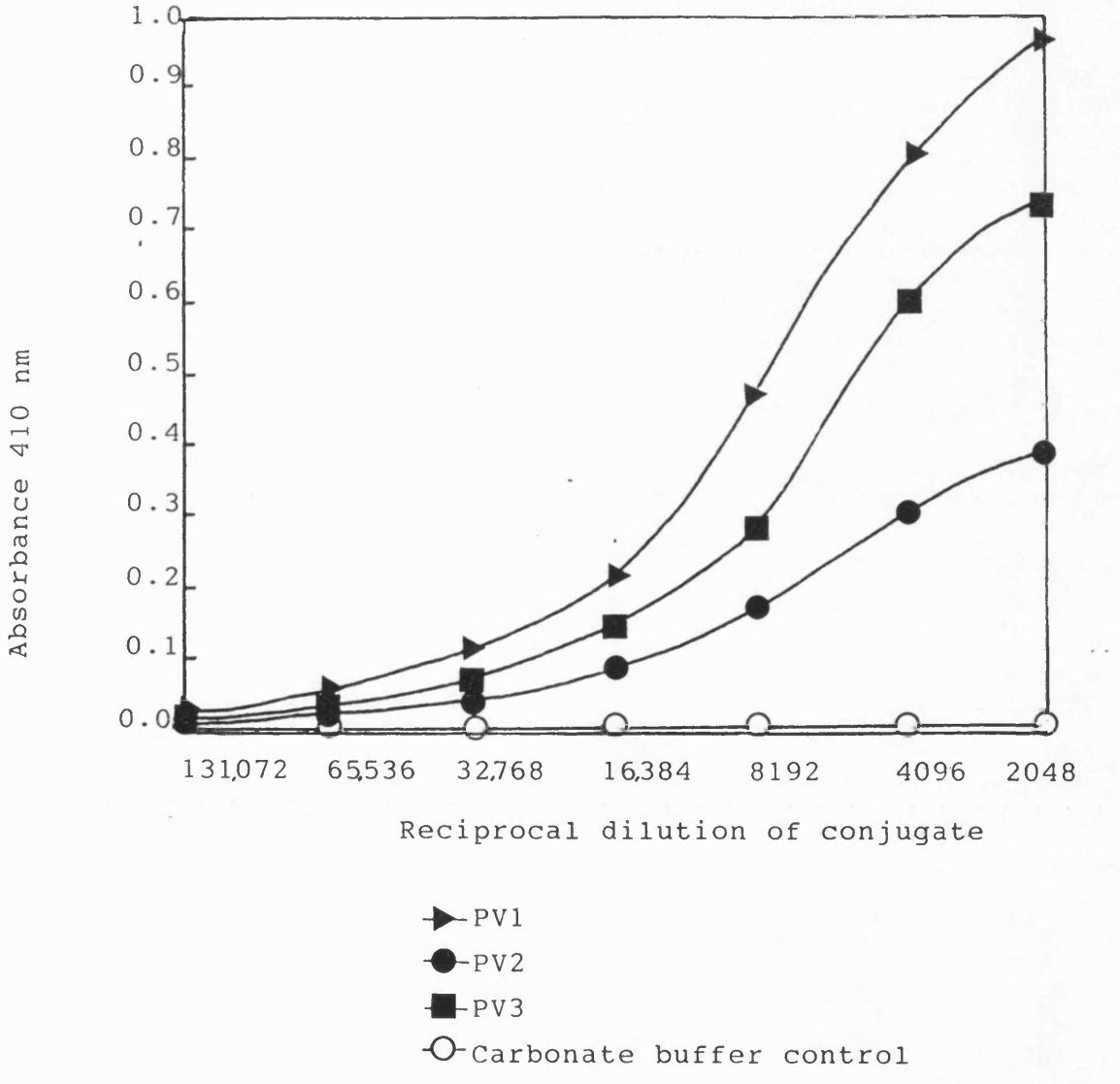
The homologous and heterologous titres of unabsorbed and cross-absorbed antiserums indicated that small antigenic differences between PV1, PV2 and PV3 may be present.

3.4.3 Comparison of PV1, PV2 and PV3 antigens using DAS-ELISA

The capacity of the DAS method of ELISA for detecting different viruses was tested with conjugates prepared with antiserum to PV1 and PV2 antibodies. Antigens of PV1, PV2 and PV3 were used in highly purified form and was used at 1 µg/ml concentration. Gamma globulins for PV1 and PV2 viruses were prepared using antisera taken at 48 and 35 days, respectively. The results are shown in Fig. 19.

According to the results (Fig. 19a), when homologous antibodies of PV1 were used both as the coating and the detecting antibody, the homologous (PV1)

Fig.19 a) Detection of PV1,PV2 and PV3 by DAS-ELISA using anti PV1 serum.
(2 hours substrate incubation)



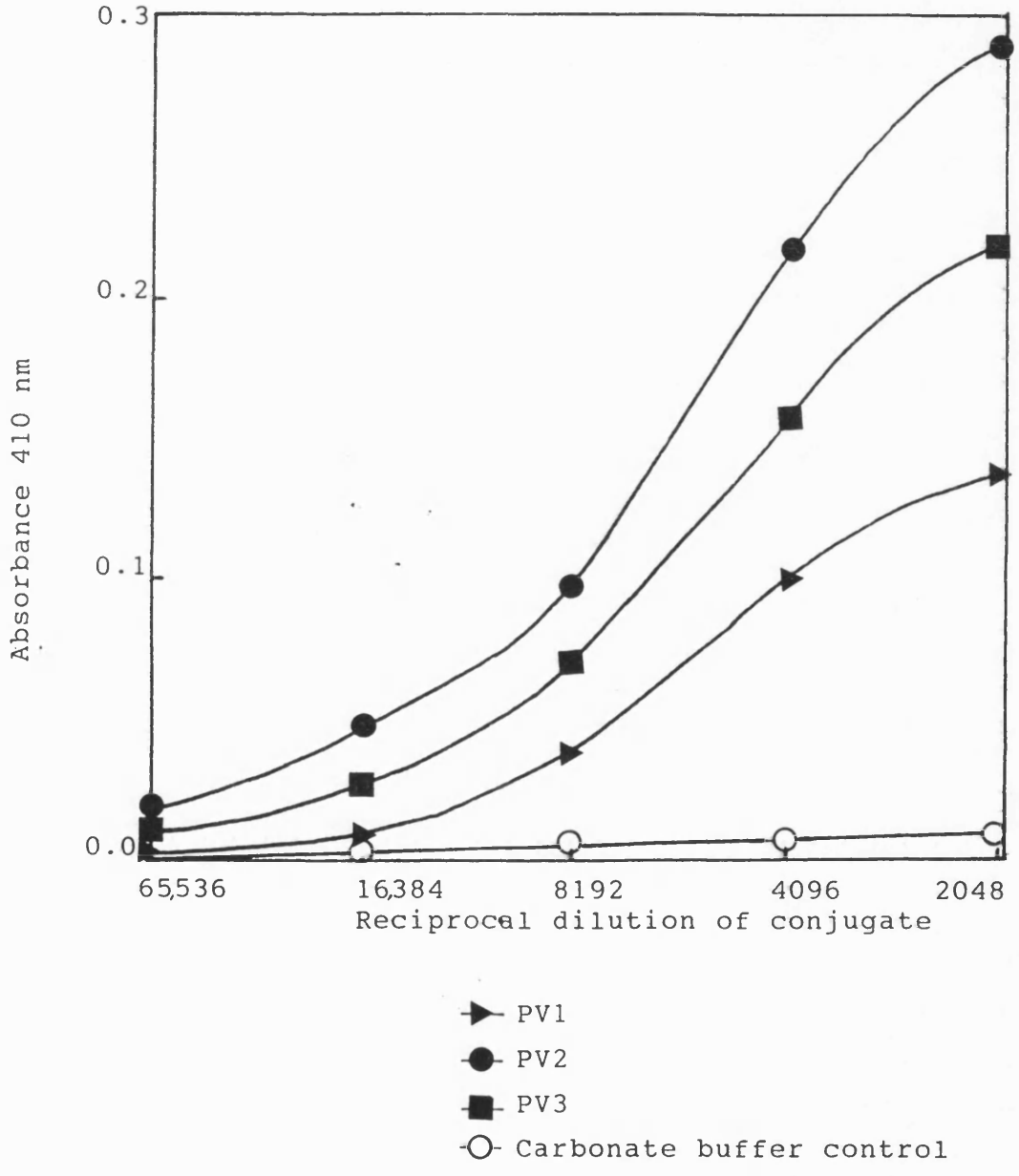
and heterologous (PV2, PV3) antigens can be detected. Moreover, Fig. 19b shows that when antibodies of PV2 were used both as coating and detecting antibody the homologous (PV2) and heterologous antigens of PV1 and PV3 were detected. However, the anti PV2 conjugate was not suitable for ELISA work as the absorbance values were very low after prolonged substrate incubation. The rabbit injected with PV2 was bled about two weeks before PV1, following the same immunization schedule. It is possible, therefore, as reported by Koenig (1978), that earlier bleedings are less suitable for conjugate preparation.

Nevertheless, the results indicated that it was possible to detect three virus isolates using one conjugate prepared to a single virus isolate. These results further confirmed that viruses isolated from passion fruits were closely related. Furthermore, results indicated that PV1 was apparently more closely related to PV3 than PV2 but isolate PV2 was apparently more closely related to PV3 than PV1.

3.4.4 Cross-reaction of PV1, PV2 and PV3 with antisera to potyviruses.

Investigations were done to study the cross-reaction between PV1, PV2 and PV3 with antisera to different potyviruses in direct ELISA as described in Chapter 2.18d. Antigens of PV1, PV2 and PV3 were used at concentration of 1 µg/ml. Antiserum dilutions of

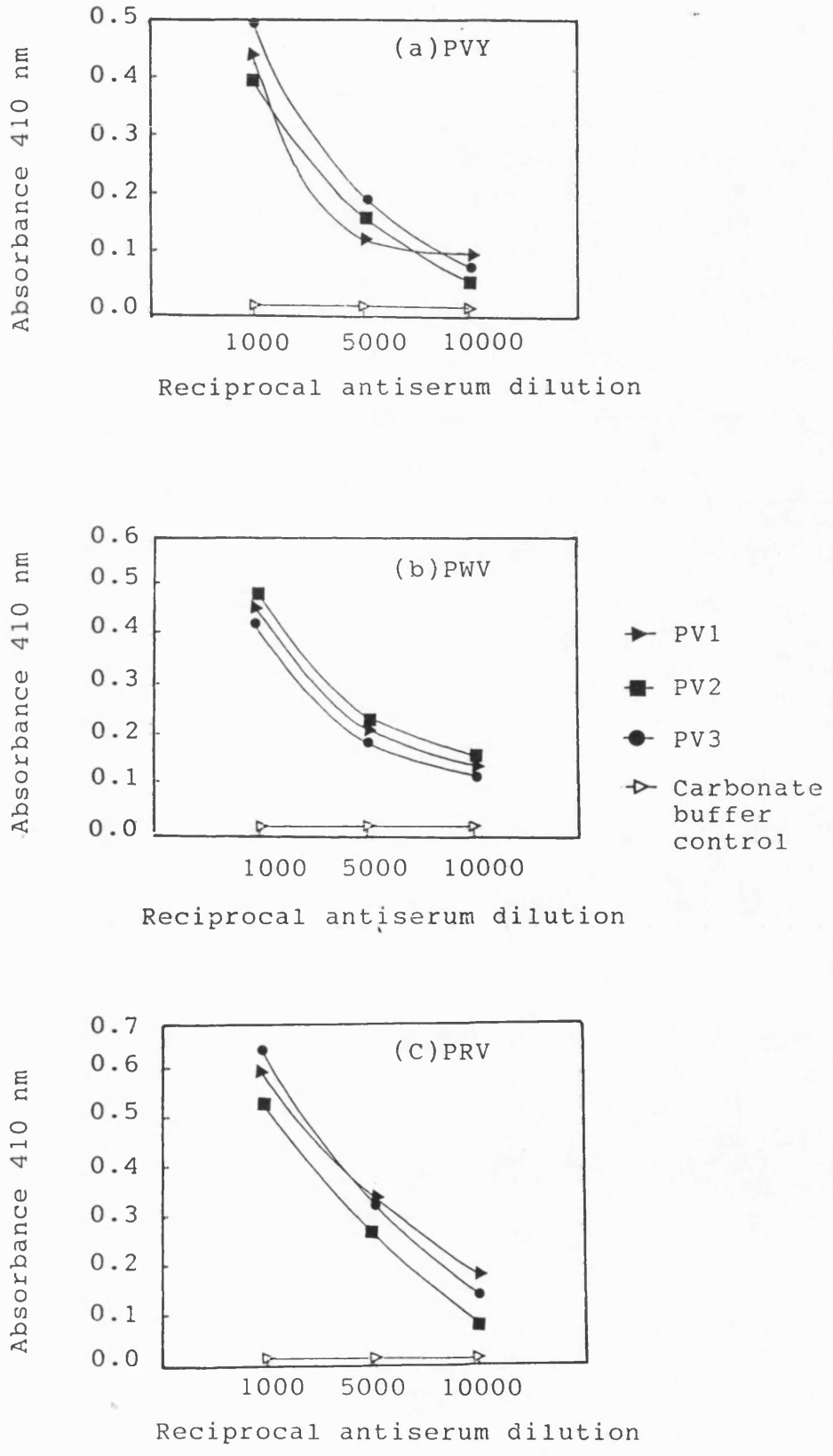
Fig.19 b) Detection of PV1,PV2 and PV3 by DAS-ELISA using anti PV2 serum.
(18 hours substrate incubation at 4 °c)



1:1,000, 1:5,000 and 1:10,000 were used. The results are shown in Fig. 20.

According to the results, PV1, PV2 and PV3 had slightly different absorbance values with the potyvirus antiserum tested. These findings further suggested that PV1, PV2 and PV3 were distinct viruses. On the other hand, PV1, PV2 and PV3 had some common antigenic determinants which can react with PRV, PWV and PVY antibody indicating that they should be included in the potyvirus group. Isolates of PV1, PV2 and PV3 did not show any serological relationship to bean common mosaic virus in a different experiment with crude sap preparations in indirect ELISA. Moreover, PV1 and PV2 gave borderline reaction with water melon mosaic virus in similar tests, while PV3 failed to show any relationship. This may probably be due to low concentrations of PV3 in extracted sap. This isolate was difficult to purify and concentrate, therefore, conclusions about the relationships of PV3 to WMV cannot be made.

Fig.20 Heterologous reaction of PV1,PV2 and PV3 antigens with antiserum against the a)potato Y virus(PVY) b)passionfruit woodiness virus(PWV) and c)passio--nfruit ringspot virus(PRV).



CHAPTER 4 EPIDEMIOLOGY OF *PASSIFLORA* VIRUSES

4.1 Studies with naturally infected field-grown *Passiflora* crops

4.1.1 Survey of host plants for possible aphid vectors

A survey of the flora for the presence of aphid colonies was undertaken in the neighbourhood of passion fruit growing areas in the low country wet zone region in Sri Lanka. Host plants for aphid vectors and the identity of aphid species found in the survey are given in Table 36.

The survey showed several plant species colonised by aphids in the vicinity of passion fruit cultivations. *Aphis gossypii* colonies were found on several plant species belonging to five families (Plate 31), whereas *Aphis craccivora* occurred mainly on the leguminous spp. (Plate 32). *A. spiraecola* was found on *Eupatorium odoratum* and *Mikania scandens* (Plate 33).

Due to the land scarcity in the wet zone region, most of the cultivations are in mixed stands, often with fruit crops, vegetables and perennial crops. Hence the cultivation system is likely to encourage aphid multiplication throughout the year.

Plate 31: Aphis spiraecola on young shoots of Eupatorium odoratum (a) and Mikania scandens (b).

(a)

(b)

Plate 32: A colony of Aphis craccivora on a shoot of Gliricidia maculata .

Plate 33: A colony of Aphis gossypii on a leaf of Solanum melongena.

Table 36: Aphids collected from their natural hosts

Host plant	Family	Aphid
Eupatorium odoratum	Compositae	*Aphis spiraecola* Patch.
Mikania scandens	Compositae	*A. spiraecola* Patch.
Vernonia cineria	Compositae	*A. gossypii* Glover.
Croton hirtus L.	Euphorbiaceae	*A. gossypii* Glover.
Hibiscus esculentus	Malvaceae	*A. gossypii* Glover.
Banana (Musa spp.)	Musaceae	*Pentalonia nigronervosa* Coquerel.
Gliricidia maculata	Leguminosae	*A. craccivora* Koch.
Vigna unguiculata	Leguminosae	*A. craccivora* Koch.
Scoparia dulcis	Scrophulariaceae	*A. gossypii* Glover.
Solanum melongena	Solanaceae	*A. gossypii* Glover.
Chilli (Capsicum spp.)	Solanaceae	*A. gossypii* Glover.
Citrus spp.	Rutaceae	*Toxoptera citricidus* Kirkaldy.

4.1.2 Aphids visiting cultivated *Passiflora*

Even though aphids on adjacent wild plants were found on passion fruit, established colonies were rarely encounted on the vines. Small colonies of *A. spiraecola* were, however, found on *P. edulis cv. flavicarpa* on very young shoots. Generally, aphids did not colonise mature vines.

4.1.3 Possible natural reservoirs for *Passiflora* viruses

Twelve weed species belonging to five families were collected from passion fruit cultivations in the wet zone region. These weed species had virus or virus-like symptoms. Mottling and mosaic were the most common symptoms observed and were found on *Passiflora foetida, Cassia occidentalis, C. tora, Crotalaria spp., Acalypha indica, Pueraria phaseoloides* and unidentified weed spp. collected from the Yattepatha area. *Centrosema pubescens* had ringspot, in addition to mosaic and mottle leaf symptoms. Other symptoms observed included vein chlorosis exhibited by *Ageratum conyzoides* and *Mikania scandens.*

The above spp. were tested for virus by grinding leaves from fully grown plants in 0.02 M tris-HCl buffer pH 7.8 containing 1 g/l Na_2SO_3 (1:10 w/v). Extracts were assayed on *P.edulis cv. flavicarpa. Aphis craccivora* was also used in isolation attempts as described in Chapter 2.22.

The inoculation tests were positive only for one sample of *P. foetida* which produced mottling symptoms on *P. edulis cv. flavicarpa.* The results suggested that alternative hosts for the passion fruit viruses were limited as no virus could be isolated from other spp. Leggat and Teakle (1975) reported *P.foetida* as a widespread potential reservoir of PWV for commercial passion fruit cultivations in Australia. Several

authors: Rosario *et al.*, 1964; Van Velsen, 1961; Wilson and Satyarajah, 1970, reported virus diseases of *P. foetida*.

Attempts to isolate virus from weed spp. by aphid transmission were negative but PV1 and PV2 in *P. edulis cv. flavicarpa* were transmitted to *P. foetida* (5/5) and *C. occidentalis* (4/5) by *A. craccivora*.

The results indicate that the high incidence of passion fruit virus diseases within the crop may be due to spread of virus between plants within the crop, rather than from external sources of infection.

4.1.4. Transmission of PV1, PV2 and PV3 by aphids

Aphid colonies found on different plant species (Chapter 4.1.1.) were raised in the insectory at the Central Agricultural Research Station, Gannoruwe, Sri Lanka to study their potential as virus vectors. *Myzus persicae* was collected from a healthy tobacco plant cv. White Burley and was maintained on Chinese Cabbage (*Brassica pekinensis*) in the insectory at the University of Bath. Aphids were collected from *Gliricidia maculata, Vigna unguiculata, Solanum melongena, Mikania scandens, Citrus* spp. and were reared on their natural host, in the insectory at Gannoruwe, Sri Lanka. The first generation offspring were used for transmission tests.

Aphids starved for 2 hr were allowed to feed on portions of passion fruit leaves infected with PV1, PV2

or PV3 for periods of 3, 5 or 30 min. Fifteen to twenty aphids were immediately transferred and allowed to feed on each passion fruit seedlings for another 5 min and 30 min. For *M. persicae*, a few tests were also made with 16 hr acquisition and inoculation access period. *Myzus persicae* was then again transferred to new healthy plants for a second inoculation period of 16 hr.

The results shown in Table 37 indicated that increasing the acquisition feeding time from 30 min to 16 hr had little effect on the transmission of passion fruit virus 1 and 2 by *Myzus persicae*. Increasing the inoculation period from 15 min to 16 hr also showed little effect. Furthermore, *M. persicae* failed to transmit the virus in the second inoculation access. *Aphis gossypii*, *A craccivora*, and *A. spiraecola* transmitted all three viruses to *P. edulis cv. flavicarpa*. However, *A. craccivora* transmitted PV3 to *P. flavicarpa* with limited efficiency. The results indicated that all three viruses in the present study were transmitted by aphids in a non-persistent manner. Furthermore, several aphid spp. were able to transmit PV1 and PV2 with similar efficiency, although in limited tests PV3 was more efficiently transmitted by *A. spiraecola*.

4.1.5 Comparison of aphid trapping methods

Investigations were undertaken in passion fruit cultivations to assess different aphid sampling and trapping methods. Experiments were done at the

Table 37: Effect of acquisition and inoculation period on the transmission of PV1, PV2 and PV3 by different aphid species

Aphid Species	No of aphids per plant	Acquisition feeding time	Inoculation feeding time	No. of plants infected / No of plants inoculated			Percentage Transmission		
				PV1	PV2	PV3	PV1	PV2	PV3
Myzus persicae	10	3 min	15 min	6/6	5/5	na	100	100	-
M. persicae	15	3 min	30 min	3/4	3/5	na	75	60	-
M. persicae	8	5 min	16 hr	4/6	na	na	66	-	-
M. persicae	15	16 hr	16 hr	4/6	2/4	na	66	50	-
M. persicae	15	5 min	1 hr	na	10/10	na	-	100	-
Aphis craccivora	20	5 min	5 min	10/10	7/10	na	100	70	-
A. craccivora	20	5 min	30 min	8/10	na	1/15	80	-	6
A. craccivora	20	5 min	30 min	4/10	na	na	40	-	-
Aphis gossypii	15	5 min	5 min	6/10	5/10	2/8	60	50	25
A. gossypii	20	5 min	30 min	6/8	na	na	75	-	-
Aphis spiraecola	20	5 min	5 min	5/6	5/6	6/8	83	83	75
A. spiraecola	20	5 min	30 min	5/6	na	na	83	-	-
A. spiraecola	20	30 min	30 min	3/5	na	na	60	-	-

na = not available

Horticultural Research Farm, Horana, Sri Lanka from May 1988 to September 1988. The present study deals with the

results of a five month trapping period using yellow bowl, cylindrical and flat sticky traps.

Description of the field

The passion fruit cultivation was about 4.5 hectares, with the passion fruit plants grown in terraces and 270 cm between plants. This cultivation was nearly $1^1/_2$ years old and apparently free from virus disease. The height of the land was 100 m above the mean sea level and fruits, vegetables and tuber crops were grown in the adjacent areas.

Traps were placed in the field, 150 cm above the ground with 1080 cm spacing between each trap. The traps were located in four rows with three types of traps distribution in a randomized manner. Trap supports were painted in black and traps were located on a bare soil cover throughout the sampling period. The traps were cleared at weekly intervals and alate and apterae were counted and preserved in 95% alcohol for subsequent indentification. Separate records were kept for catches from each trap. The aphids collected in the different kinds of trap are shown in Table 38a.

A total of 412, 265 and 131 aphids were collected in the yellow water pan traps, cylindrical sticky traps and flat sticky traps, respectively. The numbers of aphids collected in this experiment, however, were low. According to Table 38a, the proportions of aphid species caught varied with the type of trap used. Two aphid

species namely *A. spiraecola* (Plate 34) and *A gossypii* (Plate 35) were in sufficient number to give a reasonable comparison among traps. Approximately 82% of those collected in yellow bowl traps were *A. spiraecola,* and yellow pan traps collected significantly more *A. spiraecola* than the other two methods (Table 38b).

Table 38 a): Catches of some aphids in different traps

Methods of trapping	Total Aphid number	Aphid species (percentage from total catch) *Aphis spiraecola*	*Aphis gossypii*	*Aphis craccivora*	*Pentalonia nigronervosa*
Water bowl	412	82	0.5	5	10
Cylindrical Sticky	265	46	44	0.4	10
Flat Sticky	131	50	48	0.9	2

Table 38 b): Trapping efficiency for some aphid species in water traps and sticky traps

Type of Trap	Mean number of aphids (log n+1) *Aphis spiraecola*	*Aphis gossypii*	*Aphis craccivora*	*Pentalonia nigronervosa*
Water bowl	1.9	0.2	0.8	0.8
Cylindrical Sticky	1.5	1.5	0.1	0.9
Flat Sticky	1.3	1.4	0.1	0.2
$LSD_{0.05}$	0.28			
$LSD_{0.01}$		0.41	0.38	0.51

Plate 34: Aphis spiraecola Patch. alate : 1-antennal tubercle; 2-siphunculus; 3-cauda; 4-abdomen. Bar represents 1 mm.

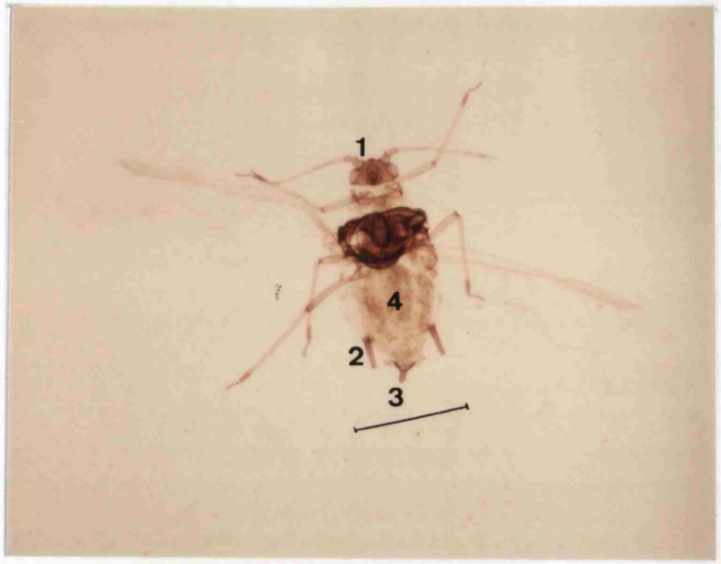
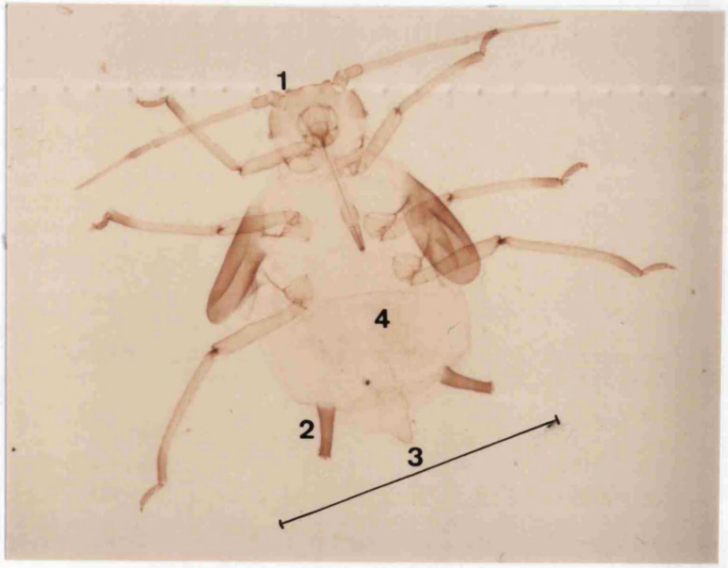


Plate 35: Aphis gossypii Glover. alate : 1-antennal tubercle; 2-siphunculus; 3-cauda; 4-abdomen. Bar represents 1mm.



Although the flat sticky traps caught fewer aphids than the cylindrical traps, there was no significant difference between the number of *A. spiraecola* caught. Heathcote (1957) reported that water traps collected more aphids than sticky traps of the same colour. A'Brook (1973) stated that covering the plastic with banding grease may reduce the reflectance of the trap. Low numbers of *A. spiraecola* in sticky traps may therefore, have been due to this effect.

Similarly, yellow sticky traps collected significantly more *A. gossypii* than water traps. Haine (1955) demonstrated that the yellow traps were most efficient on calm days or in sheltered locations, while the sticky traps were more efficient when there was air movement. The low numbers of *A. gossypii* collected in water traps were unexplained, although Halbert *et al.* (1986) reported that *A. gossypii* collected more frequently in green traps than in yellow traps.

There were no differences between the two methods of sticky trapping for the collection of *A. gossypii*. Significantly, more *A. craccivora* (Plate 36) was caught in water traps than cylindrical sticky traps and the flat sticky traps. Moreover, higher numbers of *Pentalonia nigronervosa* (Plate 37) were collected in water traps and cylidrical sticky traps than flat sticky traps. Generally, the number of aphids collected in flat sticky traps was lower than the other two methods.

Plate 36: Aphis craccivora Koch. apterous: 1-antennal tubercle; 2-siphunculus; 3-cauda; 4-dorsal abdomen with an extensive black patch.
Bar represents 1 mm.

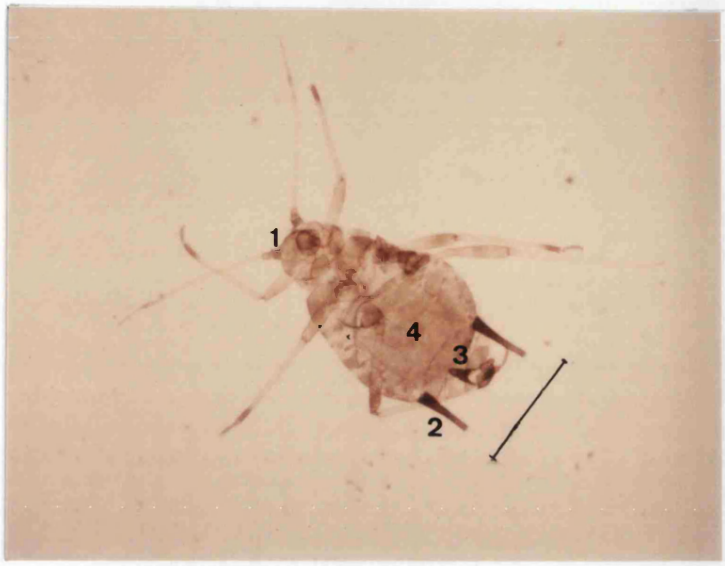
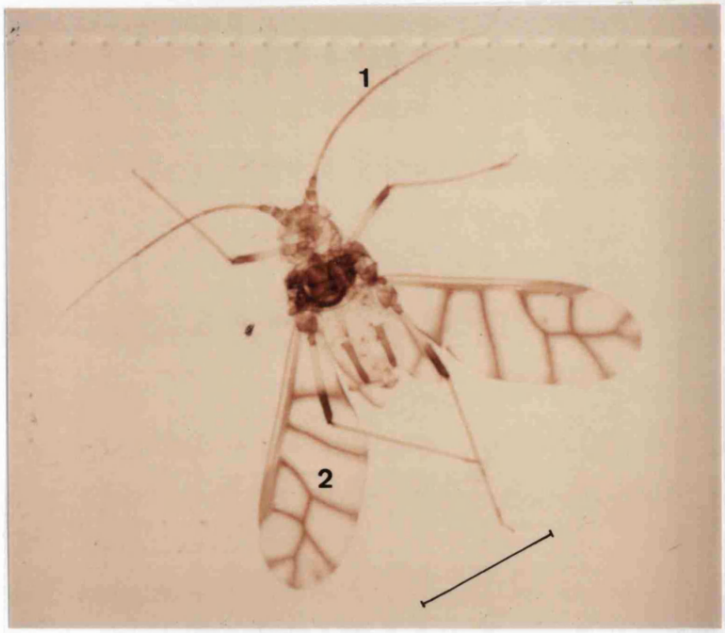


Plate 37: Pentalonia nigronervosa Coquerel. alate: 1-antennae; 2-broadly bordered wing veins with brown pigment.
Bar represents 1 mm.



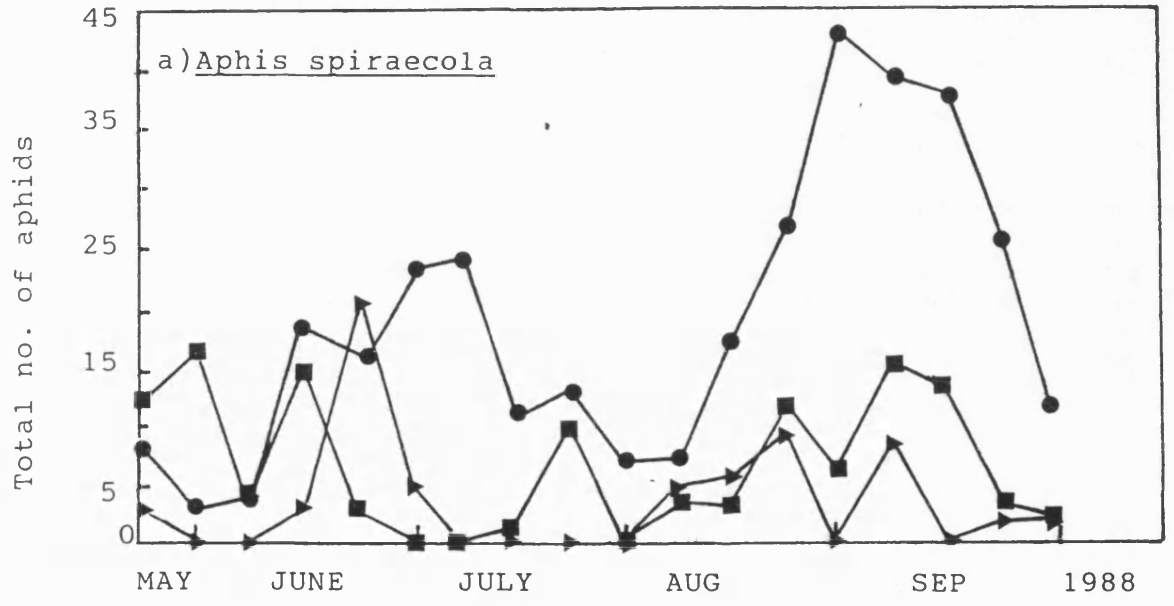
Heathcote, (1957) pointed out that horizontal traps only attract flying aphids in the 'landing' phase, and thus catch fewer aphids than the more practical vertical cylindrical traps. The number of aphid species collected in different traps is shown in Fig. 21.

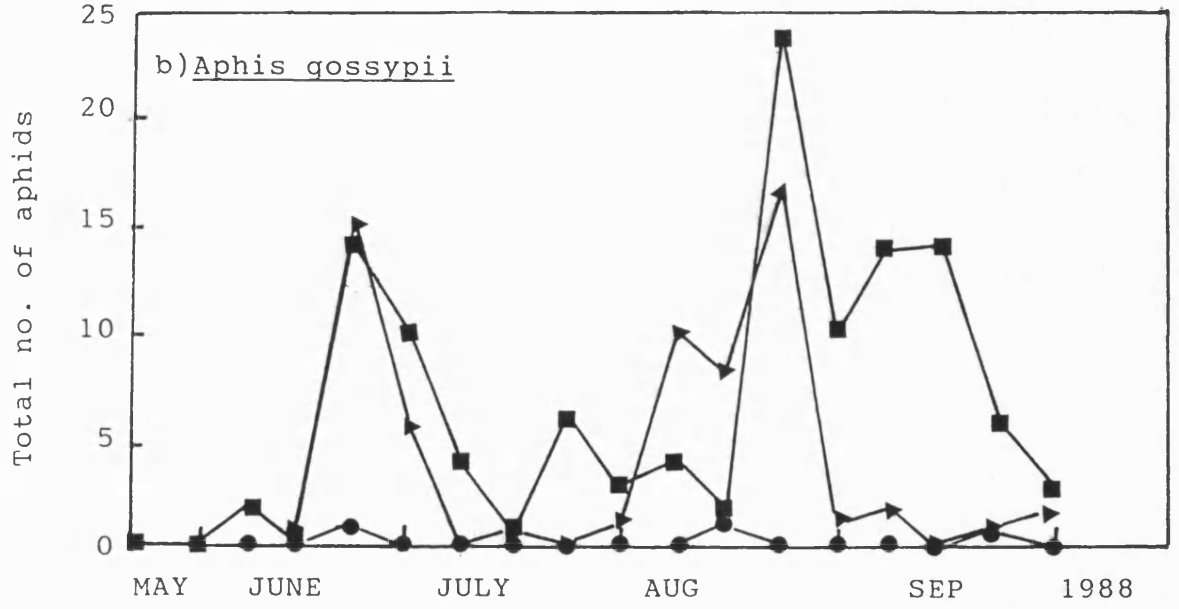
Aphis spiraecola was most abundant in yellow water traps collected in June, July, August and September, while *A. gossypii* was also abundant in sticky traps in June, August and September, but was rarely collected in water traps.

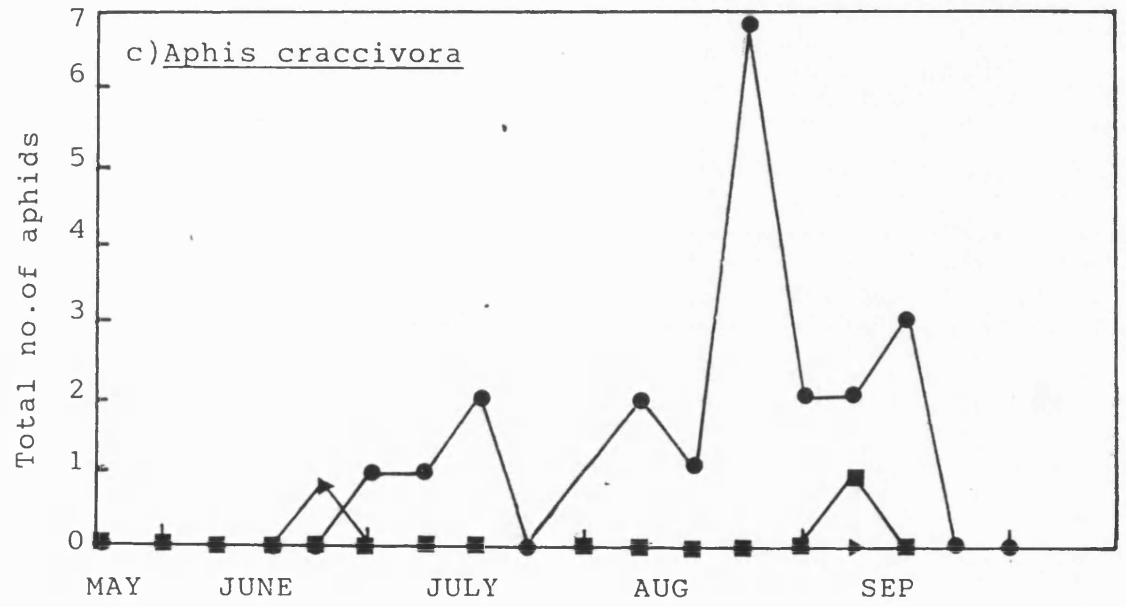
The results suggested that *A. spiraecola* was attracted to yellow traps. Most of the aphid spp. that were attracted to the yellow traps seemed to be those that feed on the foliage of herbaceous plants.

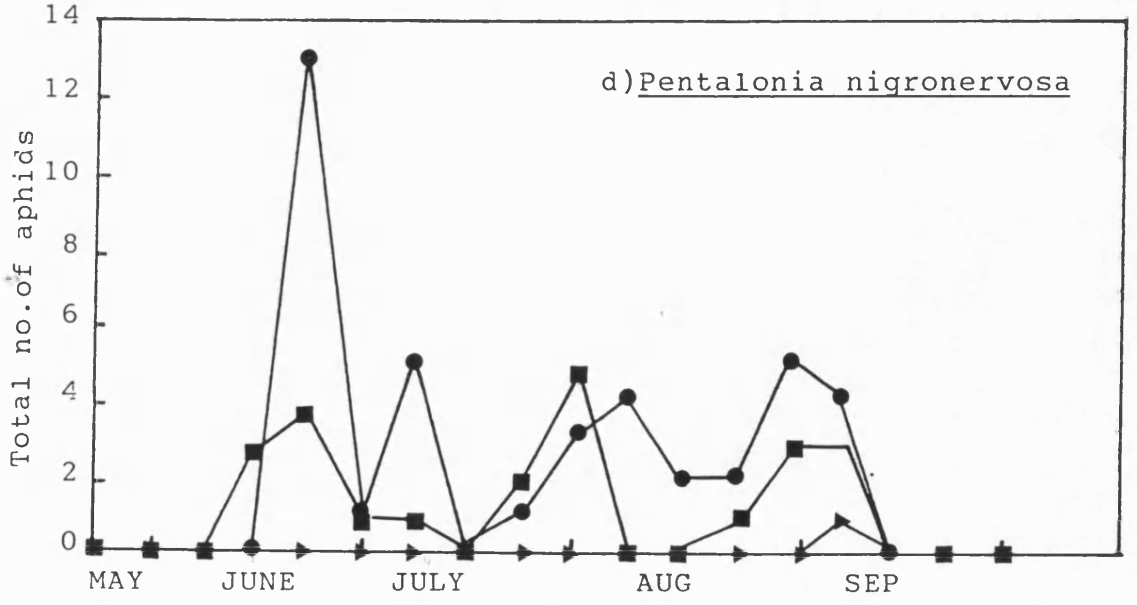
According to the results obtained in the present study, it seems that the comparative efficiency for catching each spp. of aphids differed with the type of trap. Water traps were more effective than sticky traps in catching several spp. of aphids such as *A. spiraecola, A. craccivora,* and *Pentalonia nigronervosa.* Unfortunately water traps required frequent attention, due to evaporation and overflowing of water during heavy rains, and there may also be a problem with aphid decay if traps are not cleaned frequently.

Fig.21 Weekly trap catches of aphid species collected in water bowl (●),cylindrical (■),and flat sticky traps (►).






c)Aphis craccivora
Total no.of aphids
0
1
2
3
4
5
6
7
MAY
JUNE
JULY
AUG
SEP


d)Pentalonia nigronervosa
Total no.of aphids
0
2
4
6
8
10
12
14
MAY
JUNE
JULY
AUG
SEP

4.1.6 Effect of trapping height for sampling aphids

During 1988, water traps were compared at two heights at Pahana Estate, Kalutara, Sri Lanka, from 15th May to 26th September and included a total of twenty trapping periods.

Six yellow water bowl traps were placed 75 cm above the ground another six were installed 150 cm above the ground, the vine height of passion fruit. All traps were 30 cm in diameter and 15 cm deep. Traps were serviced once a week and aphids were counted in the laboratory and placed in insect specimen bottles, in 95% alcohol, for subsequent identification.

Results are given in Table 39 together with total number of aphids caught and the percentage of the total made up of different aphid species.

Table 39: Catches of some aphid species in water traps at different heights

Height of trap (cm)	Total collection (6 traps)	Percentage of aphid species			
		Aphis spiraecola	*Aphis gossypii*	*Aphis craccivora*	*Pentalonia nigronervosa*
75	7,004	98	0.8	0.2	0.01
150	2,618	98	1.4	0.4	0.04

A total of 7,004 aphids were collected in yellow water traps at 75 cm above the ground. Whereas 2,618 aphids were caught when the traps were installed at

vine height of the passion fruit. Yellow water traps at 75 cm above the ground were considerably more effective for trapping. *Aphis spiraecola* represents approximately 98% of the total collection. Other species like *A. gossypii, A. craccivora,* and *P. nigronervosa* were low in number at either height. Heathcote, (1957) showed that for pans placed at 3 heights, significantly larger numbers of aphids were captured at ground level. Similarly, with *A. spiraecola* the lower position was significantly more effective ($P < 0.05$) at trapping aphids than those at vine height (Fig 22). The results suggested that trap height was an important parameter to consider when designing trapping methods.

4.2 Studies on experimentally infected *Passiflora*

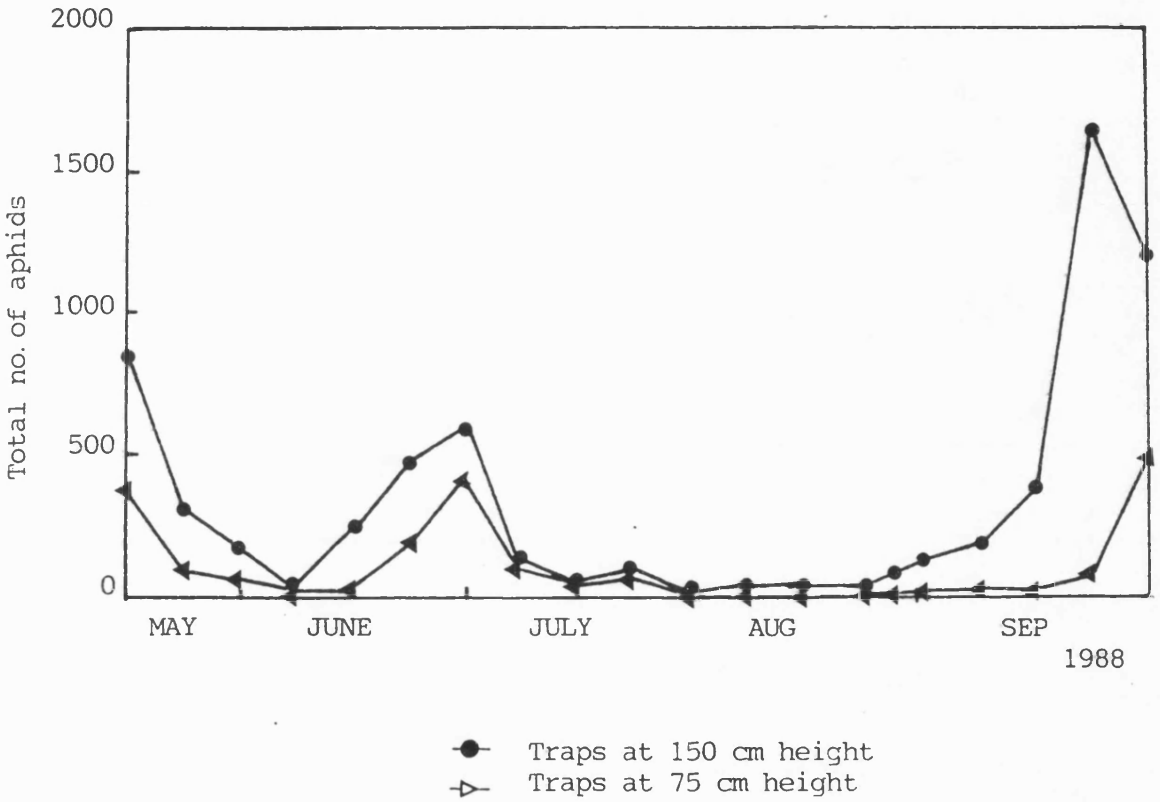
4.2.1 The seasonal occurrence of aphids in a passion fruit cultivated area using yellow water pan traps

There is little information on the ecology of aphid vectors in passion fruit crops in Sri Lanka. To obtain a better basic knowledge of aphid incidence, a trapping programme was carried out during 1988 - 1989 in Pahana Estate, Kalutara, Sri Lanka.

Experimental

Passion fruit seed of the yellow fruited cultivar (*P.edulis cv. flavicarpa*) was collected from apparently symptomless mother plants. These were sown in

Fig.22 Catches of Aphis spiraecola at two different trapping heights.



the nursery at Regional Agricultural Research Station, Bombuwela. Seedlings were grown in the nursery for 6 weeks and were planted out in the field when 10 - 12 mature leaves were present.

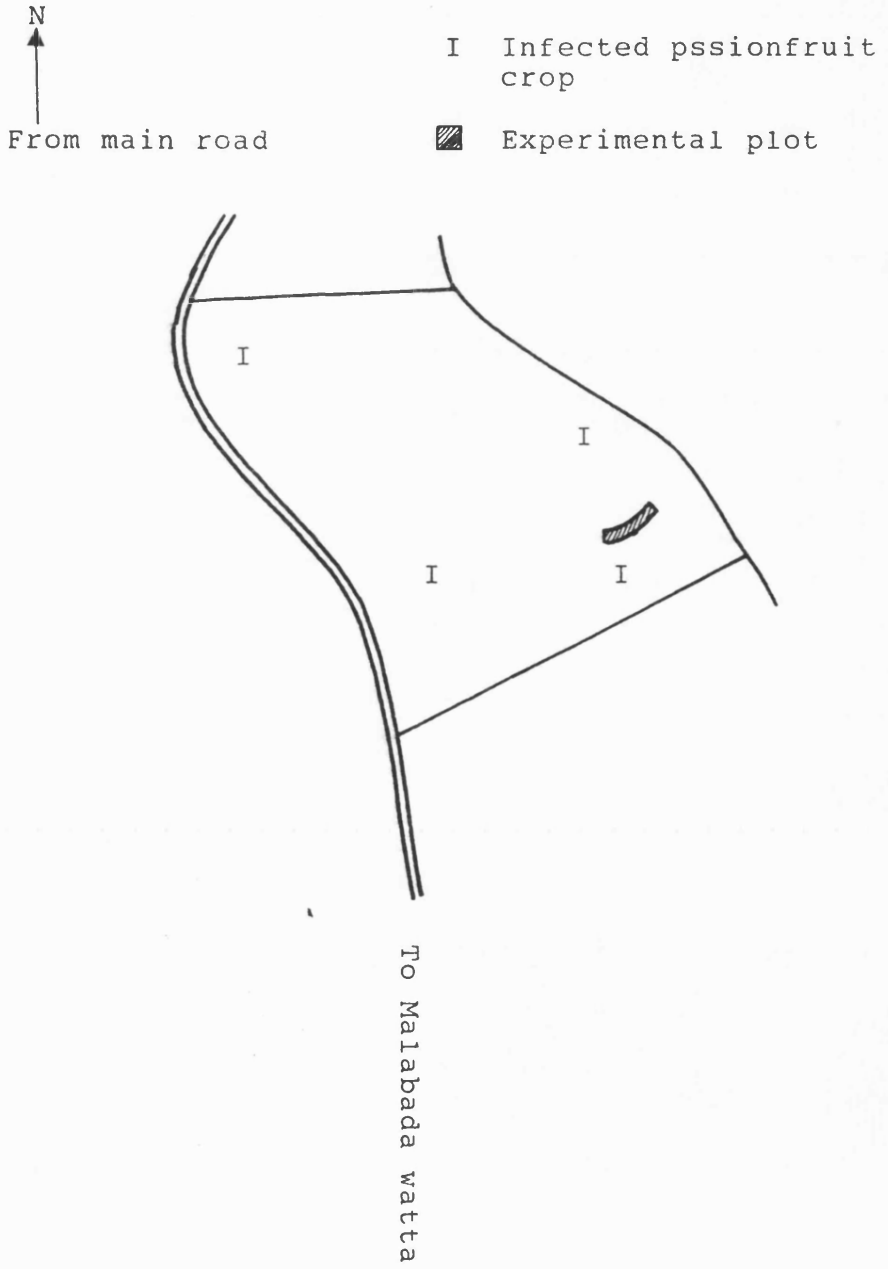
Experimental site

The experimental field at Kalutara is situated 5 miles away from the Regional Agricultural Research Station, Bombuwela, which is at an altitude of approximately 100 m. The mean annual rainfall is 2,500 - 5,000 mm, with a maximum in October and November (North-East monsoon). February and August are relatively dry, with little or no rain. The maximum and minimum temperature ranges are 28 - 35°C and 20 - 24°C, respectively.

Passion fruit Plot

The experimental plot consisted of six contours of about 20 plants each with 360 cm between the plants and approximately 210 cm between the rows (Fig. 23). A fully-grown passion fruit crop, 90% of which was infected with mottle symptoms (from visual observation) and 10% with ringspot symptoms was adjacent to the healthy plot on 3 sides. The soil cover of the experimental plot consisted of a mixture of *Pueraria phaseoloides*, *Centrosema pubescens* and *Mikania scandens*, which were removed to within a radius of 60 cm from the base of the vine, at monthly intervals. *Vigna unguiculata*, *Crotalaria spp.*, *Cassia tora*, *Mikania scandens*, *Eupatorium odoratum* were the predominant weeds in the neighbourhood of the

Fig.23 Field layout of experimental site of Pahana Estate, Dodangoda, Sri Lanka.



experimental plot. As reported earlier (Chapter 4.1.1), they were unlikely to be a source of virus but could be sources of vector aphid species. *Gliricidia maculata* was used as a 'live post' to support the adjacent passion fruit crop. Apart from above species, *Citrus* spp. and a few banana plants occurred. Normal cultural practices were followed to ensure vigorous growth of the crop.

Assessment of the abundance of winged aphids

Plastic, circular yellow traps, approximately 30 cm in diameter were set in the field to monitor the seasonal movement of winged aphids. Traps included an overflow hole on one side and a simple filter placed over the hole to allow excess water placed 900 cm apart at a diagonal in the field. These were mounted on wooden platforms, 75 cm above the ground. Traps were half-filled with fresh clean water and the trap contents were removed at weekly intervals (usually 10 - 11 am). Aphids were separated from other insect species in the laboratory and placed in 98% alcohol for preservation, before identification.

Disease incidence

This was determined by exposing the field-grown passion fruit seedlings to natural infection by aphids. Weekly counts of diseased plants were recorded on the basis of symptomatology. Infected plants were further confirmed by back inoculation to *P. foetida* and *C. amaranticolor.*

Infection rate

The rate of infection was calculated using the logistic equation (Simmonds, 1981) given by

$$\text{Log}_e \, {}^{I}/_{100-I} = rt$$

where I = cumulative percentage of infection

t = time

r = rate of infection

Weather data

Weather data were obtained from the Regional Agricultural Research Station, Bombuwela, 5 miles from the experimental plot.

Results

(i) Abundance of winged aphids

Table 40 showed that a total of 10 aphid spp. were caught in the yellow water traps during 1988 - 1989. About 90% of all aphids trapped were *A. spiraecola*. This species was trapped regularly throughout the experimental period.

Other aphid species, such as *A. craccivora, A. gossypii, Toxoptera citricidus* and *P. nigronervosa,* were low in number. *Aphis spiraecola* colonised weeds such as *Mikania scandens* and *Eupatorium odoratum* which were common in or around the experimental field. This observation was consistent with the earlier data on

Table 40: Winged aphids trapped during 1988 / 1989 *

	1988								1989				
Species	M	J	J	A	S	O	N	D	J	F	M	A	M
Aphis spiraecola	1356	1386	358	401	3428	934	244	411	307	63	5	17	18
A. gossypii	15	18	12	9	2	7	49	39	37	5	4	2	-
A. craccivora	-	3	5	5	2	-	-	15	4	3	3	1	-
Toxoptera citricidus	-	1	2	1	-	4	6	8	-	-	-	-	-
Toxoptera auranti	-	-	-	-	-	1	-	-	-	-	-	-	-
Pentalonia nigronervosa	-	1	1	-	-	1	1	-	3	-	3	2	-
Melanaphis sacchari	-	-	-	1	-	-	-	-	-	-	-	-	-
Rhopalosiphum maidis	-	-	-	-	-	1	26	13	1	-	-	-	-
Tetraneura nigriabdominalis	-	-	-	-	-	-	6	2	2	-	10	-	-
Cerataphis variabilis	-	-	-	-	-	-	2	-	-	-	1	-	-
Unidentified spp.	-	-	-	-	-	-	-	-	-	-	1	9	-

* TOTAL OF 6 TRAPS

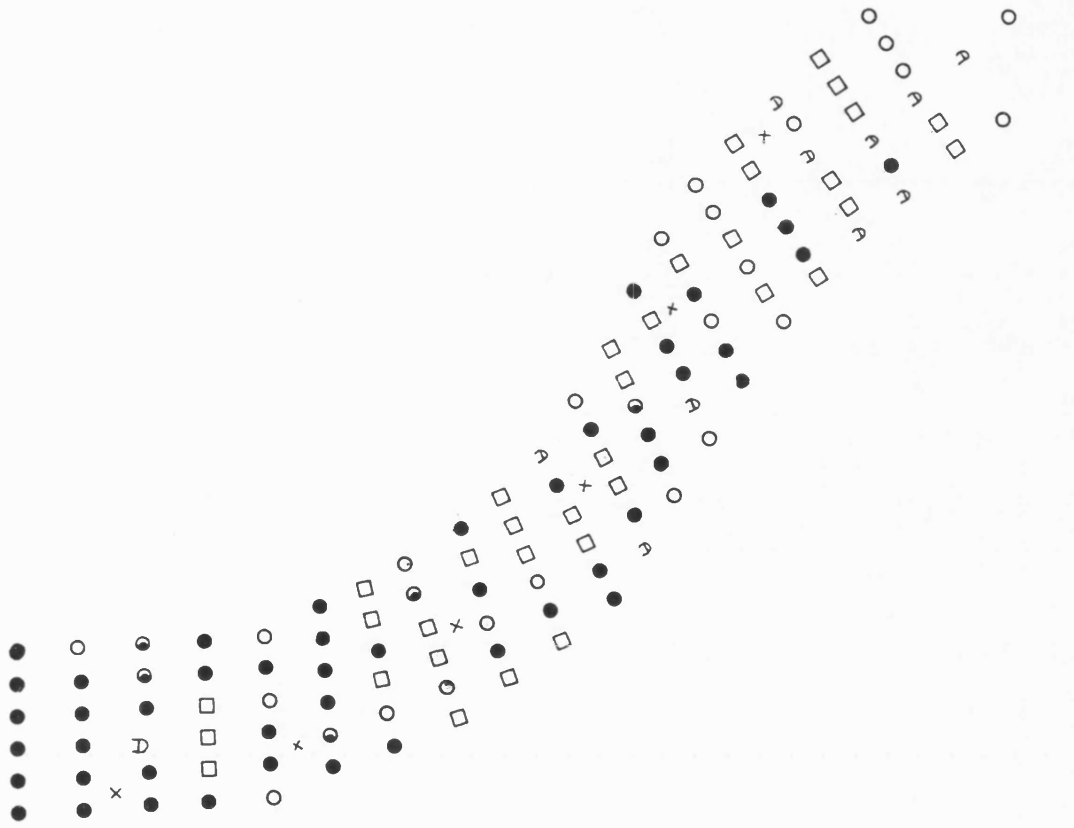
natural hosts (Chapter 4.1.1) and trap counts (Chapter 4.1.5). Colonies of *A. craccivora* were observed on *Gliricidia maculata* in the area throughout the experimental period and were high when compared to their numbers present in traps. *Pentalonia nigronervosa* and *T.*

citricidus were few presumably because few banana and citrus plants were present in the field. *Aphis craccivora, A. gossypii, T. citridus,* and *P. nigronervosa* are also important vectors of non-persistent viruses (Fritzsche *et al.*, 1972) but the numbers trapped were too low to see any relationship.

(ii) Disease incidence

The first symptoms on passion fruit seedlings were observed two weeks after field planting. Similar results were obtained under laboratory conditions when viruliferous aphids were fed on healthy plants. Initial symptoms of infection were vein-yellowing, yellow spotting and downward bending of young leaves. Later, these infected plants showed mottling, in addition to the initial symptoms. Only a few plants (Fig. 24) developed ringspots on older leaves, during five months of observation. Figure 24 shows the primary and secondary spread of the disease in the field. Infected plants were first distributed throughout the crop with no obvious gradient of infection. Clusters of diseased plants developed later along rows in each contours. This pattern of infection shows that most spread was within the crop as the initial foci expanded. Disease levels were regularly low in the early planting possibly due to defoliation of plants soon after field planting and the time taken to produce new flushes of growth, which varies with the climate. Disease level increased rapidly in July, and reached a plateau probably due to lack of new

Fig.24 The distribution of virus infected passion fruit plants at five months of growth.



● Early infected plants with mottle symptom

○ Late infected plants with mottle symptom

◒ Infected plants with ringspot symptom

D Plants died □ Plants not infected

× Aphid traps

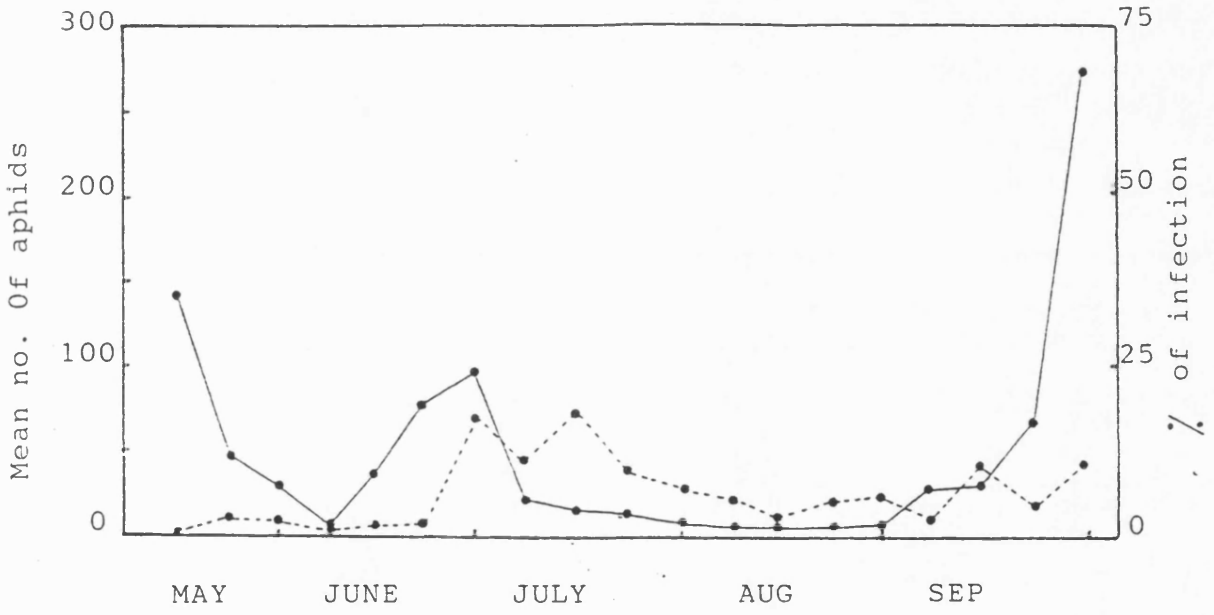
flushes during flowering stage which were less attractive to aphids; multiple probing by aphids on plants already infected may also have contributed to the plateau. There was a significant positive correlation (r = 0.57) between the number of winged *A. spiraecola* trapped and the percentage of virus-infected passion fruit plants. The percentage of infection and number of aphids trapped during a five month period are shown in Fig. 25. Numbers of infected plants were higher around the first three traps which collected more aphids than the other traps (Fig.24).

The results of the present experiment were based on 5 months period of data collection. Monitoring flights and disease incidence over several seasons would provide a more reliable picture of their relationship.

(iii) Infection rate

Van der Plank (1968) in his compound interest (CI) equation has shown a way of interpreting field data on a plot. According to this author, the rate of infection increases exponentially when only a small percentage of disease is considered. In the present study the disease spread is about 67%, therefore a modification of the CI equation has to be used. Using the logistic equation, the percentage of infection, 'I' is converted to a factor to obtain a straight line where the slope gives the rate of infection. It remains constant throughout the season. The 'S' shaped curve

Fig.25 The percentage of mottle infected passion fruit plants and number of alate Aphis spiraecola trapped during May - Sep 1988.



(Fig. 26a) was transformed to a straight line (Fig. 26b) , using the logistic equation to calculate the rate of infection. The gradient of this line, which was the rate of infection, was 0.45.

(iv) Relationship between weather data and the abundance of winged aphids

The total catch of aphids varied seasonally. To investigate the influence of weather on the seasonal fluctuations of aphids, the effect of several climatic factors on total catch of aphids was tested by regression analysis.

Of the factors tested, only rainfall proved to be strongly correlated with weekly totals of *A. spiraecola*. Fig. 27 shows the number of *A. spiraecola* trapped each week, and weekly totals of rainfall. The shape of this graph suggests that the population of aphids followed periods of higher rainfall. The effect of rainfall on aphid numbers was less obvious during February, March, April and May 1989. The effects of rainfall can last for several weeks: positive correlations were found between *A. spiraecola* upto 4th preceding week of rainfall. The correlation coefficients (r) for rainfall on aphids are given in Table 41 and the results suggested that weekly catches of *A. spiraecola* were substantially affected by rainfall.

Wet weather encourages the growth of *Mikania scandens* and *Eupatorium odoratum*, which are colonised by

Fig.26 a) Disease progress curve in passion fruit during five months of growth.

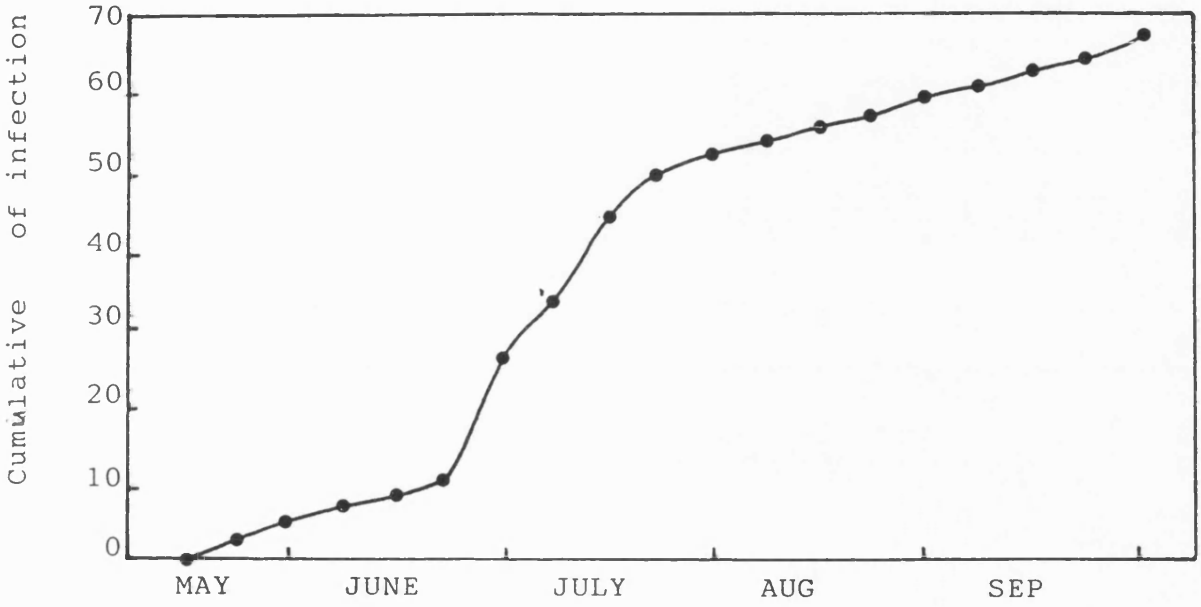


Fig.26 b) Straight line graph derived from disease progress S shaped curve in Fig.26 a.

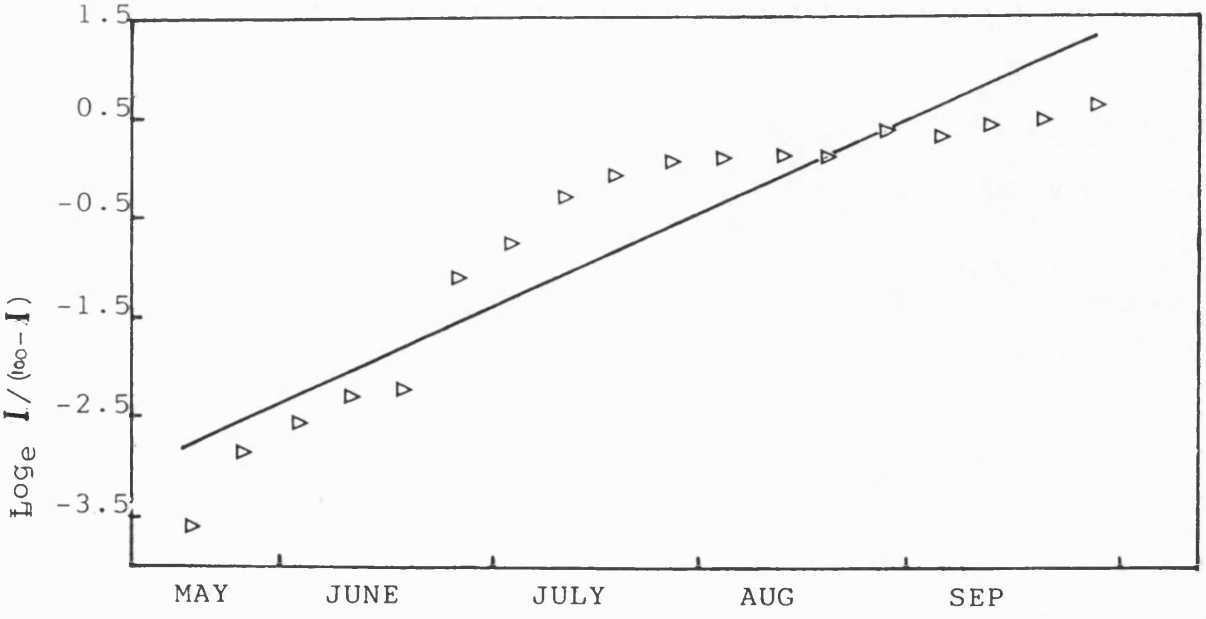


Fig.27 a) Numbers of Aphis spiraecola trapped in yellow water pans during April 1988 to May 1989.

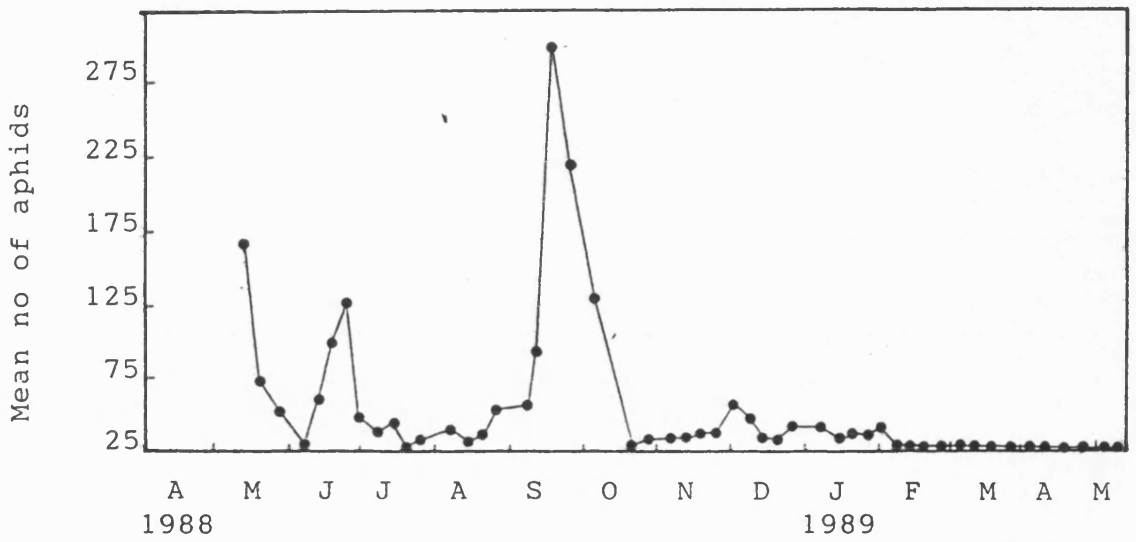


Fig.27 b) Weekly total rainfall data during April 1988 to May 1989.

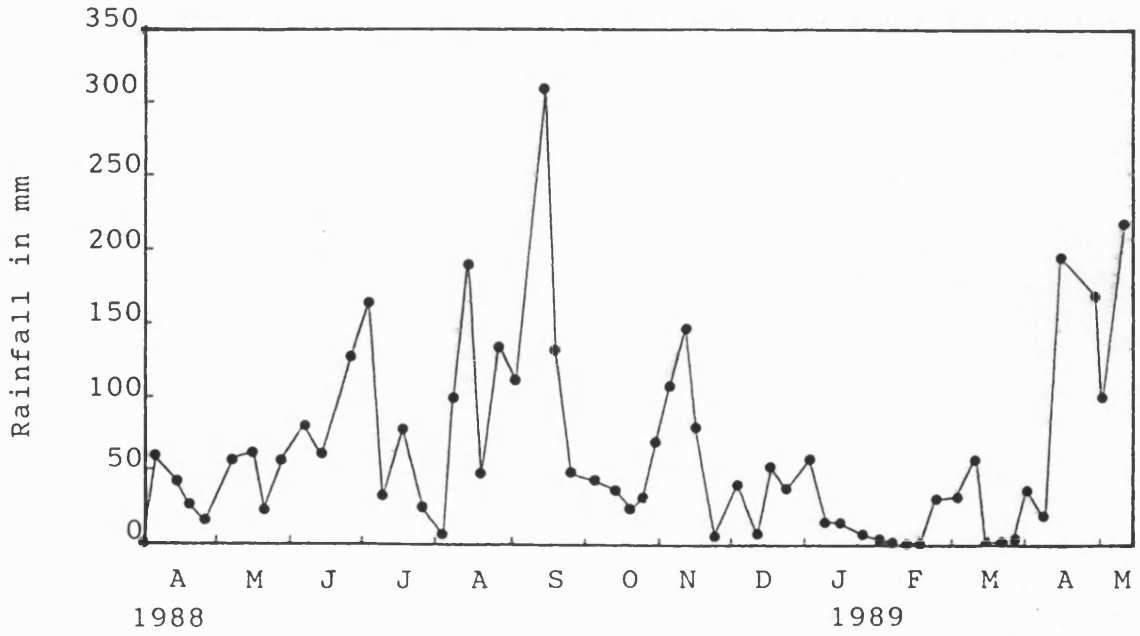


Table 41: Correlation coefficients of rainfall in mm, aphid numbers and percentage of infection in *Passiflora edulis cv. flavicarpa*

(a) *Aphis spiraecola* on percentage of infection (n=9, sig at 5% level)	0.57
(b) rain on *A. spiraecola* (n=42, sig at 5% level)	0.32
(i) rain of 1st preceding week on *A. spiraecola* (n=43, sig at 1% level)	0.42
(i) rain of 2nd preceding week on *A. spiraecola* (n=44, sig at 1% level)	0.56
(i) rain of 3rd preceding week on *A. spiraecola* (n=45, sig at 1% level)	0.46
(i) rain of 4th preceding week on *A. spiraecola* (n=46, sig at 5% level)	0.35
(i) rain of 5th preceding week on *A. spiraecola* (n=47, ns)	0.23

n = number of observation
sig = significant
ns = non significant

A. spiraecola. Rapid multiplication of aphids during optimum conditions results in overcrowding and the production of winged aphids. Therefore, trapped aphid numbers were higher following wet, rather than dry, weather. Johnson (1954) reported that numbers of aphids flying fluctuate from hour to hour as well as from day

to day. Thus, more detailed analysis would be required to separate the effects of various climatic factors on aphid movement. However, it is of some interest that the overall weekly catch of aphids was correlated with rainfall. The main peak of *A. spiraecola* occurred in September, smaller peaks were observed in May, June, December and January.

(v) Discussion

A significant positive correlation could be demonstrated between the number of winged aphids trapped and percentage of virus infection in passion fruit virus. *Aphis spiraecola* appeared to be the most important vector under local conditions as it represented 98% of the total catch of aphids. Broadbent *et al.*, (1950) demonstrated that potato virus Y was transmitted by alatae of *Myzus persicae* during the summer coming from a diseased potato field to plantings of young potatoes, when the levels of infection in adjacent fields were high. Mueller (1964) has reported that a yellow cultivar of lettuce attracted more aphids than a reddish brown one. Young passion fruit leaves, therefore, may be more attractive to flying aphids (De Wijs, 1974) because of the yellowish colour of young leaves. This may result in more landing activity and 'sap sampling' probes which would increase the possibility of transmission. New growth of passion fruit seedlings usually follows periods of rainfall so that more infection is possible following wet weather.

Weather may have an indirect effect on aphid behaviour by affecting the condition of the host plants. Aphid multiplication and morph determination are controlled by plant nutrition and the physiological condition of the plant (Lamb, 1958). The number of aphids in the air at any time depends upon a complex of factors including population density on crop, rate of alatae production and flight activity. All of these factors may be influenced by climate. Johnson (1952) pointed out that it is necessary to be very circumspect in drawing conclusions concerning the mode of action of climate on aphid transmission solely from trap data, since factors other than mere numbers may be involved.

The results indicated that the use of yellow water bowl traps was quite feasible both for survey work and ecological investigations of most aphid species. These results also suggested that winged aphids were present all the year round.

4.3 Non aphid-borne transmission

4.3.1 Pollen transmission

The pollen transmissibility of PV1 and PV2 was tested by grinding pollen obtained from infected plants with one or two drops of 0.02 M tris-HCl pH 7.8 buffer containing 1 g/l Na_2SO_3 and inoculating the extracts to *C. amaranticolor* plants.

None of the test plants showed any symptoms on inoculated leaves after 4 - 6 weeks. The results suggested that these passion fruit viruses were not transmitted by pollen.

4.3.2 Transmission through seed

Seed transmission was studied using virus-infected PV1 and PV3 seed of *P. flavicarpa* and *C. occidentalis*. This was done as follows:

(i) Twenty-five seeds were soaked in water overnight and inoculum was prepared by homogenizing seed on a 0.02 M tris-HCl buffer pH 7.8 containg 1 g/l Na_2SO_3 (1:10 w/v), which was then assayed on *C. amaranticolor*.

(ii) Fifty seeds of PV1 and PV3 selected at random were grown in a nursery and seedling leaves used to prepare inoculum as described above.

The inoculated plants did not show any symptoms and suggested that PV1 and PV3 were not seed transmitted.

(iii) To investigate seed transmission of PV1, further studies were done with direct-ELISA. Immature seeds, and the fruit pericarp of PV1-infected *P.foetida* were used. Infected leaves were used as a control. Equal weight samples of diseased and healthy tissues were ground in mortar and pestle with PBS-TPO buffer. The

extracts were then strained through muslin and antigen dilutions were made using the same buffer. The results are presented in Fig 28.

Virus was detected in leaves and fruit but not in immature seeds. A weak positive reaction was obtained for the sap extracted from fruit skin at $^1/_5$ dilution. The results indicated that PV1 infected seeds were apparently free of virus.

4.3.3 Transmission by grafting

This study was made by wedge grafting shoots of PV1, PV2 and PV3-infected *Passiflora edulis cv. flavicarpa* to *P. ligularis*, and *P. flavicarpa*. All the grafted plants showed virus symptoms 2 - 4 weeks after grafting (Plate 38). Infection was confirmed by back inoculation to *C. amranticolor*.

This experiment has indicated that passion fruit viruses could be transmitted by grafting. Graft transmission of viruses in nature is probably uncommon, but may occur through chance grafting of roots as they grow together (Walkey, 1985). Apple mosaic virus has been reported to be transmitted this way in apple trees (Hunter *et al.*, 1958).

4.3.4 Transmission through pruning secateurs

The possibility of contact / mechanical transmission by infected sap or tissues on the blades of

Fig.28 Comparative absorbance curves obtain by DAS-ELISA for different type of tissues.

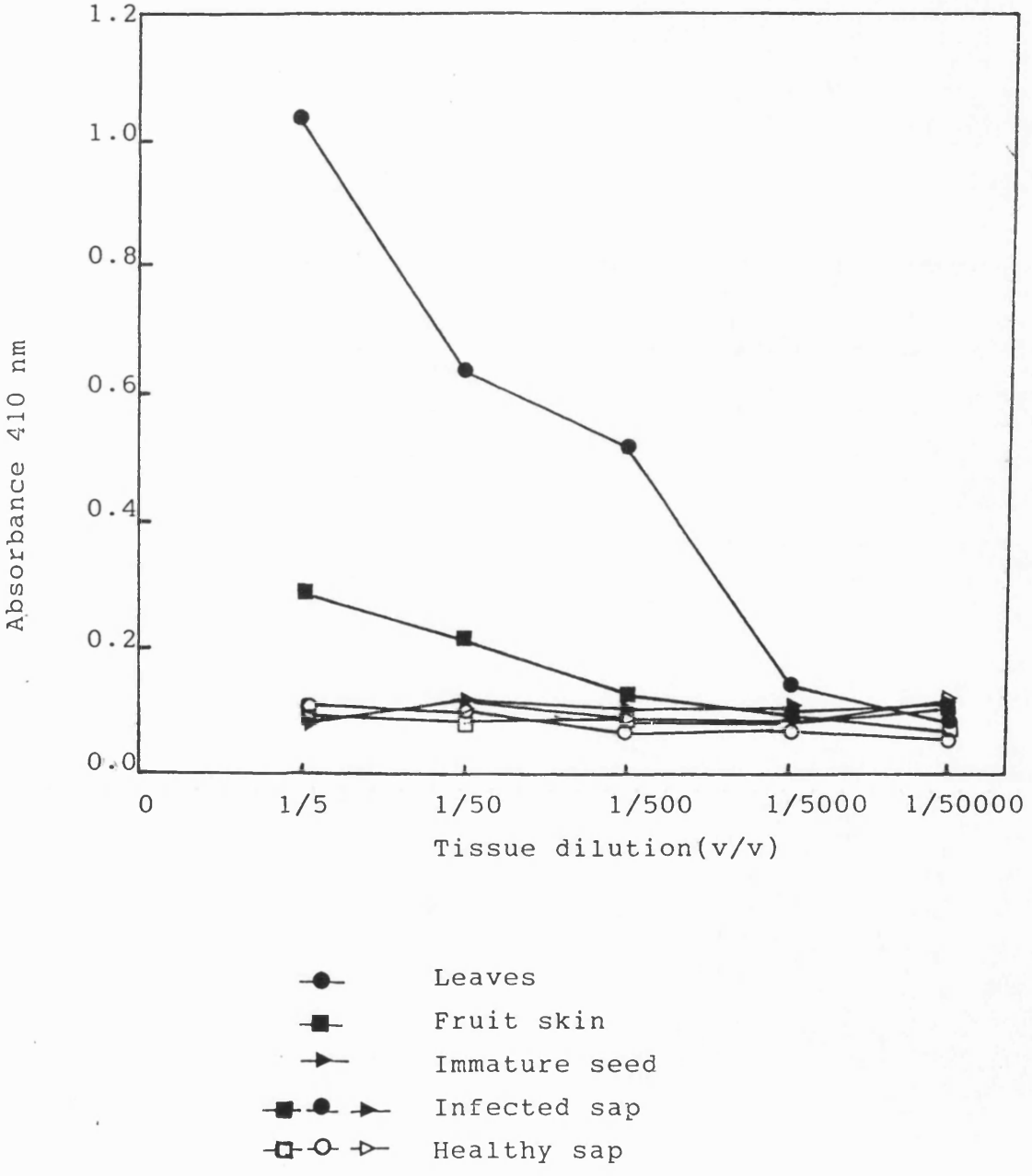


Plate 38: The transmission of PV1 by Wedge graft; S - infected scion, R- rootstock. Note the symptoms induced in Passiflora edulis cv. flaviccarpa rootstock two weeks after grafting.

secateurs was investigated. Twenty, apparently virus-free passion fruit plants were pruned heavily with pruning secateurs that had been earlier used to prune severely infected plants infected with PV1. Pruning was repeated twice during a 2 months period. Plants which developed symptoms were back tested on *C. amaranticolor*.

Three plants (15%) developed virus symptoms two weeks after the second pruning. Back tests confirmed infection, indicating the possibility of transmission of PV1 through pruning secateurs. Seneviratne and Wickramasingha (1972) earlier showed the possibility of transmission of mottle virus through pruning secateurs.

4.3.5 Transmission by contact of diseased and healthy vines

Healthy plants kept side by side with PV1- and PV2-infected plants in a glasshouse, were allowed to 'intertwine' with each other for a 2 months period. The virus was not observed to be transmitted to healthy plants in this manner.

CHAPTER 5 ECONOMIC IMPORTANCE OF PASSION FRUIT VIRUSES

5.1 Geographic distribution and incidence of virus diseases affecting passion fruit plants in the low country wet zone of Sri Lanka

A survey of passion fruit (*Passiflora edulis cv. flavicarpa*) was conducted in June and September, 1988 in Kalutara, Colombo, Galle and Gampaha districts in the wet zone of Sri Lanka (Fig. 29). Passion fruit cultivations were randomly selected from a list of growers, maintained at Agrarian Services Centres in each segment within the district. Disease incidence was assessed by visual observation. Infected plants in each cultivation were counted and the percentage infection and disease severity recorded (Table 42).

High levels of passion fruit mottle symptoms were observed in the Kalutara and Colombo districts. According to the results, the disease incidence had reached 30 - 80% and 5 - 100% in Kalutara and Colombo districts, respectively.

Sporadic occurrence of mottle infection was observed in Galle and Gampaha districts with disease incidence of 1 - 10%, or less. Most farmers in these districts had adopted a stringent field sanitation programme which included roguing of plants with symptoms.

Fig.29 Geographic distribution and incidence of Passion fruit virus diseases in the Low Country Wet Zone of Sri Lanka

Table 42: Distribution of passion fruit viruses in four districts of Low Country wet zone, Sri Lanka

District	Number of Cultivations Examined	Disease Symptoms	Disease Incidence (%)	Disease Severity Rating *
Colombo	5	mottling	5 - 100	2.7
		mottling, severe leaf distortions, fruit with brown corky growths.	1	5
Galle	10	mottling and yellow spotting	0 - 10	2
		mottling and ringspot	1	1.5
		mottling, leaf crinkling, abnormal growths on fruits.	1	5
Gampaha	8	mottle.	1	<1
Kalutara	11	mottling and spotting	30 - 80	3.5
		mottling and ringspotting	2 - 30	1.0

* Disease severity based on a scale:
0 - no symptoms
1-2 - milder symptoms
2-4 - moderately severe symptoms
5 - severe symptoms

* Mean value

Apart from the widespread mottle symptom of passion fruits, yellow circular rings were also observed in some crops. Ringspotting together with mottling and dotting symptoms were of recent occurrence and only 2 - 20% and 0 - 1% disease incidence were recorded in Kalutara and Galle districts, respectively.

A few cultivations had very severe disease symptoms, with mottling of leaves, leaf crinkling, small brown fruits with corky growth.

The results of this survey show that the virus or virus-like diseases have reached epidemic proportions in parts of Colombo and Kalutara district and seem to be spreading rapidly. The same diseases, however, have not yet become well established in surveyed areas of Galle and Gampaha districts. Surveyed areas are given in Fig 29.

5.2 The effects of passion fruit virus infection on growth and yield in a yellow passion fruit species (*P. edulis cv. flavicarpa*)

Passion fruit is a very profitable crop to cultivate, and viruses may cause severe economic losses (Dassanayake unpublished observations and Simmonds, 1959), so the effects of PV1 (Homagama A and B) and PV2 on growth and yield were studied.

Passion fruit seeds were collected from apparently healthy, high-yielding, mother plants in the

Low Country wet zone. Seeds were then sown in the nursery and, later, repotted into 10 cm diameter pots in the glasshouse at the University of Bath. Seedlings at the six leaf stage were inoculated with an extract from *P. foetida* infected with PV1 (Homagama A and B) and PV2 prepared in 0.02 M tris-HCl pH 7.8 buffer containing 1 g/l Na_2SO_3 (1:10 w/v).

Three weeks after inoculation plants were repotted in 30 cm diameter pots and were arranged in a randomized block design with five replicates. Each replicate had four treatments including apparently healthy controls, with two plants each treatment.

Effects on growth, due to virus infection were recorded at two week intervals up to three months. Once the infection was stabilized about four months after inoculation, the severity was rated on a zero to five scale based on several characteristics.

<u>The parameters measured were:</u>

(i) Plant height, (Stunting)

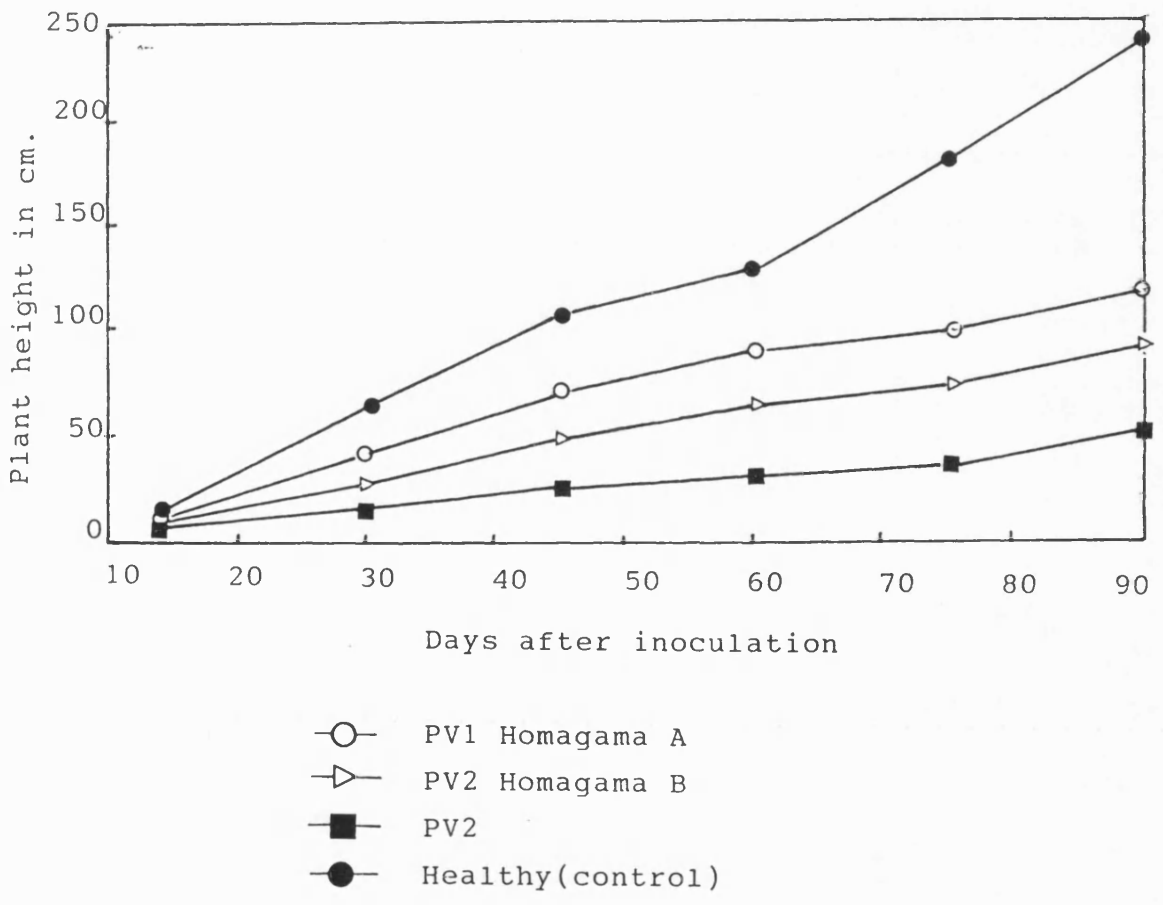
(ii) Growth, (Size of the canopy)

(iii) Leaf lamina reduction or distortion

Scale 0 (No symptoms) to 5 (Severe symptoms)

The results of experiments done to study the effect of some of these parameters are given in Fig 30 and Table 43. Prior to analysis log transformation was

Fig.30 a) Effect of passion fruit viruses on plant height. (mean).



used on total number of leaves, shoots and flowers in order to equalise the variance.

Passion fruit virus infection resulted in a marked setback in growth components of infected plants. According to the results shown in Fig. 30a, the mean plant height of artificially infected plants was less than that of healthy plants. Healthy plants had a significantly higher plant height ($P < 0.05$) at 45, 75 and 90 days after inoculation.

With leaf number, only those plants inoculated with PV2, 75 and 90 days after inoculation, showed a significantly ($P < 0.05$) lower leaf number. Passion fruit virus 1 - infected plants (Homagama A and B), had the next lowest leaf number and these plants initially showed defoliation (Fig 30b).

Although the number of shoots were numerically fewer in healthy controls at 30, 45 and 60 days after inoculation, the differences with infected plants were not significant ($P > 0.05$). At 90 days, however, the numbers of shoots were highest in the healthy control. Plants infected with PV2 (but not PV1) had a significantly lower number of shoots ($P < 0.05$) than controls after 90 days (table 43a).

The number of flowers after one fruiting season are summarised in Table 43b. Healthy controls had more flowers than virus-infected plants, but this difference was not significant ($P > 0.05$). Fruit counts were low

Fig.30 b) Effect of passion fruit viruses on number of leaves (mean).

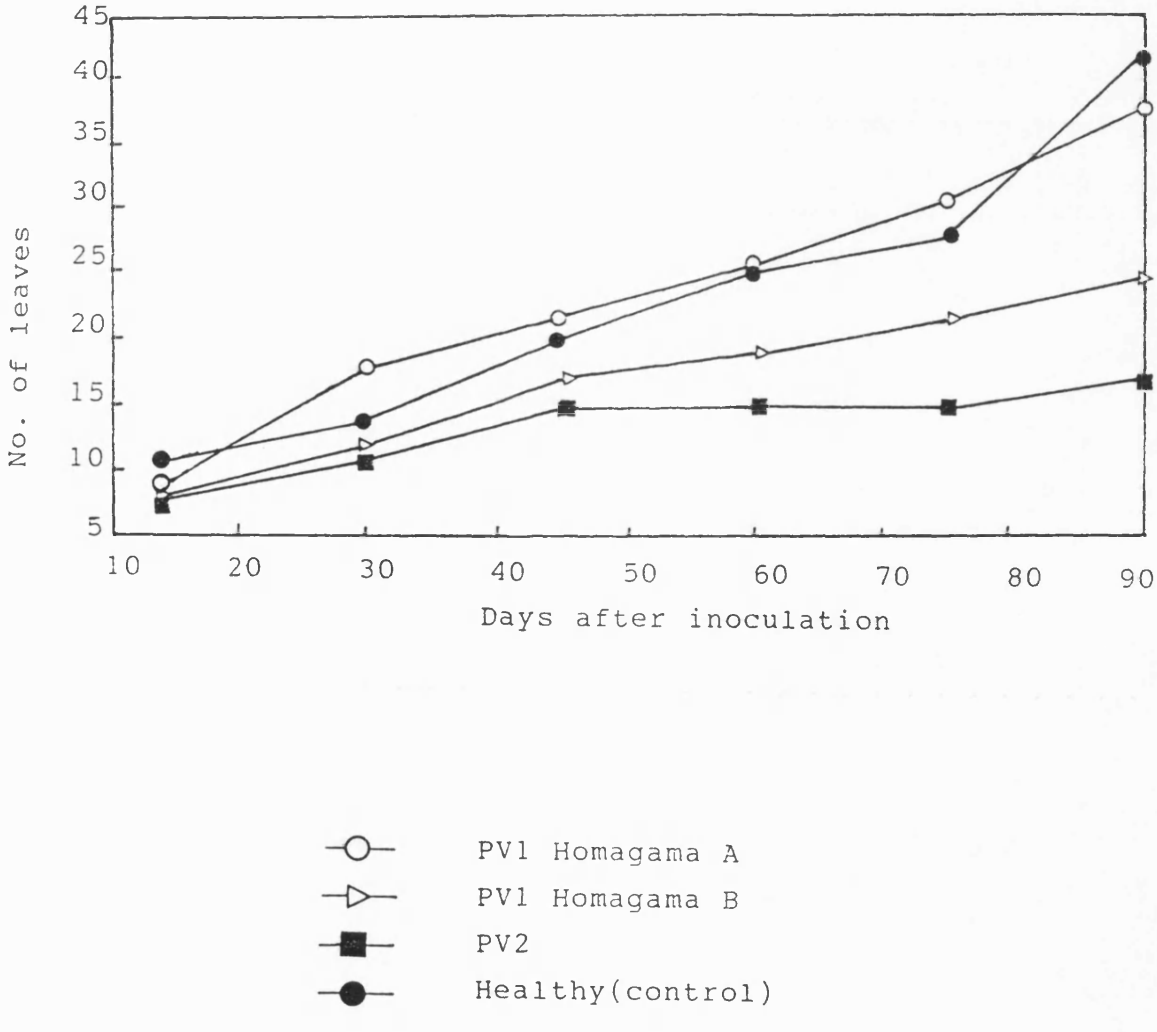


Table 43: Effect of passion fruit viruses on growth and fruiting parameters

(a) Mean number of shoots

Virus isolates	Day after inoculation					
	14	30	45	60	75	90
PV1 Homagama A	1	2.3	2.4	2.5	4.4	5.4ab
PV1 Homagama B	1	2.2	2.3	2.3	2.9	4.5abc
PV2	1	2.2	2.3	2.3	2.4	3.6bc
Healthy control	1	1.5	2.2	2.2	2.5	7.9a
	NS	NS	NS	NS	NS	

(b) Mean number of flowers and fruits

Virus isolates	No. of flowers	No. of fruits	Fruit weight (g)	Brix value
PV1 Homagama A	23	3	21	12
PV1 Homagama B	16	1	42	13
PV2	10	-	-	-
Healthy control	45	5	63	12
	NS			

NS = not significant

(c) Mean disease index value

Virus isolate	Mean disease index value*
PV1 Homagama A	7.95
PV1 Homagama B	9.85
PV2	12.53
Healthy control	0

* Mean disease index value from 10 plants

and, therefore, it was difficult to make any reliable comparison between diseased and healthy plants. The results indicated, however, that healthy controls had more fruits and a higher mean fruit weight than those of diseased vines. There was no such difference between diseased and healthy vines for brix value which was a measure of total soluble solids.

Poor fruiting and flowering in both diseased and healthy vines could be due to prevailing microclimatic conditions during the growing season. These climatic factors may influence flowering and fruit setting of plants as well as the course of the infection.

According to the disease index values it seemed that PV2 virus more severely affected plant growth than PV1 - Homagama A and B. Passion fruit virus 1 Homagama B isolate was also more severe than PV1 Homagama A.

5.3 Effect of virus infection on quality of passion fruit (*P. edulis cv. flavicarpa*)

Preliminary investigations were done at the Adoptive Research Unit, Homagama, Sri Lanka to study the effect of virus infection on marketable quality of passion fruit (*Passiflora edulis cv. flavicarpa*). Fruits were collected during the fruiting season from a four year old passion fruit cultivation where all the plants showed symptoms of mottle.

The fruits were mostly mature and green when picked. These fruits were then brought into the laboratory and allowed to ripen before readings were taken. Fruits were hand graded for the presence of mottle, abnormal growth with hard pericarp and unmarketable appearance. The fruits were analysed using the following parameters:

(i) Presence of mottle on pericarp

(ii) A hard pericarp which was difficult to cut through with a knife

(iii) Weight of fruit

(iv) Cavity diameter

(v) Fruit diameter

(vi) Rind thickness and brix value

A hand refractometer was used to measure the level of total soluble solid which is an indication of total sugars in the fruits.

Fruits were graded in the following groups:

Group A	Mottle and hard pericarp, unmarketable fruits.
Group B	Mottle pericarp, unmarketable fruits.
Group C	Fruits without any symptoms, marketable fruits.
Group D	Fruits without any symptoms, hard pericarp, marketable.

Four picks of fruit were analysed over two months period. At least 80 - 100 fruits were included

for each analysis. Results are summarised in Table 44 (mean of four picks).

Table 44: Effect of virus on quality of passion fruits

Group	% of fruits	wt of fruits (g)	Fruit diam. (cm)	Cavity diam. (cm)	Rind thickness (mm)	Brix value
A	45	75.5	5.7	4.2	6	17.2
B	29	70.5	5.6	4.3	4.8	16.9
C	17	68.8	5.6	4.3	4.3	17.3
D	8	80.5	5.8	4.7	4.5	17.5

The percentage of marketable fruits was low when compared to unmarketable fruits in each pick. Results indicated that unmarketable fruits exceeded 70% in total harvest over 2 months. Unmarketable fruits had green spots and tiny pimples on pericarp (Plate 39), and were difficult to cut through using a knife. However, some fruits were free of any symptoms, but had slightly thicker pericarps. Reduced fruit cavity was not observed in any of the four groups examined. Rind thickness varied from 4 - 6 mm in each group but infected fruits were free of woodiness symptoms (thickened pericarp and reduced pulp cavity). There was no reduction of brix value between the diseased fruits with or without symptoms.

Plate 39: (a) A fruit of Passiflora edulis cv.flavicarpa infected with mottle symptoms in the field (left) and a normal fruit (right).
(b) Cross section of the same.

(a)

(b)

CHAPTER 6 CONTROL THROUGH RESISTANT CULTIVARS

In seeking methods of control for passion fruit viruses, identification of resistant cultivars can be regarded as important. In Sri Lanka several species of *Passiflora* are cultivated and of these, *Passiflora edulis cv. flavicarpa* (yellow cultivar) and *P. edulis* (purple cultivar, are the two recommended cultivars. Apart from these cultivars, several other species of *Passiflora* are grown in various parts of the country including *P. mollissima, P. van volxemii, P. edulis f. edulis* and *P. ligularis* (Plate 40) in the up country Nuwara Eliya area, and *P. foetida, P. suberosa* (Plate 41) and *P. quadrangularis* in the low country wet zone.

Screening for resistance was done with 10 different *Passiflora* spp. collected from different parts of Sri Lanka. Seeds were planted in 12 x 9 cm germinated trays in a glasshouse at the University of Bath. Seedlings were transplanted into 8 cm diameter plastic pots.

Apparently healthy seedlings of similar size from the various spp. of *Passiflora* described below were used for the screening test. Inocula were prepared using PV1 and PV2 infected leaves of *P. foetida* and leaves were ground in 0.02 M tris-HCl pH 7.8 plus 1 g/l Na_2SO_3 (1:10 w/v). The results are summarised in Table 45.

PLate 40: Passiflora suberosa L.

Plate 41: Passiflora ligularis Juss.

The resistant reaction

Inoculated plants were grown in the glasshouse with average temperatures of about 19°C and a light intensity of 3,000 - 3,500 Lux. Back tests for infection were made to *C. amaranticolor* three and eight weeks after inoculation.

Out of 10 species of *Passiflora* screened against PV1 and PV2 only *P. suberosa* showed resistance to infection. *Passiflora ligularis* reacted with mild symptoms and may be considered as a tolerant species. *Passiflora edulis cv. flavicarpa, P. quadrangularis* and *P.foetida* were highly susceptible while the other species were also susceptible.

The observations suggested that passion fruit species varied in their susceptibility to PV1 and PV2. The response of *Passiflora* spp. to the two passion fruit viruses showed that resistance may be available in some wild *Passiflora* species. These species could, therefore, be used in future hybridization programmes to improve virus resistance in cultivated passion fruits.

6.1 Confirmation of the virus resistance in *P. suberosa* to PV1

Screening of different spp. of passion fruit showed that *P. suberosa* was resistant , possibly immune, to PV1 virus to sap inoculation. However, further studies were carried out in Central Agricultural Research

Table 45: Susceptibility of *Passiflora* spp. to PV1 and PV2

Species	No of plants infected / No of plants inoculated		Percentage infection		Disease severity	
	PV1	PV2	PV1	PV2	PV1	PV2
Passiflora edulis f. edulis	9/10	8/10	90	80	s	vs
P. edulis cv. flavicarpa	20/22	14/20	80	70	s	vs
P. edulis (hybrid)	9/18	3/18	50	16	s	vs
P. caerulea	3/10	na	33	na	m/mo	na
P. foetida	20/20	20/20	100	100	mo	vs
P. ligularis	10/15	5/20	66	25	m	mo
P. mollissima	24/24	3/18	100	16	mo	vs
P. quadrangularis	5/5	5/5	100	100	s	vs
P. van volxemii	10/15	3/18	66	16	mo	vs
P. suberosa	0/15	0/15	-	-	-	-

Abbreviations

vs = very severe, s = severe, m = mild,

mo = moderate, - = no reaction

Institute, Gannoruwe, Sri Lanka to examine the resistance of *P. suberosa* to PV1. This virus was readily transmitted by *Aphis craccivora* under laboratory conditions as well as by grafting. Attempts were, therfore, made to transmit PV1 from *P. edulis cv. flavicarpa* to *P. suberosa* by these methods.

6.1.1 Graft transmission (Wedge grafting)

Two month old apparently virus free *P. suberosa* plants were used as rootstocks, and PV1 virus-infected *P. flavicarpa* shoots were used as scions. For grafting, scion shoots with symptoms were selected and the wedge grafting technique was used to transmit the virus (Chapter 2.22d). Graft union and the scions were covered with a transparent polythene bag to improve the healing of the bud union. One month after grafting, *P. suberosa* plants were indexed by inoculating to *C. amaranticolor* and *P. flavicarpa*.

6.1.2 Aphid transmission

First offspring generation of *Aphis craccivora*, reared on *Vigna unguiculata*, was used to transmit the virus from *P. edulis cv. flavicarpa* to *P.suberosa*. Different acquisition periods (5 - 15 min) and inoculation feeding periods (5 - 30 min) were used with 15 - 20 adult wingless aphids for each transmission. Twenty-five *P.edulis cv. flavicarpa* plants were inoculated. After inoculation, these were maintained in an insect protected glasshouse. To test for PV1 infection in passion fruits, back inoculations were made to *C. amaranticolor*.

Wedge grafted *P. suberosa* plants showed a vein yellowing, four weeks after grafting. These symptoms, however, faded with subsequent growth of plant and virus could not be detected when young leaves were back tested on *C. amaranticolor*. Plants inoculated by *Aphis*

craccivora did not show any virus-like symptoms, and virus could not be detected in back tests to *C. amaranticolor.*

These results suggested that either the plants supported no virus replication ('immune'), or virus replication if present was too low to be detected ('resistance').

6.2 Aphid resistant properties in *Passiflora* species with special refernce to the glandular hairs

Aphid resistance can be based on a combination of several mechanisms of a biochemical or physical nature. Aphid resistance has been reported by Gibson (1974, 1979) and Gunenc and Gibson, (1980) in a large number of *Solanum* spp. as being due to trichomes on the aerial surface of leaves. Gibson (1971) pointed out that specific aphid resistance mechanism, such as pubescence, was a more effective form of resistance than that based on a nutritional mechanism. Preliminary studies were, therefore, undertaken to search for 'hairs' on the leaf surface of selected *Passiflora* spp.

These were: *P. caerulea, P.edulis cv. flavicarpa, P. ligularis, P. foetida* and *P. suberosa.* The presence of hairs and trichomes were determined using a scanning electron microscope.

Passiflora foetida

Passiflora foetida had dense hairy stems and leaves. Under the scanning microscope, three types of hairs were observed in this species. One type, in large numbers , had longer hairs which tapered from the base and terminated in a sticky glandular tip (Plate 42). This type resembled the type B glandular hairs described by Gibson (1976). The second type of hairs were longer and ended with a pointed tip (Plate 43). The third type was low in number, and consisted of single lobed glandular hairs, although the presence of needle like projections from the head was unusual (Plate 44).

Passiflora suberosa

The presence of hairs on leaves was not visible to the naked eye. In the scanning electron microscope, however, it was found that *P. suberosa* also had glandular hairs which differed those present in *P. foetida*. These hairs had short stalks with elongated heads (Plate 45). *P. suberosa* was less pubescent than *P. foetida*.

Other species

Possible hair-like structures were observed in some plants of *P. flavicarpa*, and *P. ligularis* although their shape was not clear.

This brief study has shown that the type of glandular hairs may differ in different species of *Passiflora*. Gilbert (1971) reported that *Passiflora*

Plate 42: A sticky tipped hair on a leaf of Passiflora foetida . Bar represents 500 μm.

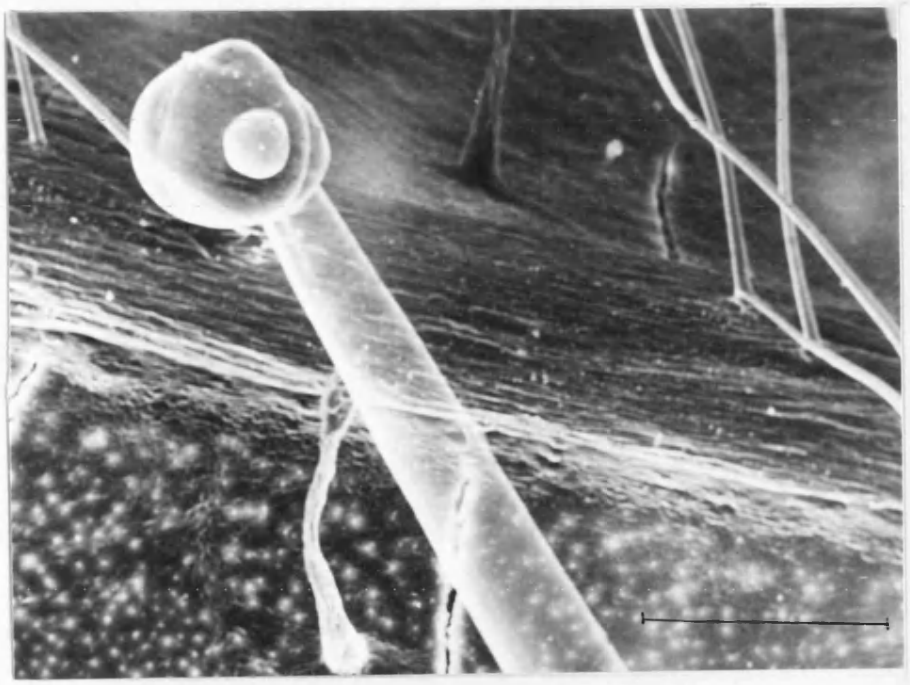


Plate 43: Two types of hairs on a leaf of Passiflora foetida . The pointed tipped hairs (marked in arrows) and a sticky tipped hair.
Bar represents 500 μm.

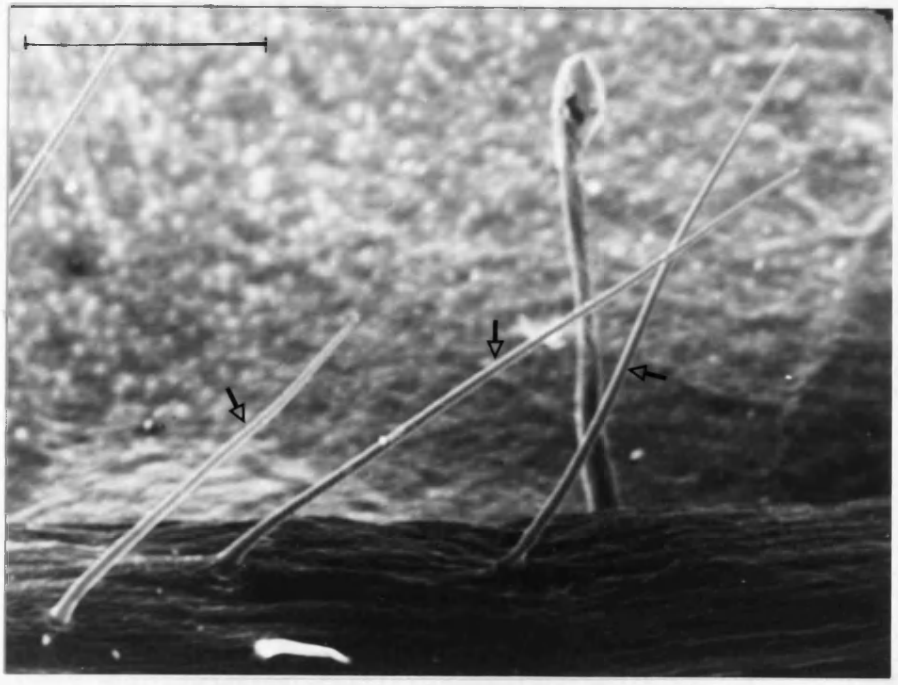
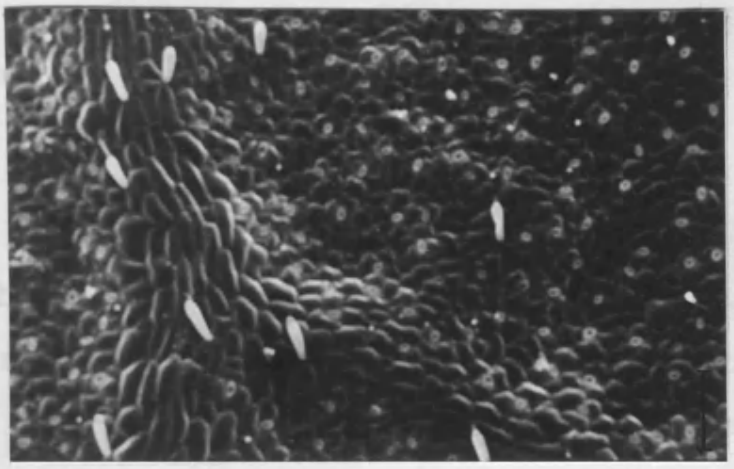


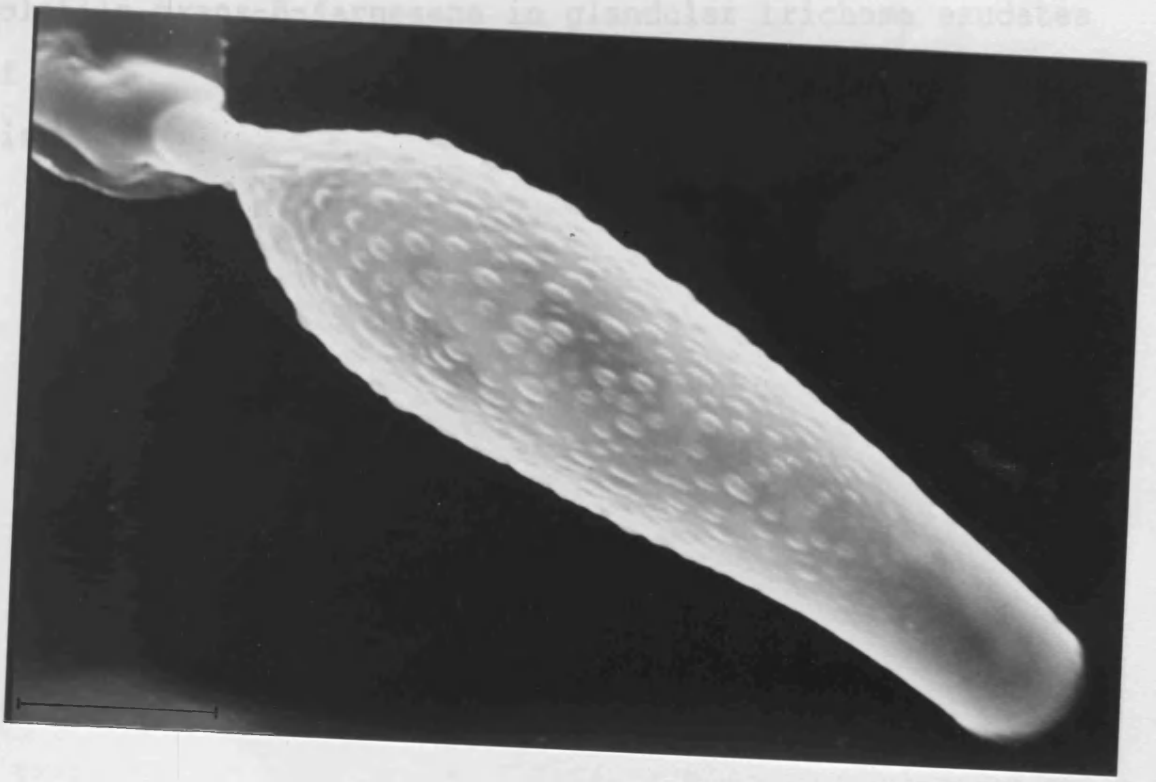
Plate 44: A pointed tipped glandular hair on a leaf of Passiflora foetida. Bar represents 100 µm.

Plate 45: (a) Glandular hairs on a leaf of Passiflora suberosa.
(b) An enlargement of a glandular hair showing more details.
The bars represents 100 μm for (a) and 10 μm for (b).

(a)



(b)



adenopoda was resistant to the larvae of Heliconiine butterflies, because the leaves were covered with hooked trichomes. McKinney (1938); Johnson (1953) and Levin (1973), described the presence of hooked trichomes in *Phaseolus* spp. which were effective for catching the tarsal claw of aphid and also prevent the colonisation of aphids on leaves.

In addition, the presence of hairs may inhibit aphid probing and the acquisition of viruses such as PVY, (Gunenc and Gibson, 1980). These authors further suggested that hairs may have the ability to restrict the multiplication of colonising aphids and the departure of alate of both colonising and non-colonising vector species. Gibson and Pickett (1983) suggested that volatile trans-B-farnesene in glandular trichome exudates of *S. berthaultii* repelled *M. persicae*, and might reduce virus spread.

CHAPTER SEVEN DISCUSSION

Three viruses were isolated from *Passiflora* spp. in Sri Lanka, designated PV1, PV2 and PV3. Passion fruit virus 1 (PV1) was isolated from *Passiflora edulis cv. flavicarpa* with mottle symptoms, PV2 from *P. caerulea* with chlorotic flecking and PV3 from *P. edulis flavicarpa* with mottle and ringspot symptoms.

In this study, passion fruit viruses were readily isolated from leaves of systemically-infected *Passiflora foetida* or *P.edulis cv. flavicarpa*. However, *P. foetida* was a more sensitive host than *P. edulis cv. flavicarpa* for isolation of PV1, PV2 and PV3. Several other workers on viruses of *Passiflora* spp. have also reported the use of either *P. foetida* (wild passion fruit virus - Rosario *et al.*, 1964; chlorotic spot virus (CSV) van Velson, 1961) or *P. edulis flavicarpa;* (passion fruit ringspot virus (PRV) De Wijs, 1974a).

Chenopodium amaranticolor was a useful local lesion assay host for all three viruses and at high dilutions produced well-separated lesions. However, *C. amaranticolor* was reported to contain high concentrations of inhibitors (Gibbs and Harrison, 1976) and these were probably the main factor limiting the mechanical transmission of single lesion isolates on *C. amaranticolor,* to either *P. foetida* or *P.edulis cv. flavicarpa,* in this study. This problem was overcome by

transmission first to *C. quinoa* and then *C. quinoa* to *P. foetida* or *P. edulis cv. flavicarpa*.

In the present work, the number of lesions produced on *C. amaranticolor* increased slightly at low dilutions of *P. foetida* sap, which suggested the presence of inhibitors of infection in the inoculum. From the study of sources of the inoculum, it was found that intermediate or young leaves, with symptoms provided a more virulent inoculum for PV1 and PV2 than older leaves possibly due to lower tannin content in young leaves than the older parts of the plant (Fulton, 1964). Alternatively, such leaves may have supported higher levels of virus replication. Neither PV1 nor PV2 could be detected in flower petals of *P. foetida* and *P. caerulea* respectively, suggesting an uneven distribution of virus in these hosts. Petals are apparently a better source of inoculum for some viruses (Gilmer, 1967).

The isolation of all three viruses was favoured by the tris-HCl or phosphate buffer with molarity of 0.02 M and 0.05 M respectively, and pH values of 7 - 8. Many woody plants contain substances such as tannic acid and polyphenoloxidases, which directly or indirectly can inactivate virus or inhibit infection (Fulton, 1966; Matthews, 1981). The effect of these sap components may be overcome by the use of high pH (8 - 8.5) buffer or the addition of reducing agents, chelating agents or protein binders to the grinding medium (Gibbs and Harrison, 1976). Using tris-HCl buffer, infectivity was enhanced

by preparing inoculum with sodium sulphite. De Wijs (1974a) also used sodium sulphite in the extraction buffer with PRV to overcome the effect of inhibitors. Sodium thioglycollate and PVP were also found to improve infectivity in the present study. However, polyethylene glycol (Ramaswamy and Posnette, 1971), and bentonite were not suitable additives to overcome inhibitors in passion fruit extracts.

Pre-inoculation treatments such as growing test plants at lower temperatures (18°C) and keeping plants in the dark before inoculation, increased the susceptibility of *C. amaranticolor* (assay host) to PV1 and PV2 virus. Furthermore, post-inoculation treatments such as, brief rinsing, covering plants with damp newspaper, growing the plants at slightly higher temperatures (18 - 22°C) with light intensity of 4,500 - 5,000 Lux also increased the numbers of local lesions produced on *C. amaranticolor*.

Passion fruit virus 1, 2 and 3 had a restricted host range so the choice of propagation host for virus purification was limited. All three viruses were purified from *P. foetida* or *P. edulis cv. flavicarpa* leaves harvested about two months after inoculation. Higher yields, however, were obtained from *P. foetida* according to a method described by Hammond and Lawson (1988).

Antisera to all three viruses were obtained using purified virus preparations as antigen source. For

the immunization of rabbits, purified virus preparations 1mg (PV1 and PV2) to 0.2 - 0.5 mg (PV3) were given. Studies were done with absorbed sera to remove healthy antigens, where present. Serological tests were made using either microprecipitin reactions or ELISA but agar gel double-diffusion was found to be a less sensitive method for assay work. The direct antigen coating modification of the indirect ELISA system using crude sap extracts was unsuitable for quantitative assay due to a prozone effect at high sap concentrations, probably caused by inhibition of virus binding through interference from host protein (Lommel *et al.*, 1982). The effect of inhibitors may be overcome by the addition of PVP (Clark and Adams, 1977) to standard ELISA antigen extraction buffer for field indexing of virus-infected *P. edulis cv. flavicarpa*. For detection by ELISA it was found that flower petals and immature seed were apparently free of virus. These results were consistent with infectivity tests using *C. amaranticolor*. When *P. foetida* was used as the source of virus, better results were obtained by replacing the sodium carbonate antigen extraction buffer with phosphate containing sodium sulphite.

Low concentrations of dsRNA in most of the tissue infected with PV1, PV2 and PV3 was common. This may have been partly due to the presence of mucilaginous substances in the sap of some woody *Passiflora* spp. which interfered with extraction of dsRNA or binding to the

cellulose column. Alternatively, low levels of virus replication when tissues were sampled may also have contributed.

Preliminary observations of leaf squashes PV1, PV2 and PV3 in the electron microscope showed these viruses to have flexuous rod-shaped particles of 770 nm (PV3) and 840 nm - 860 nm (PV1 and PV2 respectively) size and suggested that PV1, PV2 and PV3 might belong to the potyvirus group. Several other properties - biological, physical and chemical were consistent with this identification.

Thus, all three isolates were transmitted by *Myzus persicae* (except PV3), *Aphis spiraecola, A. gossypii* and *A. craccivora* in a non-persistent manner. Aphids could acquire or transmit the viruses in probing feeds of 3 - 5 min or less. Hollings and Brunt (1981) reported that many potyviruses were transmitted by aphids in a non-persistent manner. The non-persistent mode of transmission of PV1, PV2 and PV3 was thus consistent with their potyvirus grouping. All these viruses were transmitted by several different aphid species although with different levels of efficiency, this also is typical of potyviruses (Hollings and Brunt, 1981).

Passion fruit virus 1 and 2 differed from PRV (De Wijs, 1974a) and *Passiflora* latent virus (PLV) (Brandes and Wetter, 1963) in being transmitted by *M. persicae*. Transmission experiments with *M. persicae*,

however, were not done for PV3. It would be interesting to see if PV1 or PV2 could provide 'helper' components reported with potyvirus group (Hollings and Brunt, 1981) that would enable PRV or PLV to be transmitted by *M. persicae*. *Aphis gossypii* and *A. spiraecola* were reported to be important aphid vectors for known potyviruses in *Passiflora* (PRV - De Wijs, 1974a and CSV - van Velsen, 1961). Passion fruit viruses in the present study were similar to PWV in being transmitted by *A. gossypii* (Greber, 1966) and *M. persicae* (Taylor, 1959).

The viruses in the present study had moderate to narrow host ranges and were similar to potyviruses in infecting *Chenopodium quinoa, C. amaranticolor, C. murale, Gomphrena globosa, Nicotiana clevelandii, Petunia hybrida, Phaseolus vulgaris* cv. The Prince (Hollings *et al.*, 1980). The symptoms induced by the *Passiflora* viruses under study were generally similar to the symptoms induced by potyviruses in systemically-infected leaves of dicotyledonous plants as reported by Hollings and Brunt (1981). These symptoms included vein clearing, mosaic, mottle, leaf puckering, rugosity sometimes with distortions and necrosis. Host range and symptomatology, however, are usually an unreliable guide to virus identity, although in a few instances the symptom type may be indicative of a distinct virus or virus group (Bock, 1982).

The host range of the virus isolates in the present study was different to that for known potyviruses

from *Passiflora* spp. (Appendix II), although symptoms induced in some hosts were quite similar. For example, the foliar symptoms associated with PV1, PV2 and PV3 in *P. foetida* were similar to PRV (De Wijs, 1974a). In contrast, PWV differed from PV1, PV2 and PV3 in infected *N. tabacum* cv. Xanthi. Passion fruit virus 2 was different from bean yellow mosaic virus (BYMV) isolated from *P. caerulea* (Plese and Wrischer, 1984). Unlike the BYMV isolates PV2 did not systemically infect *Chenopodium* spp. Most of the reported *Passiflora* viruses were also infected with CMV, however, while the viruses under study failed to infect some common hosts of CMV including cucumber, tomato and *Datura stramonium*. This indicated that the isolates were not CMV or did not include mixtures with CMV.

Symptom expression of PV3 in *Passiflora* and legumes was similar to that of PRV (De Wijs, 1974a). Thus, both PV3 and PRV induced chlorotic local lesions on *P. quadrangularis*, and systemic infection with chlorotic rings in *Cassia occidentalis* and ringspot in *P. edulis cv. flavicarpa*. However, PV3 was different from PRV in infecting *N. clevelandii* but not *P. suberosa* which was susceptible to PRV. The viruses under study appeared to have similar symptoms to the virus causing chlorotic spots of *P. foetida* (van Velsen, 1961), but differed in infecting *P. edulis*.

Passiflora suberosa could not be infected with any of the passion fruit viruses in the present study.

Aphid and graft transmission were also unsuccessful in transmitting PV1 from *P. edulis cv. flavicarpa* to *P. suberosa* suggesting the latter host to be immune. This indicated that PLV - carlavirus (Brandes and Wetter, 1963) and tip blight strain of PWV (Greber, 1966) were not implicated in the diseases as these viruses, like PRV, infected *P. suberosa*.

Physical properties of PV1, PV2 and PV3 showed them to be moderately stable and infectivity was lost within 5 - 7 days when stored in distilled water at room temperature. Other potyviruses isolated from *Passiflora*, like PWV and CSV, were also reported to be moderately stable (Appendix III). Passion fruit ringspot virus was apparently different from viruses in the present study in its higher LIV (12 - 14 days). The PV1, PV2 and PV3 isolates were different from CSV in having higher dilution end-points and lower LIV values. On the other hand, the DEP of PV2 was similar to that of PWV. In contrast, PV2 differed from PWV, in having a higher LIV. However, physical properties, like host reaction have limited value for identification purposes, particularly if different source plants are used (Francki and Hatta, 1980). The physical properties of the virus isolates in the present study, however, were in the broad range reported for potyviruses.

Several different buffers at a range of molarities were tested but 0.05 M tris-HCl buffer adequately preserved infectivity for purification

purposes although phosphate was probably equally satisfactory. Sodium sulphite was included as a stabilizing additive in the extraction buffer, but was probably not necessary. A comparison of the n-butanol / PEG purification method with the method described by Hammond and Lawson (1988) showed the latter method to be satisfactory for all three viruses in infectivity assay, purity in electron microscope, and UV absorption. Subsequently, passion fruit isolates (PV1, PV2 and PV3) were all purified using the same protocol for potyvirus purification decribed by Hammond and Lawson (1988).

This method involved extraction of virus in high ionic strength and high pH phosphate buffer used previously by other workers on potyviruses, (Shepherd and Pound, 1960; Maat and Mierzwa, 1975). Triton X-100 used by De Wijs (1974a) for PRV, was used to clarify the sap and minimise aggregation (Hollings and Brunt, 1981). Other studies on *Passiflora* have used different clarification agents including n-butanol (Taylor and Kimble, 1964) and carbon tetrachloride and diethyl ether (Bakker, 1974) for PWV from New South Wales, and East Africa, respectively. The passion fruit viruses in the present study were concentrated from clarified extracts by PEG precipitation. This method has been used successfully for a number of potyviruses although it can induce serious aggregation with some viruses (Hollings and Brunt, 1981).

Further purification of PV1, PV2 and PV3 was done by caesium sulphate density gradient centrifugation. This method has been widely used for further purification of many potyviruses (Hammond and Lawson, 1988). However, further purification of PRV (De Wijs, 1974a) and PWV (Taylor and Kimble, 1964) and other potyviruses (Hollings and Brunt, 1981) has also been done by sucrose density-gradient centrifugation. Purified viruses (PV1, PV2 and PV3) were resuspended in 0.1 M BK buffer pH 8 and could be stored at -20°C for periods of 4 - 6 weeks without significant loss.

The absorption spectrum of purified PV1 and PV2 commonly produced a tryptophan shoulder at about 290 nm similar to those of pepper veinal mottle virus (Brunt and Kenten, 1971) and tobacco etch virus (Damirdagh and Shepherd, 1970). Based on published extinction coefficients for potyviruses (Hammond and Lawson, 1988), the yields of *Passiflora* viruses were satisfactory for PV1 and PV2 (about 10 - 15 mg/100g) but were low for PV3 (0.2 - 0.5 mg/100g). The variable yields obtained may indicate different susceptibilities of *P. foetida* to the three viruses, or may reflect differences in stability under the same purification conditions. Hollings and Brunt, (1981) noted that different strains of many potyviruses frequently differ considerably in their ease of handling.

In electron microscopy studies, negatively stained leaf squash preparations, contained flexuous rod

particles, with a normal length of about 840 - 860 nm for PV1 and PV2 repectively, and about 770 nm for PV3, when magnification of the electron microscope was calibrated against polystyrene latex spheres. The width of the virus particles of all three virus was about 12 - 13 nm. The particle length range of potyviruses is now considered to be 680 - 900 nm (Matthews, 1979). The particle lengths of the viruses under study, therefore, were consistent with a potyvirus group classification. Carlaviruses (Wetter and Milne, 1981) are often less flexuous than typical potyviruses and have a normal length of 620 - 690 nm, while closteroviruses are, generally, much more flexuous and those in sub group II are not usually aphid-borne (Lister and Bar-Joseph, 1981).

The modal length of PRV (De Wijs, 1974a) was 810-830 nm when negatively stained with uranyl acetate or uranyl formate at 56,000 magnification, without any size standard. On the other hand, the normal length of PWV was 745 nm and 730 nm with TMV (300 nm) or polystyrene latex shperes (264 nm) respectively as size standard. *Passiflora* isolates in the present study would appear to be distinct from PLV which has a particle length of only 650 nm (Brandes, 1963) and probably belongs to carlavirus group.

Some leaf squash preparations of PV2 had aggregates of particles similar to tulip breaking virus, narcissus yellow stripe virus and carnation vein mottle

virus (Hollings and Brunt, 1981). However, unlike these viruses, membranes were not detected in association with the particles, possibly due to damage during the preparation of the specimens.

Thin sections of leaf tissues of *P. edulis cv. flavicarpa* infected with PV1 and PV2 revealed the presence of pinwheels, scrolls and laminated aggregates, typical of potyviruses (Christie and Edwardson, 1977). The pinwheel inclusions present in PV1 or PV2 infections were similar and were composed of curved plates. From these findings it could be established that PV1 and PV2 viruses probably belong to the potyvirus group and could be further characterized as the sub group III potyviruses (Edwardson, 1974) because the cytoplasmic inclusions were seen as 'pinwheels', bundles, tubular and laminated aggregates. Passion fruit woodiness virus also belong to sub group III according to Edwardson's (1974) classification. Mophologically similar inclusions are induced by some other unclassified, flexuous rod-shaped viruses but can be distinguished from the potyviruses by the lack of aphid transmissibility (Hollings and Brunt, 1981).

Protein preparations of three *Passiflora* viruses migrated as two bands in SDS-PAGE, with a slower migrating band of molecular weight about 33,000 daltons. The polypeptide of potyviruses group members have been estimated to range from 32,000 for tobacco etch virus TEV (Hiebert and McDonald, 1973) to 36,500 for maize dwarf

mosaic virus MDMV (Hill *et al.*, 1973). Most capsid polypeptides, however, are intermediate between those two extremes. For example, a molecular weight of 33,000 was reported for BYMV, BCMV and PWV (Moghal and Francki, 1976).

Apart from the slow-migrating component, most of the preparations also had a faster migrating polypeptide with a molecular weight of 25,000 daltons. This value was slightly lower than the reported values for the faster migrating component (26,000 - 28,000) of potyviruses (Brunt and Kenten, 1971; Huttinga and Mosch, 1974; Michelin-Lausarat and Papa, 1975; and Hill and Benner, 1980). A number of viruses in the potyvirus group have shown capsid protein heterogeneity when analysed by SDS-PAGE (Hiebert and McDonald, 1973; Huttinga and Mosch, 1974; Taiwo and Gonsalves, 1982). Capsid protein heterogeneity, therefore, can be considered as common with viruses of the potyvirus group. In addition to proteolysis of the native protein, Hill and Benner (1980), suggested the heterogeneity may also be due to variations in the disulphide bridge patterns of the protein.

For viruses that provide detectable levels of dsRNA in plant tissues the number and size of the dsRNA can provide useful diagnostic information. At least one major dsRNA segment with molecular weights of about 6.9 - 7.0 x 10^6 was detected with all three viruses which would give a single stranded genomic RNA size of about

3.4 - 3.5 x 10^6. A major dsRNA in a similar size range (5.4 - 7.0 x 10^6) was reported for the potyviruses such as bean mosaic, potato y, tobacco etch and turnip mosaic virus (Valverde *et al.*, 1986).

Antisera obtained from bleedings taken 4 weeks after first injection had titres of at least 1:8,192 for all three viruses in microprecipitin grid titration reactions under paraffin oil, using purified virus preparations as antigen sources. Hollings and Brunt (1981) reported that potyviruses differ considerably in their immunogenicity although the majority of potyviruses are strongly immunogenic. In this respect, immunogenicity of PWV (1/1,024) was similar to that of viruses under study (Taylor and Kimble, 1964) although the routes of injection and the amounts given were somewhat different.

Agar gel double-diffusion was found to be unsatisfactory for virus detection. Chemical and physical virus disruptive methods such as sonication, SDS, pyrrolidine and 3, 5, diiodosalicylic acid (lithium salt) failed to give satisfactory results with either crude sap extracts or purified preparations of PV1. Similar difficulty has been reported for many potyviruses (Hollings and Brunt, 1981). In contrast, sonicated PWV gave an homologous titre of 1:128 in agar double-diffusion tests (Moghal and Francki, 1976). The weak positive reactions in the present study may be due to a large proportion of antigenic determinants in the

disrupted virions not reacting to antibodies prepared against intact virus.

The serological relationship of PV1, PV2 and PV3 to other potyviruses was determined by direct antigen coating indirect ELISA, as it is reportedly less strain specific (Koenig, 1981) with crude sap or purified virus as antigens. All three viruses cross reacted with antisera to potyviruses PVY, PWV (definitive members), PRV (possible member). The reactions of PV1 and PV2 with antiserum to watermelon mosaic were borderline. No cross-reaction was found between PV1, PV2, PV3 and an antiserum to bean common mosaic virus. Taylor and Kimble (1964) also found no cross-reaction between PWV antiserum and BCMV or BYMV.

Antisera prepared against PWV from Australia, PRV from Ivory-Coast were the only two antisera to *Passiflora* viruses used in cross-reactivity studies. The results showed that PV1, PV2 and PV3 viruses were related to PWV and PRV. The degree of relationship to PV1, PV2 and PV3 could not be determined, however, because the homologous antigens were not available for comparison. The relationship of the viruses under study to other potyviruses isolated from *Passiflora* is, therefore, uncertain. Whether these differences reflect genuine differences between the viruses or merely differences at the strain level or in technique is not known. Further serological characterisation of the *Passiflora* viruses is needed using antisera to a broad range of virus isolates

in the potyvirus group, and, possibly, monoclonal antibodies produced against specific antigenic determinants on the viruses.

A number of criteria suggested that PV1, PV2 and PV3 were all members of the potyvirus group. The most important of these were the shape (flexuous rods) and size (normal lengths 770 - 860 nm) of apparently intact particles and the serological cross-reactivity of the isolates with known members of the potyvirus group. The presence of pinwheel inclusions was also considered diagnostic for potyviruses when accompanied as in this case, by aphid transmissibility (Hollings and Brunt, 1981). Other supporting evidence were the molecular weights of the capsid protein and double-stranded RNA which fell within the range expected for typical potyviruses. Other properties, which related to host plant reaction or *in vitro* properties, were not diagnostic enough for group identification but may be of value in strain or within-group differentiation. Determinations of amino acid sequence homologies and nucleic acid hybridisation studies will be needed to define the affinities of the *Passiflora* isolates, with other group members.

The question arose as to whether the three *Passiflora* isolates, PV1, PV2 and PV3 were distinct viruses within the potyvirus group, strains of existing viruses or even identical viruses. In an attempt to answer this question, comparisons of PV1, PV2 and PV3

were made with respect to their biological, physical and chemical properties. To increase the validity of the observations most tests with the three isolates were done at the same time, under the same conditions, although they were described separately, in the text.

Firstly it was noticed that all three isolates had similar but not identical host ranges and reactions (Table 11, 28 and 32). Symptoms induced by PV1, PV2 and PV3 were compared in selected differential hosts and although symptoms were greatly influenced by environmental conditions, clear differences in symptomatology between the three isolates were observed. *Passiflora quadrangularis* was the most useful spp. for distinguishing the three isolates. Thus while PV1 induced vein clearing initially and mottle with slightly deformed leaves, PV2 produced a much more severe disease with symptoms which included vein and stem necrosis, severe stunting, and a premature collapse of the leaves. The production of chlorotic local lesions on inoculated leaves was the main difference between PV3 (lesions) and PV1 (no lesions) but the systemic disease with PV3 was also more severe than that of PV1.

The initial symptoms of PV1, PV2 and PV3 on *P. foetida* was vein-clearing. Passion fruit virus 2, however, subsequently produced severe leaf curling, epinasty, stem necrosis, vein necrosis and premature defoliation, whereas PV3 induced vein-clearing on both inoculated and non-inoculated leaves. Secondary

chlorotic dotting symptom was common with all three isolates, but, PV3 induced milder symptoms on this host than PV1.

In *Passiflora edulis cv. flavicarpa*, PV2 induced vein-clearing, leaf crinkling, defoliation and vein necrosis. Symptoms produced by PV3 on this host were similar to that of PV1 except that in the early stages of infection, it induced chlorotic rings. These symptoms however were diffuse under the prevailing environmental conditions. It was also found that *P. edulis cv. flavicarpa* infected with PV2 had reduced plant height, number of leaves and number of shoots (Fig 30, Table 43) when compared to PV1. Although these two viruses both suppressed growth compared to healthy plants.

Cassia occidentalis was also a useful diagnostic plant. Passion fruit virus 3 induced chlorotic rings and mottle in systemically-infected leaves, whereas PV1 and PV2 induced systemic mottle only. In *Chenopodium quinoa* PV3 induced chlorotic local lesions while PV1 and PV2 induced only chlorotic mottle on inoculated leaves. Isolates PV1 and PV2 induced local and systemic chlorotic lesions on *Nicotiana clevelandii* while PV3 produced symptomless local infection only. However, this species was not equally susceptible to all isolates and sometimes PV3 did not infect the host.

These studies indicated that PV1, PV2 and PV3 while similar in their host ranges, differed in the detailed symptom expression they produced in different species. On this basis, they could be considered as distinct viruses or strains or 'pathotypes' of one virus. Matthews (1981) stated that many strains of virus may have similar host range but also that symptom differences often reveal existence of a strain when no other available criterion will do so. The extent of differences in disease symptoms, however, may be a quite unreliable measure of the degree of relatedness between different members of group of strains (Siegel and Wildman, 1954).

Based on their physical properties determined with extracts of *P. foetida*, PV1, PV2 and PV3 were also distinct isolates. Thus PV1, PV2 and PV3 had TIP respectively 70 - 75°C, 60 - 65°C and 50 - 60°C and DEP of 10^{-5} - 10^{-6}, 10^{-4} - 10^{-5} and 10^{-3} - 10^{-4}. The longevity of PV1 in crude sap extracts was between 6 - 7 days when the solution was kept at room temperature and 5 - 6 and 4 - 5 days for PV2 and PV3 respectively. Babos and Kassanis (1963b) pointed out that different strains of a virus may have different thermal inactivation points, although properties in vitro are not generally a very reliable diagnostic tool (Francki and Hatta, 1980). In the present study, the differences between the isolates seemed more clear cut for PV1 and PV3 than PV2 which had 'intermediate' properties.

In purification experiments PV1 and PV2 gave average yields of 10 - 15 mg/100g tissues which was rather higher than that obtained with PV3 (0.2 - 0.5 mg/100g). The differences were, however, consistent with the properties *in vitro* of these viruses. Walkey (1985) noted that different strains of the same virus may require specifically different treatments, at any stage in their purification.

In electron microscopy, negatively stained 'squash' preparations contained flexuous rod-shaped particles with a normal length of about 840 - 860 nm for PV1 and PV2 respectively. The normal length of PV3 was shorter (770 nm), under the same preparative conditions. Differences in rod length were frequent between strains of helical viruses such as tobacco mosaic virus and tobacco rattle virus (Cooper and Mayo, 1972) and barley stripe mosaic virus(Chiko, 1975).

In serological tests between PV1, PV2 and PV3 (Chapter 3.4), the antisera titres in indirect ELISA were much higher with the homologous antigen than with the heterologous antigen. That some cross-reaction occurred indicated that the isolates were related to each other but the reactions were not identical (in reciprocal tests) and this suggested that the isolates were serologically distinct. The closeness of the relationship was, however, indicated by the average SDI for the three isolates - PV1/PV2 1.7; PV1/PV3 0.78; PV2/PV3 1.45.

The antigenic differences of the particles of viruses were also measured by cross-absorption tests. These tests measured the proportion of antibody that reacted specifically with determinants on the homologous antigen, following absorption with heterologous virus (Gibbs and Harrison, 1976).

The results of cross-absorption experiments (Table 35) tended to support the relationships indicated by homologous and heterologous cross-reaction studies. Kassanis (1961) and Babos and Kassanis (1963a) reported that when cross-absorption with a related virus isolate leaves the titre against the homologous strain unchanged, the relationship between the two strains was distant, whereas, when the homologous titre decreased as a result of cross-absorption, the two viruses should be considered closely related. In the present study, when antisera specific for PV1, PV2 or PV3 were cross-absorbed with PV1, PV2 or PV3 antigens, the titres of the respective antisera were reduced in each case (Table 35). This indicated that PV1, PV2 and PV3 should be considered 'closely related'. The results also suggested, however, that PV1 had a closer serological affinity to PV3 than PV2 and conversely that PV2 was serologically more closely related to PV3 than PV1.

Conjugate prepared with antibody to PV1 effectively detected both PV2 and PV3 in DAS-ELISA. This could also indicate a close serological relationship

between PV1, PV2 and PV3, because several authors have reported that enzyme conjugates prepared from antibodies against one virus strain do not react with closely-related strains (Koenig, 1978; Bar-Joseph and Salomon, 1980; Barbara *et al.*, 1978; Lister and Rochow, 1979; Uyemoto, 1980; and Kelly *et al.*, 1978). However, the degree of specificity apparently differs with different viruses. Thus , enzyme-labelled antibodies to one strain of bean yellow mosaic virus (Stein *et al.*, 1979), potato virus Y (Koenig, 1978; Weidemann and Koenig, 1979), citrus tristeza virus (Bar-Joseph *et al.*, 1979) reacted strongly with other serologically distinct strains of the same virus. Stein *et al.*, (1979) reported that the strain specificity of DAS-ELISA may be quite pronounced with some isometric or short elongated viruses, but less so with long flexuous-rod type viruses.

Although the serological evidence indicated that these isolates from *Passiflora* were related but not identical, some caution is warranted. Firstly in the present study only one 'polyclonal' antiserum to each virus was produced. According to Matthews (1981), the extent of cross-reaction can be affected by the variability of antisera, both in successive bleedings from the same animal and in sera from different animals. This author suggested testing successive bleeds from many animals, and then pooling the results. Another source of variability may be the proportions of free or aggregated protein and intact virions in the antigen preparations

used for injection, and in the samples used to coat ELISA plates. Thus, Moghal and Francki (1976) found that antisera to certain intact potyviruses reacted poorly or not at all to dissociated, homologous virus.

A more critical assessment of the serological relationships of PV1, PV2 and PV3 will require the production of several antisera and a range of monoclonal antibodies to each virus.

Most groups of virus strains appear to have coat proteins of very similar size, for example, tymovirus (Symons *et al.*, 1963); tobacco mosaic virus (Wittmann and Wittmann, 1966); aucuba mosaic virus (Kraal, 1975) and some potyviruses (Makkouk and Gumpf, 1975) but significant differences have been noted for legume carlaviruses (Veerisetty and Brakke, 1977). Coat protein results of present study indicated that PV1, PV2 and PV3 could not be clearly separated on the basis of size (25,000, 33,000) or number (two) of polypeptides in the protein coat.

Two different dsRNA patterns were detected for PV1, PV2 and PV3 after PAGE of nucleic acid extracts of *P. foetida* or *P. quadrangularis*. The dsRNA patterns of PV1 and PV3 were the same and produced single segment of dsRNA of similar molecular weights (7 - 6.9 x 10^6). In contrast extracts from PV2 infected *P. foetida* plants produced two segments of dsRNA (molecular weights of 7.3 and 5.0 x 10^6). This band pattern provided further

slight evidence that PV2, at least was distinct from PV1 or PV3. Efforts to improve the yields of dsRNA from infected *Passiflora* or other hosts may show further differences of value in strain differentiation. Using the dsRNA technique, Gildow *et al.* (1983) separated barley yellow dwarf virus isolates into two groups based on both number and size of dsRNA produced in infected oats.

The passion fruit viruses were transmitted from diseased to healthy *P. edulis cv. flavicarpa* or *P. foetida* plants by several species of aphids, *Aphis craccivora, A. spiraecola, A. gossypii* and *Myzus persicae* under laboratory conditions. However transmission efficiency was lower when *A. craccivora* was used to transmit the PV3 from *P. flavicarpa* to the same host, (Table 37). This may be due to lower concentrations of the viruses in the source plant or genuine difference in vector efficiency. Different transmission efficiencies have been reported for strains of potato virus Y (Simons, 1969) and watermelon mosaic virus (Karl and Schmelzer, 1971).

It is clear from the above comparisons that the *Passiflora* virus isolates were similar in many of their properties; for example, serological relationships, host range and physical properties. In other respects, however, the isolates appeared to differ particularly in symptom expression on common host plants. On the basis of these differences, therefore, the isolates should

probably be regarded as strains or 'pathotypes' of one virus. Further work is needed to determine whether the isolates represent strains of a 'new' potyvirus or are merely strains of an existing one.

The pathogenicity of PV1 in *P.edulis cv. flavicarpa* was confirmed by inoculation of pure cultures to healthy plants which produced original symptoms of the disease in the field. A virus with the same properties as PV1 was isolated from diseased plants. Naming of this isolate, for example, 'passion fruit mottle virus' must await a clearer definition of its relationship to other viruses.

A causal association of PV2 with disease in the natural host could not be confirmed due to lack of healthy *P. caerulea* plants. This spp. does not form fruits under tropical climatic conditions in Sri Lanka, and fruits were also not set under glasshouse condition at the University of Bath throughout the study period. In the case of PV3, re-inoculation to *P. edulis cv. flavicarpa* failed to reproduce typical ringspot symptoms observed in the field. Ringspot symptoms did appear transiently at an early stage of growth under glasshouse conditions at Bath, but mottle and chlorotic spot symptoms predominated. The aetiology of the ringspot symptoms, thus remains unproven until the pure healthy culture can be returned to healthy plants, and evaluated for symptoms under local climatic conditions in Sri Lanka.

Epidemiology and control

The *Passiflora* virus isolates were apparently not transmitted through seed of most of the reported passion fruit viruses namely, PWV (McKnight, 1953; Taylor and Kimble, 1964), PRV (De Wijs, 1974a), WPV (Rosario *et al.*, 1964) and Martini (1962) are also not seed-borne. Moreover, PV1 and PV2 were not transmitted by contact if vines were allowed to intertwine, similar to that of WPV (Rosario *et al.*, 1964). Transmission was demonstrated, however, via pruning knives and wedge grafting.

In the present study, it was found that PV1, PV2 and PV3 could be spread from diseased to healthy plants by contact or vector transmission. Aerial vectors, such as aphids, probably play a major role in disease transmission. Generally, in passion fruit cultivations, aphids do not colonise, but they have been detected as 'visitors'. Large numbers of plants can now be rapidly indexed for virus infection by sensitive serological techniques such as ELISA (Clark, 1981). Progress has also been made in detecting viruses by serologically within individual vectors collected in the field (Harrison, 1981). The applications of less sophisticated methods, however, also produced interesting observations. In 1988/89, trapping experiments showed that alatae population of *A. spiraecola* reach their peaks in May, June and September and remained low for the rest of the period. *Aphis spiraecola* appeared to be the most

important vector in the spread of PV1, although *A. gossypii* and *A. craccivora* may also be involved.

Comparison of different trapping methods showed that water traps were more effective in catching *A. spiraecola* than *A. gossypii*. On the other hand, sticky traps caught more *A. gossypii* than water traps. Therefore, in the search for potentially important virus vectors, it is suggested that several different methods of sampling, be used to collect aphids since species not readily collected by one technique, may be collected by another (Zettler *et al.*, 1967).

There was a significant correlation between the numbers of winged *A. spiraecola* trapped and the percentage of mottle infected passion fruit plants. A significant correlation has been demonstrated between the numbers of winged aphids trapped, and the percentage of virus infection, for a variety of crops and viruses (Bacon *et al.*, 1976; Broadbent, 1950; De Wijs, 1974b; Gonzalez and Rawlins, 1969; Heathcote, 1974; Knoke *et al.*, 1974; and Watson *et al.*, 1953). The abundance and proximity of virus source plants rather than populations were singled out as important for the spread of watermelon mosaic viruses 1 and 2 (Adlerz, 1974). The particular species of aphids trapped, rather than total numbers, may be a significant factor (Broadbent *et al.*, 1951; Dickson *et al.*, 1956 and Dickson and Laird, 1959).

Temperature and rainfall are the most important regulating factors in moist tropical climates on the seasonal cycle of insects (Broadbent, 1967). The temperature fluctuations in the low country wet zone of Sri Lanka seemed not to be a limiting factor for aphid reproduction and plant growth. Of the other climatic factors, rainfall proved to be an important factor determining the number of *A. spiraecola*. Positive correlations were found with *A. spiraecola* trapped and weekly total of rainfall, for several weeks. The most important hosts of *A. spiraecola*, weeds such as *Mikania scandens* and *Eupatorium odoratum* are available all the year round if there is some rain during the dry season. De Wijs (1974b) reported *Eupatorium conyzoides* as a suitable host for *A. spiraecola* and rainfall directly influenced the population of *A. spiraecola*. A positive correlation between rainfall and flight of *A. spiraecola* has also been noted by Van Hoof (1962) in Surinam.

According to the survey conducted in several passion fruit cultivations in the wet zone, aphids have often been observed on adjacent crops in passion fruit cultivations. *Eupatorium odoratum* and *Mikania scandens* (Theobald, 1929) were the only important host plants readily colonised by *A. spiraecola*. On the other hand, *A. gossypii* colonised several plant species, such as *Scoparia dulcis*, *Croton hirtus*, *Hibiscus esculentus*, *Capsicum* spp. and *Solanum melongena*. According to Lefroy and Houlett (1909), this aphid spp. has wide host range.

Aphis craccivora reportedly colonised mainly legume spp. (Lefroy and Houlett, 1909).

A preliminary survey in the wet zone area showed virus diseases to be common in many passion fruit crops. In the present study, it was not possible to evaluate losses in yield caused by viruses under field conditions. However, under glasshouse conditions infected plants of PV1 and PV2 had markedly reduced development of growth and yield in comparison with apparently healthy plants. Moreover, field collected diseased fruits were unmarketable due to a mottled pericarp. Woody fruits with reduced pulp cavity were not a characteristic feature of viruses under study. In this respect the *Passiflora* viruses differed from the PWV (Taylor and Kimble, 1964).

Direct chemical control of virus diseases is not yet possible on a large scale (White and Antoniw, 1983). Therefore, the main approach to their control has always been preventative. Hence a sound understanding of the epidemiology and ecology of plant virus disease should be the key to effective control, especially for aphid-borne viruses.

Experiments showed that passion fruit viruses can be spread from diseased to healthy plants by sap transmission. Further means of infection might involve contamination with infective sap during cultural operations such as training and pruning of the vines.

Basic farm / nursery hygeine may reduce virus spread and pruning knives and other tools used during these operations should be thoroughly cleaned between cuts by dipping in a strong detergent.

In 1989, a field survey indicated that recovery of virus from wild plants in or around passion fruit cultivations was rare. Virus was recovered from one sample of *P. foetida* even though it is a common weed grown in many infected passion fruit cultivations in the wet zone. Nevertheless, clean culture by keeping weeds down would reduce possible reservoirs of the virus or vectors in weed hosts, and the subsequent transmission of the virus to the healthy vines. Preventing a build up of aphids by good husbandry may be a worthwhile preventive measure.

However, since passion fruit is a 'long term' perennial crop, perpetuation and spread of virus in the field, should be minimised. Infected plants should be replaced soon after they are observed. Roguing as a control measure has long been used in potato 'seed' crop production in the Netherlands, Scotland and Germany (Walkey, 1985) and for control of papaya mosaic virus (Holtzmann and Ishii, 1964). Removal of infected plants is also used to control cacao swollen shoot virus in West Africa (Kenten and Legg, 1971).

Sticky yellow polythene sheets erected vertically in the field (Cohen and Marco, 1973) may be a

more practical and economical preventive measure to reduce the spread of virus diseases in passion fruit fields in Sri Lanka. Although some workers have attempted to control virus vectors by means of predators, parasites or pathogens, such measures have met with little success (Harpaz, 1982). No detailed observations were made on predators and parasites of *A. spiraecola*, but, larvae of coleoptera were found predating the apterous aphids of *A. craccivora*. More research on biological control methods is needed and such methods may be useful within an integrated control programme (Harpaz, 1982).

Cross-protection has been practised in Australia to avoid yield losses by severe strain of PWV (Simmonds, 1959; and Greber, 1966). Similar studies made by Seneviratne *et al.* (1983) in Sri Lanka showed that a mild strain protected clone will perform well if grown under conditions of good management, with adequate irrigation, and if the weed hosts of the virus are controlled. According to the author's knowledge, so far there is no such cross-protection programme practised on a large scale in Sri Lanka.

No control has been developed for the passion fruit virus diseases in Sri Lanka and resistance to more common viruses such as PV1 has not been found. Several cultivated and uncultivated species of genus *Passiflora* have been identified in Sri Lanka. It is apparent that different species do vary significantly in their reaction

to the viruses. It was found, for example, that *P. suberosa* was immune to all three virus strains. It has also been observed in passion fruit cultivations that individual plants within a given planting show a wide range of reaction to the virus. However, breeding for resistance requires detailed knowledge of the viruses involved in combination with a suitable method of screening and evaluation. Aphid resistance due to the presence of glandular hairs on the surface of leaves may also be important characteristics for evaluation. These characteristics could, therefore, be used in a breeding programme to upgrade the cultivated species.

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APPENDIX I

Medium used for the *in vitro* culture of woody plants (Murashige and Skoog, 1962)

a. Mineral Salts

Major Elements (mM):	NH_4NO_3	41.2
	KNO_3	18.8
	$CaCl_2.2H_2O$	3.6
	$MgSO_4.7H_2O$	1.5
	KH_2PO_4	1.25
	Na_2EDTA	0.2
	$FeSO_4.7H_2O$	0.1
Minor Elements (μM):	H_3BO_3	100.0
	$MnSO_4.4H_2O$	100.0
	$ZnSO_4.4H_2O$	30.0
	KI	5.0
	$Na_2MoO_4.2H_2O$	1.0
	$CuSO_4.5H_2O$	0.1
	$CoCl_2.6H_2O$	0.1

b. Organic constituents (mg/l):

myo-inositol	100.0
glycine	2.0
nicotinic acid	0.5
pyridoxin-HCl	0.5
thiamine-HCl	0.1

Other organic constituents (sucrose, growth regulators and agar) were as described in the text.

APPENDIX II

Comparison of the host range of several potyvirus isolated from *Passiflora* spp. with PV1

Plant species	PWV	PRV	CSV	BYMV	PV1
Amaranthaceae					
Gomphrena globosa	(L)	(L)	na	L	L
Chenopodioceae					
Chenopodium album	L	L	na	na	L
C. amaranticolor	L	L	na	S	L
C. foetidum	na	L	na	na	L
C. quinoa	na	(L)	na	S	L
Leguminosae					
Centrosema pubescens	S	na	-	na	-
Phaseolus vulgaris	S	L	-	L	L
Vigna unguiculata	-	-	-	-	L
Passifloraceae					
Passiflora edulis	S	na	-	na	S
Passiflora edulis cv. flavicarpa	S	S	S	na	S
P. foetida	na	S	S	na	S
P. ligularis	na	S	na	na	S
P. mollissima	na	-	S	na	S
P. quadrangularis	na	S	na	na	S
P. suberosa	-	S	-	na	-
Solanaceae					
Nicotiana benthamiana	na	S	na	na	na
N. clevelandii	(S)	-	na	S	S
N. glutinosa	na	(S)	-	-	na
N. megalosiphon	na	na	na	-	-
N. tabacum	(L)	na	-	-	-
N. sylvestris	na	na	S	na	na
Datura stramonium	na	-	na	na	-
Petunia	(S)	na	-	-	(L)
Cucurbitaceae					
Cucurbita pepo	na	(L)	-	-	-
Cucumus sativus	(L)	na	-	-	-

Abbreviations

PWV	- Passionfruit woodiness virus	(McKnight 1953, Taylor and Kimble 1964; Teakle *et al.*, 1967).
PRV	- Passionfruit ringspot virus	(De Wijs, 1974a).
CSV	- Chlorotic spot virus	(van Velsen, 1961).
BYMV	- Bean yellow mosaic virus from *P. caerulea*	(Plese and Wrischer 1984).
PV1	- Passionfruit virus 1	

L - Local infection (L) - Local, symptomless infection
S - Systemic infection (S) - Symptomless systemic infection
- - no infection na - not available

APPENDIX III

Comparison of physical properties between reported potviruses isolated from *Passiflora* spp. with viruses under study

Physical Properties	PWV *Phaseolus vulgaris*	PRV *Passiflora edulis*	CSV *Passiflora foetida*	PV1	PV2	PV3 *Passiflora foetida*
LIV	3-4 days	12-14 days	3 days	6-7 days	5-6 days	4-5 days
TIP	50-60°C	65-70°C	65°C	70-75°C	60-65°C	50-60°C
DEP	10^{-4}-10^{-5}	10^{-6}-10^{-7}	10^{-7}-10^{-8}	10^{-5}-10^{-6}	10^{-4}-10^{-5}	10^{-3}-10^{-4}

Abbreviations

LIV - Longevity in vitro

TIP - Thermal inactivation point

DEP - Dilution end point

PWV - Passionfruit woodiness virus (McKnight 1953; Taylor and Kimble, 1964)

PRV - Passionfruit ringspot virus (De Wijs, 1974a)

CSV - Chlorotic spot virus (van Velsen, 1961)

PV1, PV2 and PV3 - Passionfruit viruses under present study