STUDIES ON THE FILAMENTOUS VIRUS ASSOCIATED WITH VEIN CLEARING DISEASE OF CITRUS IN INDIA

By

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on a filamentous virus associated with vein clearing disease of citrus in India"** , submitted to the faculty of Post- Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Plant Pathology by **Abdulrahman Abdulfatah Ahmed Al-shami** embodies the results of the *bona-fide* research work carried out by him under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that the help or success of information used during the course of investigation have been fully acknowledged.

YSAhlawat

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INTRODUCTION

In India, total area under cultivation is 165.5 million ha, of which 15.3 million ha is under fruits and vegetables. The area under citrus cultivation in India is 3.8 lakh ha and the annual estimated total production of citrus fruits is 28.22 lakh tones. Therefore citrus is considered second priority crop in India in view of its area and production. Globally its priority is for health benefits. The committee on Diet and Health, Food and Nutrition Board of the National Research Council, USA, recommended in 1989 that one should "Every day eat five or more servings of a combination of vegetables and fruits, especially green vegetables and yellow citrus fruits." Initially, the significance of citrus fruits was for its vitamin C and folic acid content but Scientists in USA found therapeutic usefulness of citrus pectin's and flavonoids. It is now established that grapefruit pectin can significantly lowers the plasma cholesterol levels and produce a significant improvement in LDL/HDL ratio. Recently it has been shown that citrus flavonoid *tangeretin* has the ability to prevent invasion of normal tissues by cancer cells. It was further shown that citrus *hesperidins, tangeretin,* and *nobiletin* have anti- inflammatory and anti- allergic activities. These flavonoids also improve circulatory system. Thus per capita consumption of citrus fruits needs to be increased to improve nutritional status of the population.

Citrus belongs to the family *Rutaceae* and there are about 150 genera and 1600 species in tropical and subtropical regions of the world , specially in the arid zones. The Rutaceae is divided into seven subfamilies comprising 11 tribes and 93 genera (Engler, 1931). Genera of three of these

subfamilies, *Aurantioideae* (33 genera), *Rutioidaea* (30 genera), and *Toddlioideae* (25 genera) are found in south east Asia. The four remaining subfamilies can be seen in the new world , Australia and other parts of Asia (Fardoss Rustem , 1998). *Aurantioideae* , the orange family is classified into two tribes, *Clauseneae* and *Citreae* and six subtribes which contain over 215 species and 65 varieties of plants (Swingle and Reece, 1967). *Fortunella, Eremocitrus, Clymenia, Poncirus* and *Microcitru* are the Close relatives of the genus citrus. " True Citrus Fruits " belong to the tribe *Citreae* and subtribe *Citrineae. Fortunella* (Kumquats), *Poncirus* (trifoliate orange) and citrus, (eight species) are the ones that are commercially important (Chapot , 1975). Among them *Citrus reticulata* Blanco (mandarins), *C. grandis* (L) osb. (shaddok or pumello), *C. paradisi* Macf. (grape fruit), *C. aurantifolia* (christm.) Swing (acid lime), *C. limon* (L.) Burm. f. (lemon) and *C. medica* L. (citron) are the species of economical value. The exact number of citrus species are unclear due to the presence of natural hybrids and the data pertaining to chemistry and molecular biology tend to change the understanding of the taxonomy of citrus species and their relatives (Jones, 1990). The true citrus fruits are native to Himalayan foot hills of North - east India, North central China, Eastern Phillippines, Burma, Thailand and Indonesia. Citrus culture as a garden industry existed for centuries in India from where it moved through traders to China, Pakistan, Egypt, Israel, Italy, Morocco,Algeria, USA, Spain, Mexico, Preaxial, Australia, African and south American countries, and Japan. Brazil is the major producer of citrus in the world (FAO, 1998) (Table 1). In India the major citrus producing states are Andra Pradesh, Bihar, Gujarat, Maharashtra, Punjab, Karnataka,Tamil Nadu and Madhya pradesh (Mankad, 1994). Total

Table 1. **Major producers and exporters of fresh citrus in the world (FAO, 1998)**

(Metric tonnes)

Country	Total Citrus	Sweet Orange		Tangerine/ Mandarin		Lime/ Lemon		Grape fruit	
	Prod.	Prod.	Exp.	Prod.	Exp.	Prod.	Exp.	Prod.	Exp.
Brazil	**17872**	16973	82	670	9	780	-	62	-
USA	**14689**	10761	601	539	23	768	123	2619	440
China	**7991**	1850	17	5730	135	203	-	207	-
Mexico	**4950**	3500	-	260	-	960	150	230	-
Spain	**4418**	2248	**1162**	1509	**1109**	641	**386**	20	17
Italy	**3401**	2180	89	504	27	713	31	2	-
India	3152	-	-	2080	-	-	-	-	-
Egypt	2817	1910	258	360	-	544	-	-	-
Argentina	2126	703	89	336	-	871	178	216	34
Turkey	1795	840	60	490	131	385	123	80	54
S. Africa	1221	970	68	-	-	74	44	177	105
Morocco	1192	766	301	403	212	20	-	3	-

Prod. = Production; Exp. = Exports

production of citrus fruits in the world during 1993 was 77,322 thousand tonnes of Which sweet oranges were 57,520, mandarins 9,010 , lemons and limes 7,708 and grape fruit and pumelo were 5,280 thousand tones (Mankad,1994).

Four agro - climateic zones have been identified in India for the purpose of citrus cultivation, these are :

1) **North-west zone :** This zone comprises of Punjab ,Hariyana , Rajasthan and part of Gujarat. Kinnow mandarin, blood red malta,

pinapple, jaffa, hamlin and valencia late sweet oranges, kagzi lime and lemon cultivars such as galgal , Italian lemon and Baramasi are the type that are cultivated here.

2) **North-eastern zone :** Assam, Meghalaya, Manipur, Tripura, Nagaland, part of Orissa and West Bengal belong to this region. Here mostly seedling trees of *Khasi* or *Sikkim* mandarins and *Assam lemon* are cultivated.

3) **Central zone :** Nagpur mandarin is major crop grown in this region. Vidharbha region of Maharashtra and part of Madhya pradesh come under this region.

4) **South zone :** Part of Andhra pradesh ,Karnataka ,Tamil Nadu and Kerala form part of this region . Sathgudi and Batavian are the main sweet orange grown in this region, coorg mandarin and acid lime are also cultivated in this zone.

India's climate is so diversified that the country offers potentiality for growing all kinds of fruits and citrus is the most important among them as citrus fruits are liked universally by people all over the world . Citrus fruits and their byproducts are being used in traditional medicine, perfumery and cosmetics. Carbonate citrus drinks are popular and lemons are used for flavoring vegetable dishes. Mandarins are used for making jams and jellies.

In India, major part of the citrus produce is consumed as fresh fruits. Only 10 -11 thousand tonns which is about 0.6 % of the total produce is being processed in the form of marmalade, squash, juice, and pickle. With this negligible processing of citrus, the country has

to import about 152 tonnes of essential oils and 600 tonnes of pectin every year (Dass , 1989). The main reason for less processing is the poor quality of the fruits in relation to the juice contents for which biotic stresses, specially of viral origin have been attributed (Pant, 1995).

Among virus and virus - like diseases citrus ringspot, tristeza, mosaic, yellow coky vein and greening were considered to be more important as they cause considerable losses to Indian citrus industry (Ahlawat, 1997). But during surveys in 1996 - 1997, a disease causing yellow vein symptoms on sour orange, etrog citron, lemons, *citrus pectinifera* and several citrus hybrids was recorded in Punjab. The affected trees showed very poor growth and only a few fruits were developed on such trees showing the disease of equal economic importance as were identified by Ahlawat *et al.* 1998) later surveys revealed the distribution of this disease in Gujarat and also in North - eastern states of India. Since this disorder of citrus was not known to occur in India or elsewhere earlier and its wider distribution in the country, this disease was identified for investigation with the following objectives :

1) To characterize the associated virus by biological and serological methods

2) To develop sero - diagnostic probes for detection of the virus in the planting material.

3) To establish serological relationship of the virus with known viruses, affecting citrus.

REVIEW OF LITERATURE

Citrus species are affected with several diseases caused by fungi, bacteria, Viruses, viroids, phytoplasma , spiroplasma, and fastidious bacteria (Table 2). Among them virus diseases are the most important as they are difficult to manage and no viricide is known for their control. These diseases spread in the nature inadvertently through propagation from contaminated planting material. In India the present status of virus diseases of citrus is given in (Table 3). A disease sharing symptoms of citrus psorosis, ringspot and citrus vein clearing virus was first observed from Abohar in punjab and later to other parts of India. Initial studies had shown that the disease is of economic importance. Therefore a detailed study has been made and comparatively reviewed in this chapter on various aspects of virus characterization and disease diagnosis.

Virus transmission

Mechanical transmission

Transmission of viruses from citrus spp to herbaceous hosts has been achieved in limited cases. Timmer *et al* (1978) mechanically transmitted citrus Ringspot virus Florida isolates from citrus to *Chenopodium quinoa.* Garnsey and Timmer (1980) mechanically transmitted psorosis B isolate from California to *Chenopodium quinoa.* Similar observations came forth from several workers (Bouhida, 1984; Garnsey and Timmer, 1988; Levy and Gumpf, 1991; daGraca *et al.*, 1991 and Navas - Castillo *et al.*, 1991). Indian citrus ringspot virus (ICRSV) is also transmitted mechanically to phaseolus *vulgaris* var. *saxa, Chenopodium quinoa* (Pant and Ahlawat,

Table 2. Current status of virus and virus-like diseases of citrus

A. Diseases of known etiology

Disease	Causal Agent	Transmission	Distribution	Reference
		A.1 Virus diseases		
1. Psorosis/ Ringspot	*Two flexuous and filamentous virus ** Linear-spirovirus *** Ophiovirus	G, MI, D	World wide, imp. In Florida, California, Argentina, Brazil, Texas, Israel, Spain, Vietnam, Central America, Australia, U.S.A., France, Italy, Iran	Swingle and Webber (1896), Fawcett (1932), *Bouhida (1984), Roistacher (1993), ** Derrick *et al.* (1993), *** Milne (1996)
2. Blind pocket	*Sobemovirus	G	California, Florida, Chile and Mediterrean basin	Fawcett and Lee (1926), *Navas-castillo *et al.* (1995)
3. Crinkly leaf	*Polyhedral virus	G, S, MI	California, Australia, India	Fawcett and Lee (1926), *Yot-Dauthy and Bove (1968), Ahlawat and Sardar (1976)
4. Leprosis	*Bacilliform-particles like Rhabdovirus	G, I, MI,	Florida, South America, Brazil	Knorr (1968), Lovisolo *et al.* (1996)
5. Infectious variegation	*Polyhedral particles ** Ilarvirus	G, MI	California, Sicily, Florida, India, Algeria, Argentina, Israel, Italy, Uruguay, U.S.A.	Fawcett and Klotz (1939), *Yot-Dauthy and Bove (1968), **Garnsey (1974), Yora *et al.* (1977)
6. Tristeza	*Flexouous rod **Closterovirus	G, MI, I	World wide distribution nearly in all citrus growing countries	Moreira (1942) *Kitajima *et al.* (1964) ** Gonsalves *et al.* (1978), Vasudeva and Capoor (1958), Chakraborty *et al.* (1993)

7. Satsuma dwarf	Isometic virus, *Serologically related to como- and nepo-viruses	G, MI	Widespread in Japan, China, Korea, Turkey	Yamada and Sawamura (1952), *Iwanami and Ieki (1995)
8. Vein enation and woody gall	*Spherical virus	G, S, I	California, S. Africa, Peru, Japan, Florida, India, China, New Zealand, Nepal, Phillippines, Australia, Spain	Wallace and Drake (1953), *Iwanami *et al.* (1992), **Philemon (1994), Mali *et al.* (1976b)
9. Citrus mosaic	*Spherical virus	G, S, I	Japan	Ishigai and Jinno (1958), *Tanaka and Imada (1976)
10. Citrus yellow mosaic	**Badnavirus	G, M, I	India	Dakshinamurty and Reddy (1975), **Ahlawat *et al.* (1996a and c)
11. Tatter leaf	* *Flexuous rod, Capillovirus	G	California, Nepal, China, Japan, Taiwan, Korea, S. Africa, U.S.A., New Zealand	Wallace and Drake (1962), *Kawai *et al.* (1995)
12. Rumple of lemon	*Virus ds RNA (?)	G	Florida, Italy, Turkey	Knorr *et al.* (1963). *Davino *et al.* (1995)
13. Citrus leaf rugose	**Ilarvirus*	G, MI	Florida, Argentina	Miyakawa *et al.* (1977), *Garnsey (1975)
14. Indian citrus ringspot	*Capillovirus	G, D, MI	Wide spread in India	Ahlawat (1989), *Byadgi and Ahlawat (1995), Pant *et al.* (1997)
15. Yellow vein clearing of lemon	*Filamentous particles **Capillovirus	G	Pakistan	*Catara *et al.* (1993) **Grimaldi & Catara (1996)

A.2 Viroid diseases

1.	Xyloporosis	*Citrus viroid (s)	G, S	Wide spread in Mediterranean countries, Brazil, Argentina, South Africa, Florida, Taxes, Phillippines, U.S.A., India	Reichert and Perlberger (1934), *Semancik & Duran-Vila (1991), Nagpal (1959)
2.	Exocortis	*Viroid, 371 nts and its **sequence variants (370-375)	G, D, CPT	Wide spread in over 44 countries	Fawcett and Klotz (1948), *Semancik & Weathers (1972), **Visvader & Symon (1985), Patil and Warke (1968) Ramachandran *et al.* (1993)
3.	Cachexia	*Citrus viroid 299 nt	G	Wide spread in Mediterranean countries, Brazil, Argentina, South Africa, Florida, Taxes, California Mexico, India, Nepal, Venezuela	Childs (1950), *Semancik (1986), Semancik *et al.* (1988), Levy and Hadidi (1993)
4.	Gummy bark	*Citrus viroids	G	Egypt, Saudi Arabia, Sudan, Turky, S. Africa	Nour-Eldin (1956) *Onelge *et al.* (1996)
.	Gum pocket	*Viroid	G, SSI	S. Africa, Argentian, Australia	Schwarz and McClean (1969), *Marais *et al.* (1996)
.	Yellow corky vein	*Viroid	G,MI	India	*Rustem Ali (1998) Reddy *et al.* (1974)

A.3 Diseases caused by mollicutes

	Stubborn	*Spiroplasma citri bacterium*	G, D, I I, SSI	California, Mediterranean countries, Middle east, North Africa, Western U.S.A.	Fawcett (1946) *Igwegbe & Calavan (1970)

2.	Rubbery wood	*Phytoplasma	G	India,	Ahlawat & Chenulu (1985), Ahlawat (1987)
3.	Witches broom of lime	*_Phytoplasma aurantifolia_	G, D	Omen, United Arab Emirated	Bove _et al._ (1988) *Bove _et al._ (1995)
			A.4 Diseases caused by fastidious bacteria		
1.	Greening	*(1) _Liberobacter asiaticum_ (2) _Liberobacter africanum_	G, I G, I	(1) In India, Nepal, Sri Lanka, Vietnam, Cambodia, Malaysia, Indonesia, Phillippines, Taiwan, China and Saudi Arabia (2) S. Africa, Zimbabwe and Yemen Both (1) and (2) are present in Mauritius island	Oberholzer (1947), *Jagoueix _et al._ (1995) Bove _et al._ (1993), Varma _et al._ (1993)
2.	Variegated chlorosis	*_Xylella fastidiosa,_ bacterium	B, I	Brazil	Lee _et al._ (1991) *Garnier _et al._ (1993)
3.	Citrus shoot yellowing	Citrus shoot yellowing organism (CSYO)	G	China	*Dewang _et al._ (1995)
			B. Diseases of uncertain etiology		
1.	Concave gum	Virus-like (?)	G	California, India, Japan, Vietnam	Fawcett and Lee (1926) Mali (1979)
2.	Impietratura	Virus-like	G	Mediterranean basin, Iran, Venezuela, S. Afrca, India California	Ruggieri (1961) Ahlawat _et al._ (1984)

3.	Yellow vein	Virus (?)	G	California	Weathers (1957)
4.	Bud-union crease	Virus-like (?)	G	Palestine, Florida, California, Egypt, S. Africa, Brazil, India, Spain, Texas, Israel, U.S.A., Japan	Grimm *et al.* (1955) Bakhshi and Dhillon (1964)
5.	Leaf curl	Virus (?)	G	Florida, Brazil, India	Salibe (1959). Mali *et al.* (1976a)
6.	Tarocco pit	Virus (?)	B	Sicily	Russo and Klotz (1963)
7.	Cristacortis	*Virus (?) **Viroid (?)	G	Mediterranean basin	*Vogel and Bove (1964) **Vogel and Bove (1968)
8.	Multiple sprouting	Phytoplasma (?)	TW	Rhodesia, S. Africa, India	Searle (1969) Ahlawat and Chenulu (1985)
9.	Blastomania	Phytoplasma (?)	B	India	Mali *et al.* (1975)
10.	Leathery leaf	Virus (?)	G, MI, I	India	Ahlawat (1975)
11.	Leaf yellow mid vein	Viroid (?)	G, MI	India	Sharma and Pandey (1983)
12.	Citrus chlorotic dwarf	Virus (?)	I, SSI	Turkey	Kersting *et al.* (1996)

G = Grafting, MI = Mechanical inoculation, D = Dodder, I = Insect vector, S = Seed, CPT = Contaminated pruning tools, SSI = Stem slash inoculation, B = Budding, TW = Top working.
*, **, *** : Causal agent and corresponding reference

Table 3. Virus and virus-like disorders known to be present in India

No.	Disease	Important characteristics
1.	**Tristeza**	First reported in Maharashtra state (*Nagpal, 1959) and later on, it has been recorded in other parts of the country (*Reddy and Rao, 1961, *Cooper, 1963, *Nariani *et al.*, 1965)
		Indicator host : Kagzy lime (*Capoor, 1961)
		Transmissioin : Grafting and dodder (*Nariani and Raychaudhuri, 1970).
		Vectors : *Toxoptera citricidus* (*Vasudeva *et al.*, 1959), *Aphis gossypii, Myzus persicae* (*Varma *et al.*, 1960), *a. craccivora, Dactynotus jaceae* (*Varma *et al.*, 1965), *T. aurantti* (*Capoor and Rao, 1967).
		Mode of vector transmission : Non persistent (*Capoor, 1975).
		Dodder : *Cuscuta reflexa* (*Nariani *et al.*, 1970).
		Host range : Citrus species and cultivars except trifoliate orange which is immune to CTV (*Ahlawat and *Raychaudhuri, 1982).
		Pathogen : A filamentous virus belonging to closterovirus group (*Kitajima, 1965). Tree strains have been identified. Antiserum prepared at IARI and used for CTV detection and strainal differentiation (Chakraborty *et al.*, 1993).
2.	**Greening**	First report from India in 1966 (*Fraser *et al.*, 1966).
		Transmission : Grafting and dodder (*Raychaudhuri, 1972).
		Vector : *Citrus psylla, Diapharina citri* (*Capoor *et al.*, 1967).
		Host range : All citrus cultivars except sweet lime and pummelo which are tolerant (*Nariani *et al.*, 1973).
		Pathogen : Bacteria-like organism (BLO) (Bove *et al.*, 1993; Varma *et al.*, 1993), Taxonomically placed as *Liberobacter asiaticum* (Jagoueix *et al.*, 1995).
3.	**Exocortis**	First reported from India in 1968 (*Nariani *et al.*, 1968); (*Patil and Warke, 1968).
		Indicator hosts : Etrong citro, Rangpur lime and cucumber cv. Suyo (Ramachandran *et al.*, 1993).
		Transmission : Contaminated tools and hands, graft and dodder, mechanically to petunia, natural root grafting.

		Pathogen : A viroid-low molecular weight pathogenic RNA related to Cvd-IIa and ITB vd group of viroids (Ramachandran *et al.*, 1993).
4.	**Citrus ringspot**	First reported from India in 1989 (Ahlawat, 1989) and later studied in detail (Byadgi and Ahlawat, 1995). **Indicator hosts** : Sweet orange. **Transmission** : Grafting and dodder. **Host range** : Different citrus cultivars including kinnow - mandarin - a hybrid. **Pathogen** : Two type of filamentous virus particles, one of them have identified as capillovirus (Byadgi and Ahlawat, 1995).
5.	**Leathery leaf**	Reported on mandarins in Darjeeling and Sikkim hills (Ahlawat, 1975; Ahlawat *et al.*, 1979) **Transmission** : Mechanically to citrus and non citrus hosts, grafting. **Vector** : *A. gossypii.* **Pathogen** : Not known.
6.	**Crinkly leaf and infections variegation**	First reported on lemon (*Ahlawat & Sardar, 1976; *Yora *et al.*, 1977) **Infectious variegation** : Sour orange and sweet orange. **Transmission** : Mechanical and grafting. **Pathogen** : A virus of ilavirus group both in crinkly leaf and variegation.
7.	**Vein enation and woody gall tumors**	First reported from India in 1984 on Rangpur lime and Kagzi lime (*Ahlawat *et al.*, 1984) **Vector** : *Toxoptera aurantii* (Manjunath, 1987) **Pathogen** : Isomeric virus particles in infected citrus and viruliferous aphid vector, *T. aurantic* (Maharag *et al.*, 1988).
8.	**Cachexia or Xyloporosis**	Symptoms on field trees were reported (*Nagpal, 1959; Chohan and Knorr, 1970). **Indicator host** : *Orlando tangelo, C. reticulata, C. paradisi* (Childs, 1950). **Transmission** : Bud grafts. **Pathogen** : A viroid, but no information from India on occurrence of Cacnexia viroid.
9.	**Leaf curl**	Reported from Maharashtra (Mali *et al.*, 1976) Graft transmission, affected different citrus species.

10.	**Impietratura**	Reported on pummelo fruits (Ahlawat *et al.*, 1984; Bhagabati *et al.*, 1986). **Transmission** : Graft. **Pathogen** : Not known.
11.	**Mosaic**	First reported from India on sweet orange and pummelo (*Murty *et al.*, 1975). **Transmission** : Grafting, dodder, budding (*Ahlawat *et al.*, 1985). **Pathogen** : Non-enveloped bacilliform virus particles (Badnavirus) (Ahlawat *et al.*, 1993; Ahlawat *et al.*, 1996).
12.	**Rubbery wood**	First reported in north-eastern regions on lemon, lime, mandarin and Nagpur orange (*Ahlawat *et al.*, 1985). **Transmission** : Graft. **Pathogen** : Association of MLO in sieve tubes of phloem.
13.	**Lime witches broom**	First reported from Maharashtra (Ghosh *et al.*, 1999), phytoplasma etiology of the disease has been established.

* In Chenulu & ahlawat (1994).

1997). Rustici *et al.* (2000) Transmitted ICRSV to 5 herbaceous hosts including *Phaseolus vulgaris* var.saxa.The present virus is also mechanically transmissible from citrus to herbaceous hosts.

Graft transmission:

Bud grafting

The citrus vein-yellowing virus is graft transmissible as in the case of citrus ringspot virus and other citrus viruses. Fawcett (1933) transmitted psorosis 'A' by bud grafting for the first time. Fawcett (1938) said that fusion of cambial cells of diseased bud; bark and root with that of healthy ones are necessary for the successful establishment of the graft. Bud grafting was also done by (Navas - Castillo et *al.* 1991). Thind *et al.* (1999) readily transmitted citrus ringspot virus through budwood however they failed to transmit the virus through seed and

insect vectors (*Diaphorina citri* and *Dialeurodes citri*). Lore and Cheema (2000) used bud grafting for the successful transmission of Indian citrus ringsopt virus (ICRSV). The present virus is also bud transmitted to citrus species.

Other methods of virus transmission

Garnsey and Whidden (1970) reported leaf tissue grafting a better technique for the transmission of citrus viruses in general. The transmission of ringspot disease from citrus to citrus was quicker by this method (Garnsey, 1975; Garnsey *et al.* 1976; and Timmer *et al.* 1978). Ahlawat (1989) reported that leaf patch grafting was better for the transmission of Indian citrus ringspot from mosambi to other citrus cultivars. Levy and Gumpf (1991) employed T graft technique for quicker transmission of psorosis virus from citrus to citrus. Garcia *et al.* (1991) employed bark chip inoculation for the successful transmission of ringspot and psorosis viruses. Wedge grafting however commonly being used for transmission of most citrus viruses. The present virus was also transmitted by various methods.

Insect transmission

Pujol and Benatena (1965) thought that the spread of citrus psorosis in Argentina was by some vectors, probably by sucking pests. Their observation was based on the spread of the disease naturally under field conditions. But they could not identify the reason. Portillo and Benatena (1989) reported the transmission of psorosis by aphids. Aphid species *Toxoptera citricidus, T. aurantii, A. citricola* and *A gossypii* although colonised on citrus but former three only transmitted

the disease. Timmer and Garnsey (1980) reported natural spread of psorosis in nucellar virus free trees of Texas. 35 trees were infected in seven years at the rate of five trees per year. Roistacher (1992) observed natural spread of psorosis A in Citrus Research Center at California, Riverside on 29 citrus seedling trees suggesting the involvement of some agency in the spread. Navas-Castillo *et al.* (1991) inoculated 20 sweet orange seedlings with *A . gossypii* and could not find symptoms even after 3 years. In another experiment they used *A. citricola* also, but without any success. ICRSV is also not transmitted by any insect vectors (Pant and Ahlawat, 1998; Byadgi *et al*, 1993). The present virus also could not be transmitted by insects, Aphid (*Aphis gossypii* Glover, *A. citrocila* Pach, *Myzus persicae* Sulz), white fly (*Bemicea tabaci L*) and Mealy bug (*Planococcus citri* Russo).

Seed transmission

It is well known that viruses pass through pollen and seeds of the infected plants but only few of them are seed transmitted. Bridges *et al.* (1965); Childs and Johnson (1966) reported seed transmission of psorosis (15-30 %) in Carrizo citrange at Florida . Campiglia *et al.* (1976) observed 1 % seed transmission of psorosis in trifoliate orange (*Poncirus trifoliata*) seedlings at Salto region Uruguay. Pujol and Benatena (1965) did not observe any seed transmission since the seedlings raised from seeds of infected trees did not show any symptoms in the young leaves of seedlings. Garnsey (1975) also reported that 54 Algerian Navel orange seedlings raised from infected seeds showed no symptoms. Wallace (1978) reported no seed transmission of citrus psorosis A from 20000 seedlings raised from

infected trees. Byadgi *et al.* (1993) could not get seed transmission of Indian citrus ringspot virus when he raised 945 seedlings from infected trees. Seeds collected from sour orange fruits from infected trees of the present virus did not show any disease symptoms upon germination.

Dodder transmission

A number of workers reported dodder transmission of viruses from citrus to citrus (Wealthers and Harjing, 1964; Desjardins *et al.*, 1969; Manjunath, 1989; Levy and Gumpf, 1991 and Byadgi *et al.*, 1993). Desjardins *et al.* (1969) transferred citrus ringspot from citrus to *Catharanthus roseus* and *Petunia* through dodder, but not vice versa. Levy and Gumpf (1991) transmitted an isolate of psorosis from citrus to Mexican Chilli through dodder and they also reproduced the disease back to healthy citron seedlings from infected Mexican Chilli by dodder. Byadgi *et al.* (1993); Pant and Ahlawat (1998) reported transmission of Indian citrus ring spot virus through dodder from mosambi sweet orange to sweet orange cvs. mosambi and sathgudi, kinnow mandarin and kagzi kalan. However, the virus in question, could not be transmitted through *cuscuta copestris.*

Host range studies

According to Catara and Grasso (1968); Timmer and Garnsey (1977) and Timmer *et al.* (1978), the host range of citrus ringspot virus (CRSV) is confined to the family *Rutaceae.* Indian citrus ringspot virus produced vein clearing as Young leaf symptom as reported by Byadgi *et al.* (1993) and Pant (1995). *Citrus aurantifolia, C. aurantium, C. excelsa, C. grandis, C. lemon, C. macrophylla, C. mitis, C. paradisi, C. reticulata,*

C. sinensis and poncirus trifoliata are some of the species that are reported to be susceptible to citrus ring spot virus. Herbaceous hosts like *Capsicum annuum, C. fructescens, Catharanthus rosesus, Chenopodium alba, C. quinoa, C. amaranticolor, Crotolaria spectubilis, Cucumis melo, C. sativus, Cucurbita pepo* cvs Richgreen and small sugar pumpkin, *Helianthus annus, N. tabaccum cv.* Turkish, *N. rustica, N. clavelandii, N. megalosiphan, N. glandiflora, Physalis floridana, Zinnia elegans, Gomphrena globosa, Sesamum indicum, Solanum nigrum, Petunia hybrida, Pisum sativum, Vigna unguiculata* are some of the herbaceous hosts to which citrus ringspot virus has been transmitted (Garnsey, 1975; Garnsey *et al.*, 1976; Timmer *et al.*, 1978; Levy and Gumpf, 1991 and da Graca *et al.*, 1991). Citrus ringspot virus did not go to *Lycopersicon esculentum, Nicotiana tabaccum* cv. KY 57, *Chenopodium murale, Citrullus lanatus, Cucumis pepo* cv. Zucchini (Garnsey, 1975; Garnsey *et al.*, 1976 and Timmer *et al.*, 1978). The Indian citrus ringspot virus could not be transmitted to any of these hosts, *Brassica oleracea* var. *botrytis, B. oleracea* var. capitata, *B. juncea, Citruleus vulgaris, Vigna mungo, V. sinensis, V. ungiculata, Catharanthus roseus, Cucumis melo, C. sativus, Gomphrena globosa, Dolichos lablab, Petunia hybrida, Nicotiana tabaccum, N. glutinosa, Pisum sativum and Glycine* (Pant, 1995). However, it was transmitted *to Phaseolus vulgaris var. singtamy and saxa, C. quinoa, Cowpea, Soybean* (Rustici *et al*, 2000; Pant *et al.*, 1997).

Purification and Electron microscopy

Bouhida (1984) purified citrus psorosis A virus for the first time and observed flexuous particles. Ahlawat and Chakraborty (1990) used phosphate buffer 0.05 M (pH 7.6) containing thioglycolic acid for the extraction of Indian citrus ringspot virus from infected leaves of

citrus. Levy and Gumpf (1991) used borate buffer 0.5 M (pH 8.3) containing 0.1 % sodium sulphite and 0.5 % 2 - mercaptoethanol for virus extraction. Derrick *el al* . (1991), Garcia *et al.* (1991) and Navas-Castillo *et al* . (1991) used Tris-HCl of 0.05 M (pH 8.0) containing 0.1 % ascorbic acid, 0.1 % cystein and 0.5 % 2 - mercaptoethanol as the extraction buffer. Ahlawat and Chakraborty (1990); Navas-Castillo *et al.* (1991) used 6 % n-butanol for the clarification of the extracted sap . The extracted sap was clarified with mixture of 50 % carbon tetrachloride and chloroform by Levy and Gumpf (1991). Derrick *et al.* (1991) used 8 % carbon tetrachloride and Byadgi *et al.* (1993) used 10 % n-butanol for clarification. Rustici *et al.* (2000) used 10 % chloroform for extraction of ICRSV from *saxa bean*. The concentration of the virus was done by differential centrifugation (Derrick *et al.*, 1991; Navas-Castillo *et al.*, 1991). The partially purified virus was precipitated by polyethylene glycol (MW - 6000) by Levy and Gumpf (1991) and Byadgi *et al* . (1993). Final purification of CRSV was done by density gradient centrifugation using 10 - 40 % sucrose gradients in TACM buffer (pH 8.0) at 95,000 g for 3.5 h (Derrick *et al.*, 1988; Navas-Castillo *et al.*, 1993). Derrick *et al.* (1991); Garcia *et al.* (1991); Navas-Castillo *et al.* (1991); and Byadgi *et al.* (1993) used 0 - 4.0 % sucrose density gradient centrifugation at 38,000 rpm for 150 min. Levy and Gumpf (1991) used cesium chloride density gradient at 56560 g for 15-8 h. Byadgi *et al.* (1993) used 0 - 30 % cesium sulphate gradient at 1,90,000 g for 60 min. for better purification. Rustici *et al.* (2000) used 10-40 % cesium sulphate density gradient centrifugation for further purification of the virus. The electron microscopy of leaf dip and purified preparations showed flexuous particles measuring 640 nm in length (Bouhida, 1984). Levy and Gumpf (1991) found filamentous

particles measuring 660-665 x 12 nm with psorosis isolate (p-203-m). Ahlawat and Chakraborty (1990) observed filamentous particles form psorosis - A infected citrus leaves . Filamentous and flexuous particles were also observed by Navas - Castillo *et al.* (1993). The size was 1000 x 10-12 nm. Garcia *et al.* (1994) observed two types of particles 300-500 and 1500-2500 nm in length and 10 nm in diameter. Byadgi also observed the association of two types of particles measuring 640 x 15 nm and 690 x 9 nm in Indian isolate of citrus ringspot virus infected leaves. Pant and Ahlawat (1998) also reported two types of virus particles. Derrick *et al.* (1988; 1991); da Graca *et al* . (1991) isolated two components of flexuous particles which were separated from top and bottom components of sucrose density gradients of citrus ringspot virus or psorosis at Florida. Same results were obtained by Garcia *et al.* (1991) and da Graca *et al.* (1991) of Argentina and by Navas-Castillo *et al.* (1991) of Spain. Derrick *et al.* (1988; 1992) observed two types of particles, spiral and extremely flexible measuring 300 - 500 x 10 nm and 1500 - 2000 x 10 nm. He termed the virus as 'spirovirus' on the basis of the helical morphology. Filamentous particles of unusual morphology were detected in psorosis A and B with the help of serologically specific antiserum to citrus ringspot virus (Navas-Castillo and Moreno, 1995). Catara (personal communication) reported that citrus yellow vein clearing in Pakistan seems to be different from citrus yellow vein clearing as described in California (Weather, 1960 , 1961). The nature of the disorder, its etiology, host range and its transmission by mechanical means or by insects, remain to be experimentally established.

Physico - chemical properties

Levy and Gumpf (1991) isolated double stranded RNA form (p-205-m) isolate of psorosis after 8 week of T graft inoculation. They observed high molecular weight ds RNA , showing migration with Mr of 5 -3 x 106 and two fast migrating RNAs with Mr of 4.5 x 106 and 4.1 x 106 respectively on 6 % polyacrylamide vertical slab gel. Citrus ringspot virus (CRSV) has been reported to contain single stranded RNA as its genome (Derrick *et al.*, 1991; Garcia *et al.*. 1991; Levy and Gumpf, 1991; Byadgi *et al.*. 1993). The estimated Mr of bottom component ssRNA was 3 .0 to 4 .0 x 106 (Derrick *et al.*, 1991). They also observed a single band from nucleic acid preparation of top component. Garcia *et al.* (1991) observed total loss of infectivity of citrus ringspot (CRSV) when it was incubated with RNase and reported CRSV as a ss RNA virus. Navas-Castillo *et al.* (1991) and Byadgi *et al.* (1993) failed to isolate ds RNA from young symptomatic leaves of CRSV infected plants. Levy and Gumpf (1991) reported the presence of a single polypeptide of 29 KDa in a psorosis isolate. Derrick *et al.* (1991), Garcia *et al.* (1991) and Navas-Castillo *et al.* (1991) observed 48 KDa coat protein in CRSV preparation. da Graca *et al.* (1991) observed 48 - 50 KDa coat protein in top and bottom components. Navas-Castillo *et al.* (1993) reported 38 KDa and 48 KDa protein associated with capsid protein of CRSV. Garcia *et al.* (1994) reported 48 and 50 KDa coat protein in CRSV. Byadgi *et al.* (1993) reported 29 KDa coat protein in an Indian isolate of ICRSV. A 47 kDa protein was detected in 6 ringspot isolate after partial purification. One isolate of psorosis A and one isolate of ringspot had 46 and 48 kDa protein respectively (Navas-

Castillo and Moreno, 1995). Pant and Ahlawat (1998) reported 60 and 67 KDa protein associated with thin flexuous particles associated with ICRSV.

Serodiagnosis

Levy and Gumpf (1991) prepared polyclonal antiserum against the psorosis isolate (P-203-m) in chicken. Derrick *et al.* (1991) have also prepared polyclonal antiserum in rabbit using purified virus obtained by electro elution from agar gel. Byadgi *et al.* (1993) also produced antiserum against Indian citrus ringspot virus. Levy and Gumpf (1991) detected the psorosis virus in antigen coated indirect Enzyme -linked immuno-sorbent assay (ELISA) from citron and sweet orange plants inoculated with isolate p-203-m . They found that cross absorption helps to reduce non-specific reaction . Detection of ICRSV in ELISA has been done by Byadgi *et al* . (1993).ISEM technique was used successfully for detection of CRSV in diseased trees by Derrick *et al.* (1991); Navas -Castillo *et al.* (1991); da Graca *et al.* (1991) and Ahlawat *et al.* (1995). Garcia *et al.* (1994) also decorated CRSV particles with CRSV antiserum. Derrick *et al.* (1991) and da Graca *et al.* (1991; 1992) reported western blotting technique for detection of CRSV. They detected 48 KDa protein in psorosis infected samples with CRSV-4 antiserum. Byadgi *et al.* (1993) detected 29 KDa protein from an isolate of ICRSV in western blotting. Alioto *et al.* (1999) detected citrus psorosis by an improved DAS - ELISA using polyclonal antibody. D'-Onhia *et. al.* (1998) compared ELISA with biological indexing to detect citrus psorosis and they found that ELISA correlates with biological indexing. During the present

studies antibodies against the present virus have been prepared and used for virus detection in ELISA and ISEM.

PCR detection

Fragments of cDNA from bottom components of CRSV were cloned and sequenced and primers were designed. RT-PCR experiments using these primers (5'ACAATAAGCAAGACAAC upstream and 5'CCATGTCACTTCTATTC downstream) allowed detection of citrus ringspot virus infected citrus leaves. Detection of citrus psorosis virus was less sensitive when the same primers were used (Garcia *et al.*, 1997). Legarreta *et al.* (2000) used a highly sensitive heminested RT-PCR for the detection of citrus psorosis virus. Barthe *et al.* (1998) used RT - PCR for detection of citrus psorosis virus. Sambade *et al.* (2000) used fast one-step RT-PCR to amplify citrus tristeza and citrus psorosis virus. RT-PCR has been used for detection of ICRSV (Shelly Parveen *et al.*, 1999). During the present studies ICRV primers (51-ccg gga tcc ATG AGC TTT GAC TAC ACA - 31 upstream and 51-cac ccg gga att cTT AAG TGT TGA AAG GGG-31) have been used to detect ICRSV by PCR amplification.

The progress of research on ringspot (= psorosis) related diseases and their etiological agents have been summarised in the following paragraphs :

1896 **Swingle and webber** - observed the disease in Florida for the first time and named it as 'psorosis' means - ulcer.

1908 **Smith and Butler** - Psorosis bark scaling symptoms reported from California.

1933 **Fawcett-** Transmitted psorosis by bud, described leaf and bark symptoms and provided first evidence of a transmissible virus disease of citrus.

1939 **Doidge** - failed to observe leaf symptoms on trees with bark lesions. Conducted one of the first eradication programmes and emphasised the need of rapid indexing method.

1942 **Fawcett and cochram** - First used lesion (bark) inoculum for quicker transmission of psorosis B and reported a long incubation period of 12 - 16 years development of bark lesions.

1945 **Wallace** - used sweet orange seedlings as indicator host for detection of psorosis. It was a major breakthrough as symptom developed within 6 - 10 week in inoculated sweet orange plants.

1947 **Wallace** - transmitted psorosis by leaf piece grafting of young or mature leaves as inoculum.

1953 **Wallace** - recorded 39 % disease incidence in a survey and indicated considerable reduction in yield due to psorosis disease.

1955 **Moore *et al.*** - conducted a survey and reported 8 % of 200,000 trees showing bark lesions. In some orchards it was up to 50 %.

1957 **Wallace** - used cross protection experiments and identified severe and mild forms of psorosis A and B.

1957 **Weather** - Reported yellow vein disease from California.

1959 **Nagpal** - observed psorosis - type symptoms on field trees of mosambi, in India.

1964 **Roistacher and Nauer** - observed variation in sweet orange as indicators and reported that pineapple Madam vinous olive lands sweet orange seedlings are better indicators.

1965 **Bridges *et al.*** - reported 10 % seed transmission of psorosis in carrizo citrange.

1965 **Roistacher and Calavan** - Established psorosis A and concave gum as two separate virus diseases.

1967 **Corbett and Price** - reported failure of psorosis to protect against citrus variegation virus.

1968 **Wallace and Drake** - reported citrus ring spot as a graft transmissible disorder of citrus.

1968 **Roistacher and Blue** - reported that psorosis and Dweet mottle diseases are not related.

1969 **Desjardins *et al.*** - Considered ringspot as a component of psorosis. They transmitted the virus to herbaceous hosts and back to citrus by dodder.

1970 **Garnsey and Timmer** - mechanical transmission of Texas, Florida and California strains of CRSV and transmitted three psorosis -B isolates from citrus to *C. quinoa.*

1972 **Broadbent** - considered psorosis as relatively less important disease in Australia because of less infected trees and effective quarantine.

1974 **Passos *et al.*** - reported 57 % disease in 10 year old nucellar selection and 100 % in paranucellar propagation. He also observed bark symptoms but could not observe young leaf symptoms.

1974 **Timmer** - Reported cross protection of CRSV against psorosis-B and believed that CRSV is a component of psorosis complex.

1975 **Garnsey** - Reported CRSV infection in star Ruby grapefruit illegally moved to Florida.

1976 **Campiglia *et al.*** - Reported seed transmission in Trifoliate orange up to 8 - 10 % and also found 20 % of nucellular mother trees showing psorosis young leaf symptoms.

1976 **Roistacher *et al.***- Shoot tip grafting was used to eliminate psorosis.

1977 **Timmer and Benatena** - Gave a summary of comparison of psorosis and other viruses causing leaf flecking symptoms.

1978 **Timmer *et al.*** Texas and Florida isolates of CRSV are basically similar.

1979 **Timmer and Garnsey** - Reported that CRSV distribution in plants was uneven.

1980 **Navarro *et al.*** Separated psorosis and exocortis by shoot tip grafting.

1980 **Vogel and Bove** - Observed ringspot symptoms on Parson,s special mandarin and Tangelo under warm conditions.

1980 **Roistacher *et al.***- Reported mechanical transmission of psorosis isolate from citron to citron by knife cuts.

1980 **Timmer and Garnsey** - Reported natural transmission of CRSV up to 20 % over a period of 8 years. No seed , soil or insect transmission but recorded high transmission from petal and stamens to *C. quinoa.* He also reported strong association of

bark scaling psorosis and CRSV. He believed CRSV may be synonymous to psorosis B.

1984 Bouhida - reported association of flexuous particles (640 nm) with psorosis in leaf dip and purified preparations and also reported that virus could be transmitted mechanically to *Nicotiana benthamiana* but not to citrus.

1984 Benatena and Portillo - Seedlings planted near infected tree showed transmission. He believed that victors are responsible for transmission.

1984 Brlansky *et al.* - Electron micrograph picture showed that plugs in xylem in psorosis affected plants are different from blight or concave gum.

1988 Garnsey and Timmer - Transmitted psorosis from tissue of bark lesion from sweet orange tree to sweet orange seedlings (graft) to citron (graft), *C. quinoa* mechanically and then graft transmitted back to sweet orange.

1988 Derrick - Found two fractions in differential centrifugation of mechanically transmissible ringspot virus. Both the components were essential for infectivity.

1988 Timmer and Garnsey - Transmitted CRSV mechanically to *C. quinoa.* and *Gomphrena globosa.*

1988 Derrick *et al.* - Reported spiral shaped virus particles of CRSV. He produced antiserum against CRSV - 4 isolate.

1989 **Ahlawat-** Reported psorosis - A for the first time from India as transmissible disease.

1989 **Manjunath** - Transmitted psorosis virus through dodder.

1990 **Ahlawat and Chakrabotry-** reported the association of a flexuous virus with psorosis in India.

1991 **Levy and Gumpf** - Purified psorosis isolate (p-203-m) and found association of 660-665 nm flexuous virus particles tentatively identified as carlavirus and produced antiserum. He also reported 29 KDa coat protein. He developed plate coat antigen indirect ELISA for virus detection.

1991 **Derrick *et al.*** - Separated two components of CRSV as top and bottom components by density gradient centrifugation and found 48 KDa coat protein in CRSV.

1992 **Derrick *et al.*-** He proposed new name to the virus associated with ringspot disease as 'spirovirus'.

1992 **daGraca *et al.*** Used immunoblot technique for detection of citrus psorosis virus using CRSV- 4 antiserum.

1992 **Garcia *et al.*** Suggested that viruses associated with citrus psorosis and ringspot diseases belong to new group of viruses.

1992 **Navas - Castillo *et al.*-** Described symptomatology, mechanical transmission to *C. quinoa* and reported 48 KDa coat protien.

1992 **Roistacher** - Reviewed psorosis virus complex and considered CRSV similar to psorosis.

1993 **Byadgi *et al.*-** Reported three type of particles associated with CRSV in India. A virus of capillovirus group measuring 640 x 15 nm ,long flexuous particles 690 x 9 nm and tubules 2250x 40 nm.

1993 **Navas - Castillo *et al.*-** They also established that top and bottom components are required for infection in C.*quinoa* as individual component was unable to cause infection. Both component contained 48 KDa coat protein. However, they also reported 38 KDa protein in their initial experiment and established that this protein is the degradation product of 48 KDa protein.

1994 **Garcia *et al.*-** reported two type of particles 300 - 500 nm and 1500 - 2000 nm x 10 nm as top and bottom components with CRSV. They suggested that CRSV represents a new genus (possibly family) related to tenuiviruses. But failed to established serological relationship between CRSV and tenuiviruses. Moreover, the capsid protein and host range of psorosis are quite different. They proposed the name ' Ophiovirus ' for the proposed new genus.

1994 **Kersting *et al.*-** Reported that biological indexing of citrus chlorotic dwarf virus (CCDV) in the eastern mediterranean region of Turkey was performed using rough lemon (*citrus jambhiri*) that produced distinct symptoms in very short time for indexing.

1994 **Guirado *et al.*-** Identified *Baianinha* and *Ceu* oranges for indexing of citrus psorosis virus complex and over come the temperature effect.

1995 **Navas - Castillo and Moreno** - Filamentous flexuous particles of unusual Morphology were detected in ISEM from infected with several ringspot isolates, psorosis A and psorosis B using an antiserum of citrus ringspot virus. All ringspot isolate had a specific protein of 47 KDa except in one isolate of psorosis A and one of ringspot which had 46 and 48 KDa protein respectively. The three type of protein were serologically related in western blot. They suggested that a common virus with different strains may be involved in psorosis A, psorosis B and ringspot disease.

1995 **Byadgi *et al.*** - Reported 29 KDa coat protein in an Indian isolate of CRSV.

1996 **Grimaldi and Catara** - Reported association of a filamentous virus with yellow vein clearing of lemon, from Pakistan.

1997 **Pant and Ahlawat** - Transmitted ICRSV to Phaseolus vulgaris and *C. quinoa.* The virus was purified from bean and polyclonal antibodies were produced. The virus was detected in ELISA and ISEM.

1998 **Barthe *et al.*** - used RT - PCR for detection of citrus psorosis virus.

1998 **D- onhia *et al.*** - Compared ELISA with biological indexing to detect citrus Psorosis virus and they found that ELISA can be correlated with biological indexing.

1999 **Alioto *et al.*** - Detected citrus psorosis by an improved DAS - ELISA using polyclonal and monoclonal antibodies.

1999 **Thind *et al.*** - Readily transmitted citrus ringspot virus through budwood however they failed to transmit the virus through seeds and insect victors (*Diaphorina citri* and *Dialeurodes citri*).

2000 **Legarreta *et al.***- Used a highly sensitive heminested RT - PCR for the detection of citrus psorosis virus.

2000 **Lore and Cheema** - Used bud grafting for the successful transmission of CRSV.

2000 **Sambade *et al.*** - Used fast one - step RT - PCR to amplify *cutrus tristeza* and *citrus psorosis* virus.

2000 **Rustici *et al.*** - Transmitted ICRSV to five herbaceous hosts. *Phaseolus vulgaris var. saxa* is the best systemic host. They purified the ICRSV from *saxa bean.* The virus had flexuous particle with evident cross banding and a modal length of 650 nm. The virus had 34 KDa coat protein and a single ssRNA of about 7.5 Kb. The derived amino acid sequence of the CP contained some short motifs similar to those of potex -, fovea -, carla - and allexiviruse, but otherwise no similarity to any of these groups. They concluded that this virus belongs to a new group of plant viruses.

MATERIAL AND METHODS

3.1. Source of culture

Five isolates of the virus were collected from different parts of the country viz: Abohar, Ludhiana, Ahmedabad, Anand and Pune. These isolates were grafted on healthy seedlings of 5 citrus cultivars, etrog citron, sour orange, mosambi, kinnow and pectinifera in the glasshouse. Three test plants of each cultivar were graft inoculated to have the culture of the virus. Symptoms on inoculated plants were observed following inoculation, for a period of six months

3.2. Pure culture

3.2.1. Electron microscopy of the virus isolates

In view to determine the association of a virus, the glasshouse infected isolates were examined in electron microscope and immunosorbent electron microscopy (ISEM) by the method of Byadgi and Ahlawat, 1995; Pant and Ahlawat, 1998.

3.2.2. Maintenance of pure culture

In view of the mixed infection in all the isolates, it became essential to develop pure culture of the virus by mechanical inoculation, as follows:

3.2.2.1. Preparation of the test plants

Four months old seedlings of mosambi raised from healthy seeds in the nursery were transplanted in earthen pots containing sand, compost and soil at 1: 2: 2 ratio and they were kept in an insect proof glasshouse.

3.2.2.2. Preparation of inoculum

Standard extract was prepared by homogenization of 1 gm of freshly collected young symptomatic leaves of glasshouse infected Etrog citron in a sterilized chilled mortar and pestle in the presence of extraction buffer 0.05 M, pH 7.2 in 1:1 ratio (w/v). The extract was filtered through double layers of cheesecloth and used for inoculation.

3.2.2.3. Method of inoculation

The leaves of 5 healthy test plants of mosambi of about 6 months old were dusted with an abrasive (Carborundum 400 mesh). The forefinger was dipped in the standard extract and stroked gently and unidirectional over the upper surface of the leaves. During inoculation, the leaves were supported from the lower side to avoid leaf injury and to ensure uniform pressure and spread of inoculum. After inoculation the leaves were washed with water to remove excess inoculum and abrasive. The inoculated plants were maintained in the glasshouse and a suitable control was maintained.

3.3. Transmission

3.3.1. Citrus to herbaceous hosts

Since the virus was found to be sap transmitted from etrog citron to mosambi, it was desired to see its transmission from glasshouse maintained pure culture on mosambi to herbaceous hosts Cowpea (*Vigna sinensis*); French bean (*Phaseolus vulgaris* var. singtamey, saxsa, gheusemi, alapati); Tobacco (*Nicotiana tabaccum , N.glutinosa*); Soy bean (*Glycene max* Merr); Cotton (*Gossypium hirsutum*) *Chenopodium quinoa.* Five seedlings of each plant species were raised in earthen pots in glasshouse and used for

inoculation. The preparation of inoculums and method of inoculation are same as described in 3.2.2.2. and 3.2.2.3.

3.3.2. Effect of temperature and humidity on sap transmission

In order to determine the optimum period for symptoms development effect of temperature and relative humidity was studied in phytotron. Various temperature ranging from 25-35^{0}C and relative humidity ranging from 70 %-90 % was maintained after sap inoculation of *P. vulgaris* var. singtamy plants. A suitable control was maintained.

3.4. Graft transmission

Since the disease was graft transmitted, a comparative efficacy of various grafting techniques was determined. The pure culture of the virus was used in all the experiments and a suitable control was maintained.

3.4.1. Wedge grafting

One-year-old healthy seedlings were cut horizontally and a vertical cut of about 2 cm was made through the center of the cut stem at the top and moist with a few drops of water. Scion of matching thickness from glasshouse-infected plants was removed and trimmed to a wedge of about 2 cm long and inserted in to the vertical cut of the stock. The graft was then wrapped and tied with a piece of moist polythene strip, covered with a moist polythene bag and kept in the glasshouse for observation.

3.4.2. Petiole grafting

Leaves showing typical symptoms were detached from the infected plants along with petiole and with the help of a sharp blade a wedge was made at the bottom of the petiole end. At the nodal point of the test plant

a slanting cut was made and the wedged petiole was inserted into the cut portion. The graft was rapped and tied with polythene strip. It was then covered with a moist polythene bag and the plants were kept separately in the glasshouse for observation.

3.4.3. Bud grafting

To do T-bud grafting a vertical cut was first made to the test plant (2-4 cm) and then a small slit was given on the upper end to make a T - like appearance. The bud from the desired twig was inserted in the T - like slit and wrapped immediately with plastic tape, leaving the bud exposed. The branches of the test plant were removed to force the bud to grow.

3.4.4. Optimum time required for graft transmission

It has been normally observed that the scions do not survive in transmission experiments. Therefore this experiment was conducted to determine the optimum time of the scion survival for transmission of the virus by wedge grafting. Eight sets of 5 plants of healthy mosambi seedlings of 12 months old were inoculated by wedge grafting. The scions were removed at weekly intervals from each set except the control. The inoculated plants were kept in the glasshouse for symptom expression.

3.5. Insect transmission

Insect transmission was done with 3 Aphids, *A. gossypii* Glover; *A. citricola; Myzus persicae* sulz, one mealybug, *planococcus citri* Russo and one whitefly, *Bemisia tabaci* L. from colonies maintained in an insectary (Table 4). The aphid transmission tests were done in a non - persistent and persistent manner by the method of Ahlawat and Chenulu, 1982.

Table 4. Maintenance of insects used for virus transmission

S.No.	Insect species	Source of collection	Host on which maintained
1.	*Aphis gossypii* Glover	*Gossypium sp.*	*Nicotiana tabaccum*
2.	*A. citricola* Patch	*Citrus sinensis*	*C. aurantifolia*
3.	*Myzus persicae* Sulz.	*Solanum tuberosum* L.	*N. tabaccum*
4.	*Bemisia tabaci* L.	*Solanum melongena* L.	*N. tabaccum*
5.	*Planococcus citri* Russo.	*C. paradisi*	*C. aurantifolia*

Transmission test with whitefly were conducted by the method of Varma, 1951. Whereas mealy bug transmission was done according to Lockhart and Autrey, 1988. Five plants of mosambi were used for each insect *spp.* The inoculated plants were indexed in EM and ELISA after 12 months of inoculation.

3.6. Dodder transmission

Healthy dodder, *Cuscuta reflexa* Roxb. was collected from healthy *Lantana camara* L. and was tested for virus transmission by the method of Ahlawat and Dhingra (1973). All the inoculated plants were indexed in EM and ELISA after 10 months of inoculation.

3.7. Seed transmission

Seeds were extracted from fruits collected from field-infected trees of Lemon, Sour orange, Trifoliate orange and Pectinifera. 100 seeds of each

variety were sown in earthen pots having soil, sand and manure mixture. The seedlings of this experiment were observed up to 24 months for the appearance of disease symptoms. 10 % samples were indexed in EM.

3.8. Host range

3.8.1. Citrus species

Host range of the virus was studied by wedge grafting from glasshouse inoculated mosambi plants to following citrus species: *Citrus sinensis L.* Blanco (Sweet orange) cvs. Malta , Mosambi,; *Citrus reticulata* Blanco (Mandarin) cvs. Nagpur orange and kinnow mandarin (a hybrid between king and willow mandarin); *C . limon* (Lemon) cv. Galgal; *C. grandis* Osbeck (Pummelo); *C . decumana* Murr (Decumana); *C . medica* L. (Etrong citron); *C.. aurantifolia* (Christen) Swingle cvs. Kagzi kalan, Kagzi lime; *C. paradisi* Mac. Fad cv. Rubi grapefruit; *C. karna* Rag cv. Karna khatta,; *C . jambhiri* Lush (Rough lemon); *C . aurantium* L. (Sour orange); *C. mitis* Blanco (Calamandin) and *C. Pectinifera*. The grafted plants were maintained in glasshouse and observed regularly for symptoms appearance.

3.8.2. Herbaceous host

Following herbaceous plant species were mechanically inoculated from pure culture by the method described earlier: *Chenopodium amaranticolor* Coste and Reyn, *C. murale* L., *C. quinoa* Willd, *Gomphrina globosa* L., *Nicotiana tabaccum* L., *N. glutinosa* L., *Cucumis melo* L., *C. sativa* L., *Capsicum annuum* L., *Vigna sinensis* Savi., *V. radiata* (L.) Wilczek., *Glycine max* (L.) Merrill., *Lagenaria siceraria* (Kolina) Standl., *Solanum melongena* L. and *Phaseolus vulgaris* L. The inoculated plants maintained in the glasshouse

for observation. Random samples of inoculated plants were indexed in EM for the presence of the virus.

3.9. Physical properties

The thermal inactivating point (TIP), dilution end point (DEP) and longevity in vitro (LIV) at room temperature were worked out by the method of Ahlawat and Chenulu (1984).

3.10. Virus extraction

For isolation of viruses buffers, its molarity and pH plays very important role. Therefore for extraction buffers, molarity of the most efficient buffer and pH were determined. The methods are described below:

3.10.1. Efficacy of different buffers on virus extraction

To determine this five buffers, tris - HCl, tris -citrate, sodium citrate, sodium borate and sodium phosphate were used at a constant pH 7.0 and molarity 0.1 M. The sap extracted in various buffers was centrifuged at 8000 g for 20 min. The concentration of virus particles was recorded in electron microscope in an area of 40 μm both in pellet and supernatant. The preparation of these buffers is given in Appendix - 1.

3.10.2. Efficacy of molarity of sodium phosphate buffer

To know the optimum molarity of phosphate buffer for extraction of the virus, the symptomatic leaves were homogenized in phosphate buffer (pH 7.0) with different molarities, 0.01, 0.05, 0.07, 0.1 and 0.5. The concentration of the virus was monitored in EM both in pellet and supernatant after low speed centrifugation at 8000 g for 20 min.

3.10.3. Effect of pH of 0.05 M phosphate buffer for the extraction of the virus

To determine the efficacy of pH levels of the buffer, the sap was extracted in 0.05 *M* phosphate at different pH levels such as 6.5, 7.0, 7.2, 7.5 and 8.0, centrifuged for 20 min at 8000 g and examined the virus particles from pellet and supernatant in EM.

3.11. Virus purification

Purification procedures as reported by Bar - Joseph et al. (1985), Levy and Gumpf (1991), Byadgi *et al.* (1993) and Rustici *et al.* (2000) were tried for comparative purification of the present virus. The extraction buffer with optimum molarity and pH was used for purification of the virus by various methods. However, the protocol described by Rustici *et al.* (2000) provided the best purification of the virus as determined in EM by the method of Byadgi and Ahlawat (1995). The method used is as follows: Symptomatic leaves of *Phaseolus vulgaris cv. Singtamy* were collected after 7 - 10 days of mechanical inoculation and homogenised in tenth volume (w / v) of extraction buffer (0.05 M phosphate buffer (PH 7.2) containing 0.005 M DIECA, 0.01 M EDTA, 0.02 M Na2SO3). After filtration through muslin cloth the extract was stirred with 10 % chloroform for 10 min at room temperature, then centrifuged at 12,000 rpm for 10 min in a Sorvall SS - 34 rotor at 4°C. The supernatant was then centrifuged at 45,000 rpm for 2h in a Beckman 55.2Ti rotor at 4°C. Pellets were collected and suspended in 200 - 400 µl extraction buffer and aliquots of 1.0 ml layered on a preformed 10 - 40 % cesium sulfate density gradient prepared in extraction buffer. The gradient with virus preparation at the top was centrifuged at 41,000 rpm for 3h using a Beckman SW41 rotor. A single

virus band was observed which was collected, diluted in extraction buffer and centrifuged at 70,000 rpm for 30 min in a Beckman 90Ti rotor. The virus was resuspended in half strength extraction buffer and dialyse 3 times at 6h each to remove sucrose and salt. The final preparation was examined in electron microscope for its purity and concentration.

3.11.1. Ultra - violet absorption profile of the purified virus preparation

20 µl of purified virus preparation was diluted to 29 times to make the volumes 500 µl in phosphate buffer. Both the quartz cuvette filled with phosphate buffer were calibrated to zero absorbance. After calibration, first cuvette was taken out and filled with 500 µl of the virus preparation. UV absorbtion was taken at 260 nm and 280 nm in Kontron - UV KON - 93 spectrophotometer.

3.11.2. Electron microscopy

For electron microscopy, 10 µl of purified preparation was put on carbon coated copper grids for 1 min. The grid was then washed with 10 drops of distilled water and stained with 2 % uranyl acetate. Excess of stain was removed by touching the edge of the grid with a piece of a filter paper and the grid was placed and examined in JEOL - 100 CX - 11 transmission electron microscope. Electron micrographs of virus particles were taken at 20,000 magnifications on the plate film or on 35 mm film. The micrographs were magnified according to the requirements. The measurement of length of 100 particles in mm was taken from the negative and the actual length of particles was calculated either by ultra - structure size calculator (Tedpella, Inc. P.O. box 510, Tustin, California, USA) or by using the following formula:

$$\text{Size of virus particles (nm)} = \frac{\text{Measured size in mm}}{\text{Magnification}} \times 10,00,000$$

3.12. Serology

3.12.1. Production of antiserum

Polyclonal antiserum from purified virus preparation was developed in young one-year-old white albino rabbit. 750 ml of the purified preparation and equal amount of Freund's incomplete adjuvant were mixed thoroughly and injected to the rabbit intramuscularly. Four injections were given at weekly intervals in right and left thigh of the rabbit alternatively. Fifteen days after the last injection, 15 - 20 ml blood was collected from the immunized rabbit by giving a cut to the marginal vein of the ear. The blood was allowed to clot at room temperature for 1 h and kept overnight at 40C.Next day the clear serum was collected by decantation. The serum was centrifuged at 2000 g for 10 min at 40C in sigma tabletop centrifuge. Antiserum was collected in clear autoclaved tubes and stored at 40C in a refrigerator after adding 0.02 % sodium azide or 50 % glycerol.

3.12.2. Purification of immunoglobulins (IgG)

Immunoglobulins (IgG) were purified from polyclonal antiserum as described by Clark and Adasms (1977). One ml of antiserum was taken in a glass tube and added 9 ml of distilled water and mixed thoroughly. Ten ml of saturated ammonium sulphate was then added to the diluted antiserum and incubated for 30 min at room temperature. The mixture was centrifuged at 12000 rpm for 10 min at 4^0C. The pellet containing antibodies was dissolved in 2 ml of half strength phosphate buffer saline

(PBS) and dialysed for 24 h in 500 ml half strength PBS giving three changes at an interval of 8 hour. The partially purified IgG were passed through DEAE cellulose (sephacel) column for final purification.

A Bio - Rad 7 mm I.D Econo column attached with a stopcock valve was used for preparation of cellulose column. Approximately 10 ml of DEAE - cellulose (DEAE - sephacel. Sigma chemicals) slurry was poured down from the sides of the column to avoid air bubbles and allowed the cellulose to settle. The column was calibrated by washing with half strength PBS (7.4) till the pH of ingoing buffer equals with the pH of effluent. The liquid level was never allowed to drain at the top of the cellulose and it was added carefully to avoid disturbance to the column.

Salt fractioned serum was then added drop by drop over the pre - calibrated DEAE cellulose column. The stopcock valve was opened and the serum was allowed to run through the column. One ml effluent aliquots were collected in pre marked tubes as soon as the column is started. Additional 15 ml of half strength PBS was carefully added to the column and effluent was continuously collected in separate tubes. The fractions were measured separately at 280 nm in the spectrophotometer using half strength PBS as a blank. The tubes with OD values above 0.8 were pooled and final strength of IgG adjusted to 1.4 OD using half strength PBS which is 1 mg/ml concentration of IgG in the solution. The OD values were plotted against the fractions to obtain elution curve for purified IgG.

3.12.3. Preparation of antibody - enzyme conjugate for Enzyme-Linked Immunosorbent Assay (ELISA)

One-step glutaraldehde method (Clark and Adams, 1977) was used for preparation of antibody alkaline phosphate as homologous conjugate

(virus IgG*AP). 2 ml of purified IgG (1 mg/ml) were mixed with 200 µl (= 2500 units) of enzyme alkaline phosphatase (Sigma) and made 0.05 % final concentration with fresh EM grade glutaraldehyde. The mixture was incubated at room temperature for 4 h till a faint brown colour was developed. The mixture was dialysed against three changes of 500 ml half strength PBS at 8 h interval. After dialysis, Bovine serum albumin was added to a concentration of 5 mg/ml and stored at 4^0C in a refrigerator.

3.12.4. Titre of Antiserum in Enzyme - Linked Immunosorbent Assay (ELISA)

3.12.4.1. Double antibody sandwich (DAS-ELISA)

To determine the optimum concentration of antigen, IgG and homologous conjugate for DAS-ELISA, method of Clark and Adams (1977) was used. The tests were performed as detailed bellow:

1. 200 µl of IgG diluted in carbonate buffer as 1 µg/ml and 2 µg/ml were added to each well of the micro - titre plate (Borosil) and incubate at 37^0C for 2 h.

2. After incubation, contents of plate wells were discarded and washed with PBS - T (flooding with three changes of PBS - T for three min each). After final washing, plates were shaken dry over a paper towel.

3. Test samples were extracted in antigen extraction buffer using pestle and mortar or polytron PT 3000 (KINEMATICA AG.). These preparations were used in two dilutions , 1 : 10 and 1 : 20 . Extract of healthy leaves and the extraction buffer served as negative control whereas extracts from known infected plant and partially purified

virus preparation (1: 100) served as a positive control. 200 µl of aliquots of negative and positive controls with various dilutions were also added and plates incubated overnight at 4^0C.

4. The plates were washed as mentioned in (2) and 200 µl of each dilution of enzyme conjugate from 1 : 500, 1: 1000 and 1 : 2000 was added per well and the plate was incubated at 37^0C for 2 h.

5. After washing as described in (2), 200 µl of substrate (P-nitrophenyl phosphate 0.6 mg/ml in substrate buffer) was added to each well and incubated at room temperature till the development of the yellow colour.

6. Results were recorded by measuring the absorbance of each well at 405 nm in a microplate redder (TECAN A-5082, Sun Rise, Austria).

3.12.4.2. Direct Antigen coating ELISA (DAC - ELISA)

DAC - ELISA was performed using the protocol of Clark and Bar Joseph (1984).

1. The samples were prepared by grinding tissue in PBS buffer containing 2 % PVP (MW 44,000) at 1: 20 (w/v) ratio. The homogenate was centrifuged at 5000 g for 10 min. pellet was discarded and supernatant obtained were used for coating the plate directly. Wells of the ELISA plate were coated with 200 µl and stored at 4^0C.

2. The plates were washed with PBS-T as described for DAS - ELISA.

3. After final washing, the blocking solution was added (200 µl/well) and incubated for 1 hour at 37^0C.

4. The plate was again washed as usual and virus - specific antibodies (IgG) were added 200 µl/well. Plate was incubated at 37^0C for 2 h.

5. Plate was washed after the incubation and enzyme conjugate (anti rabbit IgG - Alkaline phosphatase (Sigma Chemical Co.) was added (200 µl/well) at 1 : 5000 dilution. The plates were incubated at 37 0C for 1 h.

6. After washing 200 µl of the substrate (P - nitrophenyl phosphate, 0.6 mg/ml) was added and allowed for colour development at room temperature.

7. Intensity of colour was measured at 405 nm using a micro plate reader (TECAN A-5082, Sun Rise, Austria).

3.12.5. Immunosorbent electron microscopy (ISEM)

3.12.5.1. Determination of the titre of the antiserum by ISEM

3.12.5.1.1. Trapping

Immunosorbent electron microscopy was used to trap the virus particles with its homologous antiserum. 20 µl of virus specific antiserum in dilutions ranging from 1 : 50 to 1 : 5000 was placed on to the parafilm membrane. Carbon coated grids were then floated over each antiserum dilution in such a way that the film on grids remain down ward. The grids over the antiserum were incubated for 60 min at 37^0C. After incubation, the grids were washed with 10 drops of 0.07 M phosphate buffer (pH 6.5) and floated over a drop of extract from infected leaf tissue on a separate parafilm membrane. These were then incubated for 30 min at room temperature. The grid were finally washed with 10 drops of distilled

water, stained with 2 - 3 drops of 2 % uranyl acetate and examined under electron microscope. For control healthy plant extract was used.

3.12.5.1.2. Decoration

Tissue from symptomatic leaf were removed with blade in a drop of 0.07 M phosphate buffer (pH 6.5) on a glass slide and finally homogenized with a glass rod. The carbon-coated grids were floated over the homogenate for 5 min with support film facing downward. The grids were then washed gently by 10 - 15 drops of phosphate buffer with the help of a pasture pipette. Grids after washing were floated over 20 µl of different dilutions of virus specific antiserum ranging 1: 2 to 1: 32768 for 30 min. Each grid was then washed gently with 10 drops of water. The grids were finally stained with 2-3 drops of uranyl acetate and dried with a piece of whatman filter paper and examined under EM. The electron micrographs were taken with 2.8 sec. exposure on plate film at 20000 magnification.

3.13. Serological relationship

Studies were conducted to determine the serological relationship of the present virus with other filamentous viruses in ISEM tests. Various antisera as detailed below were used for trapping/decoration of the virus particles from known positive sources. Sources of antisera are given below:

S. No.	Antiserum	Source
1.	Citrus yellow vein clearing virus (CYVCV)	ACPV, IARI , New Delhi India
2.	Indian citrus rinspot virus (ICRSV)	ACPV, IARI , New Delhi India
3.	Garlic mosaic virus (GMV)	ACPV , IARI , New Delhi India
4.	Garlic latent virus (GLV)	D.E. Lesemann, Braunschweig, Germany
5.	Shallot latent virus (SLV)	L.Bos, Wageningen, Netherland
6.	Henbane mosaic virus (HBMV)	ACPV , IARI , New Delhi India
7.	Papaya ring spot virus (PRSV)	ACPV , IARI , New Delhi India
8.	Potato virus Y (PVY)	ACPV , IARI , New Delhi India
9.	Potato virus X (PVX)	CPRI, Shimla
10.	Carnation latent virus (CLV)	L.Bos, Wageningen, Netherland

ACPV = Advance Center of Plant Virology, IARI, New Delhi, India
CPRI = Central Potato Research Institute, Shimla

3.14. Coat protein analysis of the virus

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) was followed to estimate the molecular weight of the virus coat protein from purified virus preparation. 12 percent polyacrylamide resolving gel and 5 percent staking gel were used. Following protein standards were used from Gennei's prestained Protein Molecular weight Marker (# SM0441)

Protein	MW, KDa
β-glactisidase	118.0
Bovine serum albumin	79.0
Ovalbumine	47.0
Carbonic anhydrase	33.0
β-lactoglobulin	25.0
Lysozyme	19.5

3.14.1. Electrophoresis

For electrophoresis, Bio - Rad model No. mini Protein 3 cell serial No. 525 BR 018947, made in USA, apparatus was used . The following reagents were prepared and used in electrophoresis.

3.14.1.1. Preparation of polyacrylamide gel

3.14.1.2. Preparation of 12 % resolving gel

10 ml of 12 % resolving gel solution was prepared as noted below:

30 % Acrylamide / bisacrylamide stock	4.0 ml
1.5 M Tris- HCL, PH 8,8	2.5 ml
10 % SDS (sodium dodecyl sulphate)	0.1 ml
10 % Ammonium persulphate	0.1 ml
TEMED (Tetramethyl ethylene diamine)	0.004 ml
Distilled water	3.3 ml

After mixing the above constituents , the resolving gel solution was poured immediately between the two glass plates fixed on the gel casting stand and allowed to polymerize for about 60 min . After polymerization

the top of the gel was rinsed with distilled water and remaining water was soaked with tissue paper.

3.14.1.3. Preparation of 5 % staking gel

5 ml of 5 % staking gel solution was prepared with the following recipes :

30 % Acrylamide/bisacrylamide	0.83 ml
1 M Tris - HCL pH 6.8	0.63 ml
10 % SDS	0.05 ml
10 % Ammonium persulphate	0.05 ml
TEMED	0.005 ml
Distilled water	3.4 ml

The above constituents were mixed and the solution was poured between two glass plates on the top of the polymerized resolving gel . The teflon comb was inserted on the gel sandwich and left for polymerization for 30 min.

3.14.1.4. Preparation of the samples

50 µl of purified virus was mixed with equal volume of sample buffer and heated for 3 min in boiling water and immediately cooled in ice.

3.14.1.5. Sample loading

The comb was removed from the top of slab carefully without disturbing the wells. The tank was filled with desired volume of Tris-glycine, electrode buffer. Twenty Micro liter (20 µl) of denatured sample

and 10 µl of marker were dispensed into each well separately with the help of a micro syringe at the bottom of the well. The electrophoresis was run at 100 volts for 3 h or till tracking dye moves near the bottom of the resolving gel.

3.14.1.6. Staining and destaining of the gel

After electrophoresis was over, the gel was removed carefully from the sandwich and stained with staining and fixation solution (50mg coomassie blue, 45 ml methanol, 10 ml acitic acid and then stir for 20 min and filtrate by using whatman paper and make the volume up to 100 ml with water) and kept over night. The gel was destained with destaining solution (methanol 45 ml, acitic acid 10 ml and distilled water 45 ml) with 2 - 3 changes until the bands were clearly visible. The distance migrated by the protein bands were measured from the edge of the resolving gel to the middle part of the band. The relative mobility (Rf) was calculated by the following formula:

$$Rf = \frac{\text{Distance migrated by a protein}}{\text{Distance migrated by the dye}}$$

For estimating molecular weight of the virus coat protein the molecular weight markers were plotted against distance migrated on a semi log paper to obtain standard curve of molecular weight markers. The molecular weight of the coat protein was determined from its Rf value and also distance migrated using the standard curve.

3.14.1.7. Western blotting:

When SDS - PAGE run is going to be over, cut whatman filter paper No. 3 (4 strips) and nitrocellulose membrane (one strip) of the exact

size of the SDS - PAGE gel and mark left corner of the filter wearing sterilized gloves. Float the filter paper, membrane strip and SDS -PAGE gel on the surface of transfer buffer for 5 min. Assemble " transfer sandwich " under buffer by placing SDS - PAGE gel on the nitrocellulose membrane and sandwiching these between filter paper strips, sponge pads and the sandwich holder. Transfer the " transfer - sandwich " to the blotting apparatus containing transfer buffer with nitrocellulose on the anode side of the gel. Run at a constant voltage (20 V) for 1 -2 h. After electro - blotting for 2 h, disassemble the transfer - sandwich layer one by one and transfer the blot to a small tray containing blocking solution. Leave the blot in blocking solution with gentle shaking for 1h at RT or at 4^0C overnight. Wash the blot three times in TBS for 10 min each at RT. Incubate the blot in primary antibody of the present virus diluted in TBS at 1: 1000 for 1 - 4 h at RT with gentle shaking. Wash the blot in TBS as above. Transfer and incubate the blot in the second antibody conjugated with alkaline phosphatase diluted in TBS 1: 2000 with gentle shaking for 1 - 4 h at RT. Wash the blot in TBS three times as mention earlier. Submerge and incubate the blot in substrate prepared in substrate buffer. Substrate consists of nitro blue tetrazolium (NBT) and bromochloro - indolyl phosphate (BCIP) at 0.33 mg and 0.175 mg per ml of substrate buffer, avoided light. After 20 min, the reaction was stopped by transferring the blot to stop solution before background color becomes significant. Rinse twice in stop solution. Air - dry the membrane and store under folds of bloting paper at RTand Protect from light. The buffers used in this experiment are presented in Appendix -I

3.15. Characterization of the genome of the virus

The virus under investigation though resemble ICRSV in particles morphology, absence of reaction with ICRSV antiserum in ISEM studies clearly indicated that it is a distinct virus. Therefore it was thought desirable to characterize the genomic components of the virus. It was decided to isolate the nucleic acid (NA) and to identify its nature by various nuclease treatments, and find out the molecular weight of the NA by denaturing agarose and polyacrylamide gel electriphoresis.

For all molecular and biological work care was taken to avoid contamination of nucleases by using rubber hand gloves and all operation were done at a low temperature of 4^0C. All tools required for experimentation were treated with diethyl pyrocorbonate (DEPC), and RNase inhibitor, for overnight and then autoclaved. For this purpose, to one liter of sterilized distilled water (SDW), 1 ml DEPC was added under chemical hood and mixed with the help of a glass rod, and kept for overnight. Tools required were dipped into the solution and kept for overnight, after which they were rinsed in SDW and used.

3.15.1. Viral nucleic acid isolation

Flow chart for Extraction of viral nucleic acid

To 500 µl pure virus preparation
↓
Add 10 µl protinase K
↓
Add 5 µl 1 % SDS
↓
Mix gently by hand and keep for 1 h at 370 C
↓
Add 500 µl Phenol: chlorophorm: Isoamylalchohol (25: 24: 1)
↓

Mix gently by hand
↓
Centrifuge at 13,000 rpm for 10 min
↓
Collect upper layer carefully
↓
Add 500-µl chlorophorm: Isoamylalchohol
↓
Mix gently by hand
↓
Centrifuge at 13,000 rpm for 10 min
↓
Collect upper layer carefully
↓
Add 50 µl 3 M sodium acetate (1 / 10V) pH 4.8
↓
Add 1 ml ethanol and mix well (2.5 vol.) and keep over night at - 20^0C
↓
Centrifuge at 13,000 rpm for 15 min
↓
Throw supernatant
↓
Add 500 µl of 70 % alcohol
↓
Centrifuge at 13,000 rpm for 15 min
↓
Throw supernatant
↓
Dry at 37^0C for 5 - 10 min
↓
Suspend the pellet in 50 µl SDW and keep in - 20^0C

3.15.2. Polyacrylamide gel electrophoresis of viral nucleic acid

3.15.2.1. Preparation of polyacrylamide gel

10 ml of 30 % polyacrylamide gel was prepared as detailed in Appendix I.

3.15.2.2. Preparation of the samples

10 µl of purified virus nucleic acid was mixed with 2 µl dye and loaded on gel 4 µl of RNA marker (Ready to Use) mixed with 2 µl dye and boiled at 700 C for 10 min and immediately cooled in ice.

3.15.2.3. Sample loading

The comb was removed from the top of slab carefully without disturbing the wells. The tank was filled with desired volume of 1X TBE, electrode buffer. 12 µl of denatured sample and 6 µl of marker were dispensed into each well separately.The electrophoresis was run at 85 volts for 3 h or till tracking dye moves near the bottom of the gel.

3.15.2.4. Staining and destaining of the gel

After electrophoresis was over, the gel was removed carefully from the sandwich and stained with 50 ml SDW and 5-µl ethediumbromide (10 µg/µl) for 15 min and destained the gel with SDW for 10 min. The bands were visualized under a 2 UVTM transiluminator directly under UV - light.

3.15.3. Agarose gel electrophoresis of viral nucleic acid

3.15.3.1. Preparation of agarose gel

0.4 g agarose was mixed with 40 ml 1X TBE and boiled for 5 - 10 min untill agarose gel disolved and cooled up to 60^0C, added 1 µl ethediumbromide and immediately poured in the casting tray.

3.15.3.2. Preparation of the samples

10 µl of purified virus nucleic acid was mixed with 2 µl dye and 4 µl of RNA marker (ready to use) was mixed with 2 µl dye and boiled at

700 C for 10 min and immediately cooled in ice.

3.15.3.3. Sample loading

The comb was removed carefully without disturbing the wells. The tank was filled with desired volume of 1X TBE, electrode buffer. 12 µl of denatured sample and 6 µl of marker were dispensed into each well separately. The electrophoresis was run at 65 volts for 3 h or till tracking dye moves near the bottom of the gel. After running the gel the bands were visualized under a 2 UVTM transiluminator directly under UV - light.

3.15.4. Electrophoresis of encapsidated nucleic acid in denaturing agarose gel

3.15.4.1. preparation of denaturing agarose gel

1.3 % agarose gel was prepared in 1X MOP buffer, composition of which is given below:

Agarose	1.30 g
SDW	100 ml

Cooled up to 60 %, and added

10 X running buffer	14 ml
Formaldehyde	25 ml

Formaldehyde was added after cooling the agarose and caste the gel. Samples were prepared in sample buffer as given below:

3.15.4.2. To prepare the samples

Viral NA	9 µl
10 X running buffer	4 µl
Formaldehyde	7 µl
Formamide	20 µl

Incubated at 65^0C for 15 min added

5 X RNA dye	2 µl

3.15.4.3. Sample loading

The comb was removed carefully without disturbing the wells. The tank was filled with desired volume of 1X running buffer, electrode buffer. 20 µl of denatured sample and 10 µl of marker were dispensed into each well separately. The electrophoresis was run 100 volts for 3 h or till tracking dye moves near the bottom of the gel. After running the gel was over the bands were visualized under a 2 UVTM transiluminator directly under UV - light.

3.15.4.4. Staining and destaining of the gel

After electrophoresis was over, the gel was stained with 50 ml SDW and 5 µl ethediumbromide for 15 min and destained the gel with SDW for 10 min. The bands were visualized under a 2 UVTM transiluminator directly under UV - light.

3.15.5. Sensitivity to nucleases

In order to ascertain the nature of NA in the final preparation, the NA extract from infected and healthy plants was digested by RNase in

0.01 M NaCl, 0.3 M NaCl and DNase separately and electrophoresed through 6% polyacrylamide gel.

3.15.5.1. RNase treatment

RNase digestion was tested in two different concentration of sodium chloride (Hull, 2001). The reaction mixture of two different digestion are given below :

3.15.5.1.1. RNase digestion in low salt concentration

Viral NA	5 µl
RNase (100 µg/µl)	4 µl
0.01 M NaCl	2 µl
SDW	9 µl

The reaction mix was incubated at 37^0C for 30 min and stopped by adding loading dye and products were analyzed in denaturing PAGE.

3.15.5.1.2. RNase digestion at high salt concentration

Viral NA	5 µl
RNase (100 µg/µl)	4 µl
0.3 M NaCl	6 µl
SDW	5 µl

The reaction mix was incubated at 370C for 30 min and stopped by adding loading dye and products were analyzed in denaturing PAGE.

3.15.5.2. DNase treatment

Viral NA	5 µl
DNase (10µg/µl)	4 µl
SDW	11 µl

The reaction mix was incubated at 370C for 30 min and stopped by adding loading dye and products were analyzed in denaturing PAGE.

For analysis in PAGE, the enzyme treated and untreated NA samples were separately mixed with 2 µl of loading dye and loaded into separate wells of the gel at the rate of 10 µl per well. The gels were run at 85 volt till the bromophenol blue was 1 cm above the bottom of the gel (after about 3.0 h). The bands were visualized under a 2 UVTM transiluminator directly under UV - light.

3.16. Total RNA isolation from infected plant

Beside characterization of encapsidated nucleic acid,it was decided to apply PCR approach to obtain information on the genomic components of the virus under investigation. Total RNA was isolated from infected plant and attempts were made to initiate cDNA synthesis with ICRSV specific primers.

Random primers and oligo dT primers were also used to initiate cDNA synthesis and amplification was attempted with random and ICRSV specific primers. Following are the various PCR protocols.

The total RNA was isolated from infected citrus leaves with the present virus with the help of a following manufacturer's instruction (QIAGEN RNeasy plant mini kit Cat. 74904). Initially 10 mg of tissues were taken for each isolate for isolating the RNA. The powdered samples were immediately soaked with 450 (l lysis buffer (RLT buffer)(containing 3 min in waterbath), vortexed for 10 sec. and incubated at 650C for 3 min in waterbath. Clear lysate was then passed through another spin column and centrifuged at 8,000 g for 2 min in a benchtop centrifuge at room

temperature. The flow through was transferred to a new eppendorf and half volume of 225 µl chilled ethanol (absolute) was added to clear lysate and mixed by pippetting. It was then passed through another spin column (pink column) and centrifuged for 1 min at room temperature at 8000 g. Flow through was discarded and 700 µl of RW 1 buffer was added to the column and centrifuged for 1 min at same speed. Again flow through and collection tubes were discarded. 500 µl RPE buffer was added to the same column and centrifuge for 1 min. The same was repeated and centrifuged for 2 min. Finally, RNA was eluted with 30 µl sterile RNase free water by centrifuging at 8,000 g for 1 min and was collected in an eppendorf. This was used as a template in reverse transcription and polymerase chain reaction (RT-PCR). Sap extracts from non-infected tissues were used as control.

3.16.1. cDNA synthesis with ICRSV primers

The RNA isolated from infected leaves of citrus using Rneasy Kit were used as template to perform the following reaction for cDNA synthesis. The primer used to initiate cDNA synthesis is (51- cac ccg gga att cTT AAG TGT TGA AAG GGG - 31), the complementary sense primer for the C terminal region of CP gene of ICRSV.

Sterile Distilled water	1 µl
10X RT buffer	2 µl
Complementary primer (100 ng/ml)	2 µl
10 mM dNTPs	2 µl
Reverse transcriptase (M-MuLV, 20 units / µl)	1 µl
Template RNA	10 µl
RNase inhibitor	2 µl

Template RNA was incubated at 65^0C for 3-5 min, chilled on ice (3-5min) and other reactives were added to the tube. The tube was chilled on ice and given a quick spin to bring down the contents. For the first strand cDNA synthesis, the reaction mix was incubated at 42^0C for 45 min.

3.16.2. PCR Amplification with ICRSV primers

The following primers were used for PCR amplification:

(51-ccg gga tcc ATG AGC TTT GAC TAC ACA-31 upstream, viral sense, N terminal region of CP gene and 51- cac ccg gga att cTT AAG TGT TGA AAG GGG - 31 downstream, complimentary sense, C terminal region of CP gene. The anticipated size of the PCR product was 500 bp.

For PCR amplification to an aliquot of cDNA the following reactives were added.

SDW	26.50 µl
10X buffer	5.00 µl
2 mM dNTPs	1.00 µl
25 mM $MgCl_2$	2.00 µl
Forward primer (100 ng/µl)	2.00 µl
Reverse primer (100ng/µl)	3.00 µl
2.5 Unit Taq polymerase	0.50 µl
RNA template (cDNA)	10.00 µl
	50.0 µl

The reaction mix was kept in a PCR (Biorad) for amplification of the target molecules using the conditions given below:

94^0C	2 min
94^0C	30 sec, 35 cycles
57^0C	30 sec, 35 cycles
72^0C	1 min, 35 cycles
72^0C	10 min

10 µl of PCR product were mixed with loading dye and subjected to agarose gel electrophoresis.

3.16.3. cDNA synthesis with oligo(dT)18 primers

ICRSV has a poly A tail and so it was decided to use oligo dT primer initiate cDNA synthesis. The RNA isolated from infected leaves of citrus was used as template and cDNA was initiated using RevertAidTM First strand cDNA synthesis Kit. The following were reaction condition for cDNA synthesis:

RNA template	10 µl
Oligo(dt)18 primer (0.5 µg/µl)	1 µl

Mix gently and spin down for 3-5sec. in a microcentrifuge. Incubated the mixture at 70^0C for 5 min, chilled on ice and collect drops by brief centrifugation. Place the tube on ice and add the following components in the indicated order :

5X reaction buffer	4 µl
Ribonuclease inhibitor (20u/µl)	1 µl
10 mM dNTPs mix	2 µl

Mix gently and collect drops by brief centrifugation. Incubate at 37^{0}C for 5 min. Add RevertAid^{TM} M-MuLV reverse transcriptase (200u/µl) 1 µl

Final volume 20 µl

Incubate the mixture at 42^{0}C for 60 min. Stop the reaction by heating at 70^{0}C for 10 min. Chill on ice.

3.16.4. PCR Amplification with random primers

For PCR amplification with random primer to an aliquot of cDNA the following reactives were added.

SDW	29.50 µl
10X buffer	5.00 µl
10 mM dNTPs	1.00 µl
25 mM $MgCl_2$	3.00 µl
Random primer (0.2 µg/µl)	1.00 µl
2.5 Unit Taq polymerase	0.50 µl
RNA template (cDNA)	10.00 µl
	50.0 µl

10 µl of PCR product were mixed with loading dye and subjected to agarose gel electrophoresis.

The reaction mix was kept in a PCR (Biorad) for amplification of the target molecules using the conditions given bellow:

94^{0}C	2 min
94^{0}C	1 min, 30 cycles
37^{0}C	1 min, 30 cycles

72^0C	2 min, 30 cycles
72^0C	10 min

3.16.5. cDNA synthesis with random primers

The RNA isolated from infected leaves of citrus using Rneasy Kit were used as template to perform the following reaction for cDNA synthesis:

RNA template	10 µl
Random hexamer primer	1 µl

Mix gently and spin down for 3-5sec. in a microcentrifuge. Incubated the mixture at 70^0C for 5 min, chilled on ice and collect drops by brief centrifugation. Place the tube on ice and add the following components in the indicated order:

5X reaction buffer	4 µl
Ribonuclease inhibitor (20u/µl)	1 µl
10 mM dNTPs mix	2 µl

Mix gently and collect drops by brief centrifugation. Incubate at 25^0C for 5 min. Add RevertAidTMM-MuLV reverse transcriptase (200u/µl) 1 µl

Final volume 20 µl

Incubate the mixture at 25^0C for 10 min and finally at 42^0C for 60 min. stop the reaction by heating at 70^0C for 10 min. Chill on ice.

3.16.6. PCR Amplification with random primers

For PCR amplification to an aliquot of cDNA the following reactives were added.

SDW	29.50 µl
10X buffer	5.00 µl
10 mM dNTPs	1.00 µl
25 mM $MgCl_2$	3.00 µl
Random primer	1.00 µl
2.5 Unit Taq polymerase	0.50 µl
RNA template (cDNA)	10.00 µl
	50.0 µl

The reaction mix was kept in a PCR (Biorad) for amplification of the target molecules using the conditions given bellow:

94^0C	2 min
94^0C	1 min, 30 cycles
37^0C	1 min, 30 cycles
72^0C	2 min, 30 cycles
72^0C	10 min

10 µl of PCR product were mixed with loading dye and subjected to agarose gel electrophoresis.

RESULTS

4.1. Collection of virus isolates

During surveys, samples from citrus trees showing vein-clearing symptoms (Fig. 1.a, 1.b, 1.c, 1.d) were collected from commercial orchards in different states. The samples thus collected were grafted on healthy seedlings of test plants as mentioned in materials and methods. These isolates were named on the basis of their place of collection as shown in Table 5.

Table 5. Isolates of citrus yellow vein clearing virus collected from various parts of India

S. No.	Place of collection	State	Host
1.	Ludhiana (PAU germplasm collection)	Punjab	etrog citron, sour orange, hill lemon, *C. pectinfera*
2.	Abohar	Punjab	etrog citron
3.	Ahmadabad	Gujarat	lisbon lemon
4.	Anand	Gujarat	lisbon lemon, rangpur lime
5.	Pune	Maharashtra	sour orange

The symptoms induced by graft transmission to inoculated plants by Abohar isolate on five citrus cultivars are summarized in Table 6.

Fig. 1.a. Symptoms of citrus yellow vein clearing disease on etrog citron in an orchard at Abohar (Punjab)

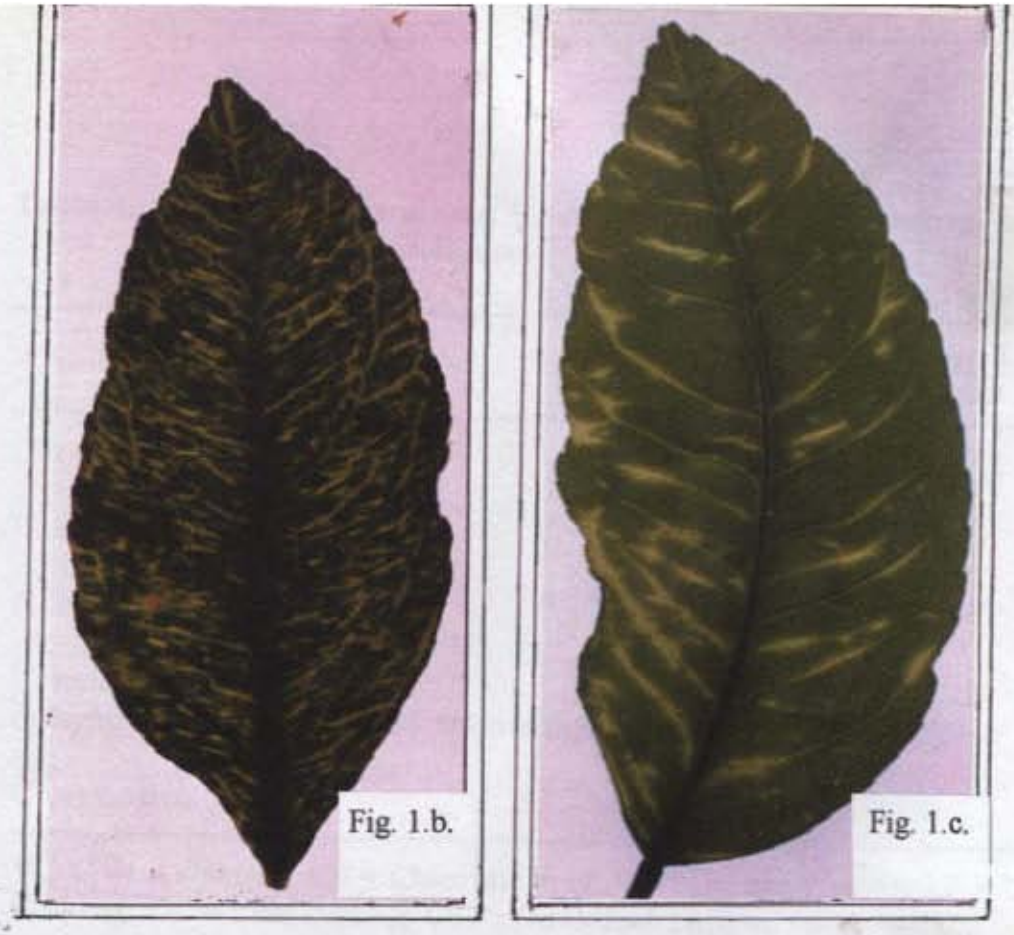


Fig.1.b. Symptoms of the disease of yellow vein clearing on etrog citron

Fig. 1.c. Symptoms of the disease on Lisbon lemon showing vein clearing and vein Banding

Fig. 1.d. Water soaked veins on the lower side of leaf of etrog citron due to yellow vein clearing disease

Table 6. Symptoms induced by Abohar isolates on a set of tests plants in Glasshouse

Test plants	Symptoms developed on inoculated plants
Citrus medica cv. Etrog citron	LF, VC, WS
C. aurantium cv. Sour orange	VC, WS, LF
C. sinensis cv. Mosambi	VC, CS, LF
C. reticulata cv. Kinnow (a hybrid between king and willow mandarins)	VC, CS, M, LF
C. pectinifera	VC,LF,OLP

VC = Vein clearing; CS = Chlorotic spot; WS = Water soaking; LF = Leaf flecking; LC = Leaf curling; OLP = Oak leaf pattern; M = mottling

Table 6 indicated that test plants showed variable symptoms on five citrus cultivars. All the isolates showed vein clearing and leaf flecking symptoms. Etrog citron and sour orange produced water socking along the veins and kinnow mandarin showed mosaic mottling, while pectinifera produced oak leaf pattern. The abohar isolate was selected for further studies.

4.2. Pure culture

4.2.1. Electron microscopy of the virus isolates

The electron microscope examination of symptomatic glasshouse inoculated plants revealed the presence of filamentous particles similar to the one reported by Byadgi and Ahlawat, 1995 for Indian citrus ringspot virus (ICRSV). Therefore,to confirm if the virions of the present virus were of ICRSV, immunosorbent electron microscopy (ISEM) was conducted

using ICRSV antiserum (source, Advanced Center for Plant Virology, IARI, New Delhi). It was found that only about 50 % of the virions were decorated with ICRSV antibodies (Fig 2). This suggested that the virus isolates collected from the field carried ICRSV and another unidentified virus.

4.2.2. Maintenance of pure culture

The Abohar isolate of the virus was mechanically inoculated to five mosambi sweet orange seedlings. After 2 months of inoculation, the test plants showed vein clearing and leaf flecking symptoms. Electron microscopy of leaf dip preparation from such plants showed presence of flexuous particles, which did not react with ICRSV antiserum in ISEM tests. This culture was therefore considered as pure culture of the virus and used in subsequent experiments. The pure culture was multiplied on mosambi seedlings in the glasshouse to avoid any contamination.

4.3. Transmission

4.3.1. Citrus to herbaceous hosts

The results of mechanical inoculation from citrus (mosambi) to test species of herbaceous plants are presented in Table 7.

The results of mechanical inoculation as presented in Table 7 showed successful transmission of the virus was obtained from mosambi to French bean (Phaseolus vulgaris var. singtamy, saxa and gheusemi) and *C. quinoa* but not to cowpea, soybean, cotton and tobacco.

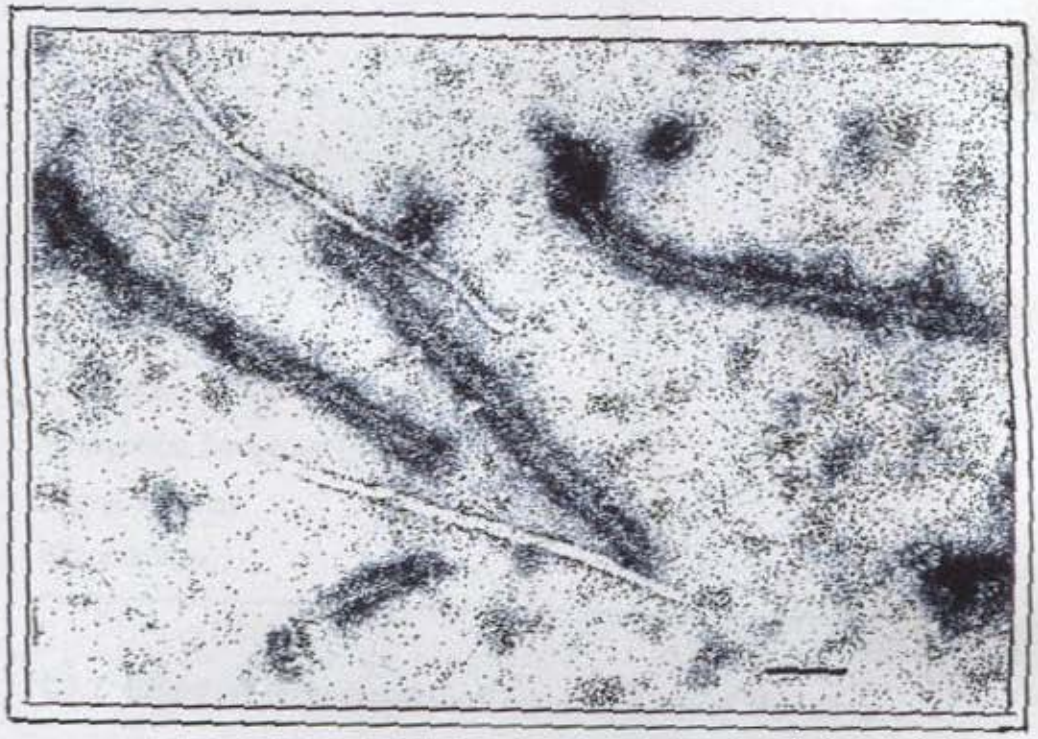


Fig. 2. Analysis of a sample from field affected etrog citron trees using ICRSV antibodies in ISEM showing decorated (ICRSV) and undecorated (virus under study) virus particles.

Table 7. Results of mechanical inoculation from mosambi to herbaceous hosts

Test plant	No. of plant infected /out of 5 inoculated	EM test	Percent transmission
P. vulgaris cvs.			
Singtamy	4	+	80
Saxa	3	+	60
gheusemi	2	+	40
Vigna sinensisa	0	-	0
Nicotiana tabacum	0	-	0
N. glutinos	0	-	0
Chenopodium quinoa	3	+	60
Gossypium hirsutum L.	0	-	0
Vigna sinensis	0	-	0
Glycine max L.	0	-	0

4.3.2. Effect of temperature on transmission of the virus

The experiment was conducted to determine the optimum temperature required for successful transmission of the disease at 90 % relative humidity (RH). The results are given in Table 8.

Table 8. Effect of temperature on transmission of the virus at 90 % RH

S. No.	Temperature (^{0}C)	No. of plants infected out of 5 inoculated	Per cent trans-mission	Symptoms appearance (days)
1.	25	5	100	5
2.	30	5	100	6
3.	35	5	100	7

It is evident from Table 8 that symptoms developed on test plants after 5 - 7 days of inoculation at 25, 30 and 35^{0}C when the RH was 90 %. There was no defference on transmission at the above-mentioned temperatures.

4.3.3. Effect of relative humidity on transmission of the virus

The experiment was conducted to know the relative humidity (RH) required for successful transmission of the disease. The results are presented in Table 9.

Table 9. Effect of RH on transmission of the virus at 30^{0}C

S. No.	RH (70%)	No. of plants infected out of 5 inoculated	Per cent trans-mission	Symptoms appearance (days)
1.	70	5	100	5
2.	80	5	100	5
3.	90	5	100	7

It is evident from Table 9 that symptoms developed on test plants after 5 - 7 days of inoculation at different RH and constant temperature 30^{0}C. So the optimum temperature and RH for the successful transmission of the virus was considered as 30^{0}C and 90 % respectively. However the results were not significant when compared to plants inoculated out of phytotron (glasshouse).

4.4. Graft transmission

The comparative efficacy of various grafting methods for the transmission of the virus is given in (Table 10, Fig. 3, 4, 5). It is evident

Fig. 3

Fig. 4

Fig. 3. Transmission of the virus by petiole grafting in glasshouse.

Fig. 4. Transmission of the by wedge grafting

Fig. 5. Transmission of the virus bud grafting.

Table 10. Efficacy of different methods of graft transmission of the virus

Method of Inoculation	No. of mosambi Plants infected /out of 10 inoculated	Per cent transmission	No. of days for symptoms development
Wedge	10	100	30 - 45
Petiole	8	80	60 - 75
Bud	6	60	90 - 115

from the results that 100 % transmission was obtained by wedge grafting followed by petiole 80 % and by bud grafting 60 %.

4.4.1. Optimum time required for graft transmission

The experiment was conducted to determine the time of scion survival required for successful transmission of the disease to mosambi sweet orange. The results are presented in Table 11.

Table 11. Time required for survival of scion for successful graft transmission

S.No.	Removal of scion after grafting (days)	No. of plants infected out of 5 inoculated	Per cent trans-mission	Symptoms appearance (days)
1.	7	0	0	-
2.	14	0	0	-
3.	21	3	60	20
4.	28	3	60	22
5.	35	3	60	25
6.	42	4	80	23
7.	49	4	80	25
Control	Not removed	5	100	23

Results of Table 11 showed that minimum 21 days are required for transmission of the virus by wedge grafting under glasshouse condition. There was no transmission on plants where scion was removed after 7 and 14 days after grafting. Up to 80 % transmission was observed when scion was removed after 42 days as against 100 % in control where scion was not removed.

4.5. Insect transmission

Insect transmission tests were conducted by three species of aphids one each of whitefly and mealy bug. These insect species failed to transmit the virus (Table 12).

Table 12. Results of transmission tests of the virus by insects

S.No.	Insect species	No. of plants infected out of 5 inoculated	Duration of observations (months)	EM test	ELISA test
1.	*Aphid gossypii* Glover	0	12	-	-
2.	*A. citaicola* Patch	0	12	-	-
3.	*Myzus persicae* Sulz.	0	12	-	-
4.	*Bemisia tabaci* L.	0	12	-	-
5.	*Planococcus citri* Russo.	0	12	-	-

Table 12 revealed that three aphid species *Aphid gossypii* Glover, *A. citaicola* Patch, *Myzus persicae* Sulz. and one whitefly, *Bemisia tabaci* L. and one mealy bug, *Planococcus citri* Russo. failed to transmit the disease. The inoculated plants did not show any symptoms even after 12 months. No virus was detected from inoculated plants when indexed by EM and ELISA.

4.6 Dodder transmission

The virus was not transmitted by dodder, *cuscuta reflexa* as the results shown in Table 13.

Table 13. Results of the dodder transmission experiment

Donor Host	Receptor plants	No. of plants infected out of 3 inoculated	EM test	ELISA test
Mosambi	Mosambi	0	-	-
	Kinnow	0	-	-

The results from Table 13 showed that the test plants did not show any symptoms even after 10 months of inoculation. EM and ELISA results were also negative.

4.7. Seed transmission

The results of Table 14 showed that out of 400 seeds sown 385 were germinated. None of the seedlings developed from these seeds had any disease symptoms up to 2 years and no virus detected in EM in 10 % samples.

Table 14. Results of seed transmission experiment

S.No.	Cultivar	No. of seed germinated out of 100 sown	No. of plants showing symptoms	% trans-mission	EM test
1.	Lemon	97	Nil	Nil	-
2.	Sour orange	94	-	-	-
3.	Trifoliate	98	-	-	-
4.	Pectinifera	96	-	-	-
	Total	**385**	-	-	-

4.8. Host range

4.8.1. Citrus species

The results of host pathogen interaction are given in Table 15. It is evident from the results that all the 16 citrus cultivars inoculated were susceptible to the virus, and showed variable symptoms (Fig. 6, 7, 8, 9, 10, 11).

Among the 13 herbaceous plant species inoculated mechanically only cvs. of *P. vulgaris* and *C. quinoa* were infected (Table 16). However, no symptoms were developed in other plant species and neither the virus was detected in EM. *P. vulgaris* cv. Singtamy and *Chenopodium quinoa* developed local lesions on inoculated leaves but systemic symptoms as severe mosaic, necrosis of vein and blotching were observed on uninoculated leaves of singtamy (Fig 12,13,14,15.a, 15.b). However, the plants of the following species did not develop any disease symptoms nor the virus could be detected in EM tests:

C. amaranticolor Coste and Reyn., *C. murale* L., *Gomphrina globosa* L., *Nicotiana glutinosa* L., *N. tabaccum* L., *Cucumis melo* L., *C. sativa* L., *Capsicum*

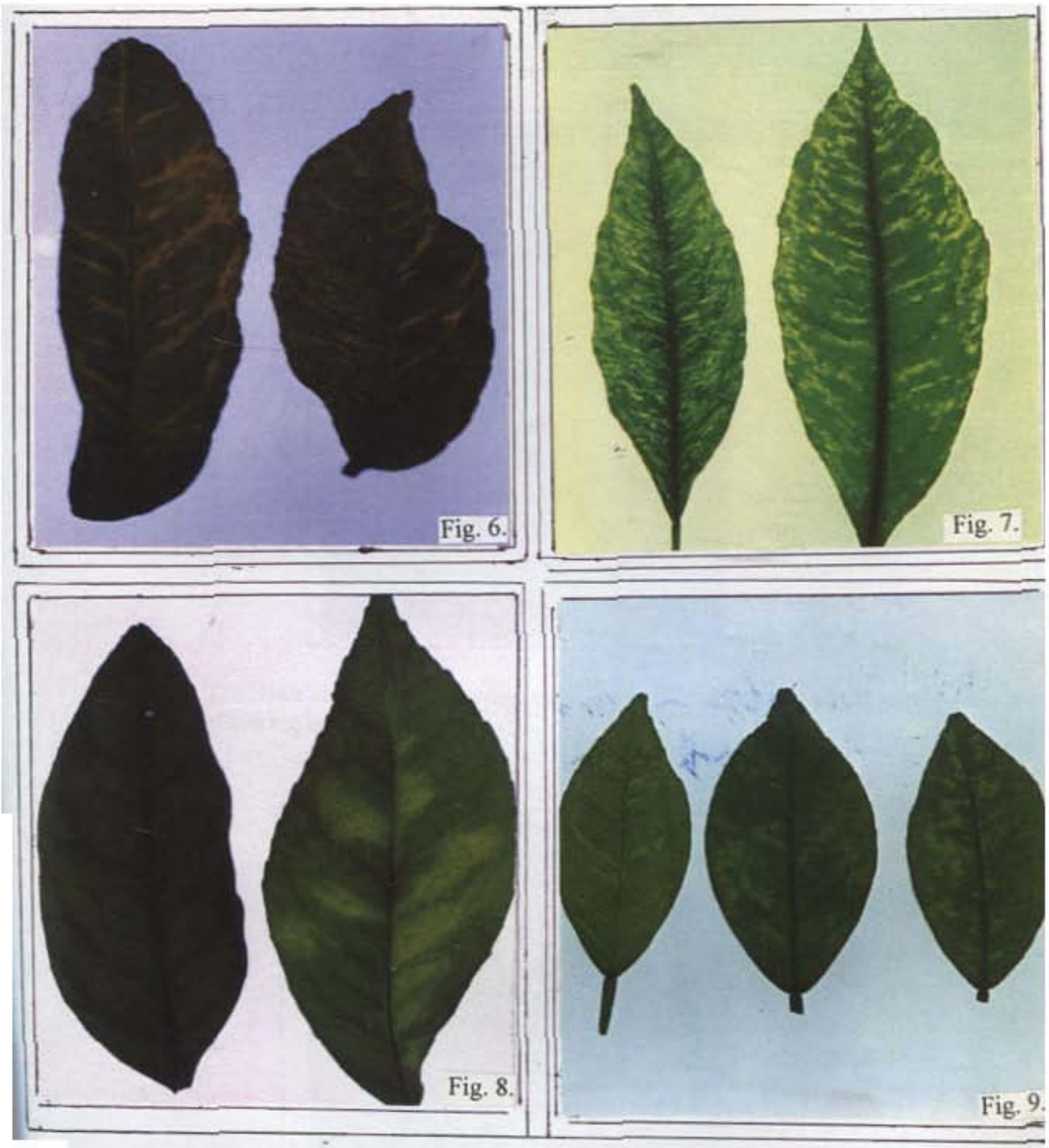


Fig. 6.Vein clearing symptoms on leaves of Lisbon lemon.

Fig. 7. Leaves of etrog citron showing vein clearing and vein banding symptoms

Fig. 8. Kinnow mandarin leaves showing mottling symptoms upon inoculation by the virus

Fig. 9. Pectinfera leaves showing vein clearing, vein flecking and mosaic symptoms upon inoculation by the virus.

Fig. 10. Trifoliate orange leaf showing light chlorotic patches and slight vein *yellowing* symptoms.

Fig.11. Rangpur lime leaves showing chlorosis spot upon inoculation with the virus

Fig.12. *Phaseolus vulgaris* var. gheusami showing veinal necrosis and necrotic spots and yellowing upon inoculation with the virus

Fig. 13. *Phaseolus* .vulgaris var. saxa showing veinal necrosis upon mechanical inoculation with the virus.

Fig. 14. Singtamy leaves showing necrotic local lesions and severe veinal necrosis and blotching symptoms

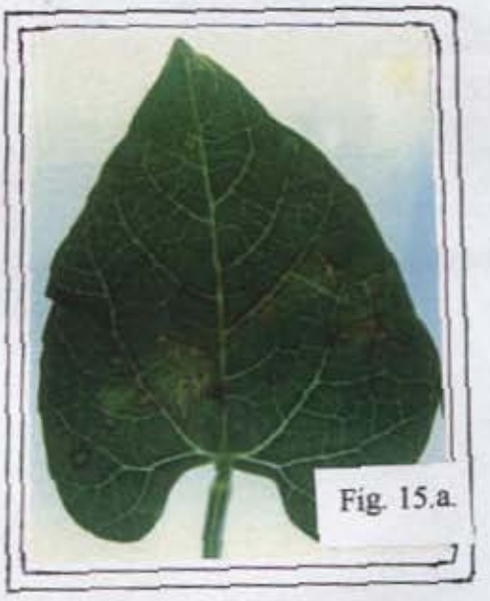


Fig. 15. a. Singtamy leaf showing necrotic local lesions and veinal necrosis symptoms

Fig. 15. b. *Chenopodium quinoa* leaf showing local lesions symptoms upon mechanical inoculation with the virus

annuum L., *Vigna radiata* (L.) Wilczek, *Glycine max* (L.) Merrill., *Vigna sinensis* Savi, *Solanum melongena* L.

Table 15. Host range of the virus on citrus species

Host	No. of plants infected out of 10 inoculated	Symptom
Citrus medica cv. Etrog citron	10	LF, VC, WS
C. aurantium cv. Sour orange	7	VC, WS, LF
C. jambhiri cv. Rough lemon	8	LC, CS
C. sinensis cvs.		
Mosambi	10	VC, CS,
Malta	4	VC, CS,
C. paradisi cv. Grape fruit	4	VC, CS, LC
C. reticulata cvs.		
Kinnow	10	VC, CS, M
Nagpur orange	3	VC, CS
C. limon cv. Galgal	7	VC, WS, LF
C. aurantifolium cvs		
Kagzi lime	5	CS, LC
Kagzi kalan	4	CS, LC
C. grandis Osbck cv. Pummelo	2	CS
C. decumana Murr cv. Decomana	3	CS, LC
C. mitis Blanco cv. Calamandin	2	CS, LC
C. pectinifera	10	VC,LF,OLP
C. karna Rag cv. Karna khatta	2	CS, LC

VC = Vein clearing; CS = Chlorotic spot; WS = Water soaking;
LF = Leaf flecking; LC = Leaf curling; OLP = Oak leaf pattern;
M = mottling.

4.8.2 Herbaceous host

The following herbaceous plant species were mechanically inoculated by sap prepared from *phseolus vulgaris* cv. Singtamy infected leaves. The results summarized in Table 16.

Table 16. Host range of the virus in herbaceous plants

Host	No. of plants infected out of 5 inoculated	Symptom
Citrus Phaseolus vulgaris L. cvs.		
singtamey	5	L, S
saxa 5	S	
gheusemi	3	S
Chenopodium quinoa Willd.	5	L
C. amaranticolor Coste and Reyn.	0	-
C. murale L.	0	-
Gomphrina globosa L.	0	-
Nicotiana glutinosa L.	0	-
N. tabaccum L.	0	-
Cucumis melo L.	0	-
C. sativa L.	0	-
Capsicum annuum L.	0	-
Vigna radiata (L.) Wilczek	0	-
Glycine max (L.) Merrill.	0	-
Vigna sinensis Savi	0	-
Solanum melongena L.	0	-

L = local lesion symptoms
S = Systemic symptoms

4.9. Physical properties

4.9.1. Thermal inactivation point (TIP)

Thermal inactivation point (TIP) was conducted by inoculation *phaseolus volgaris* var. singtiamey after giving various temperatures to the inoculum. The results are presented in Table 17.

Table 17. Determination of TIP for the virus

S. No.	Temperature (^{0}C)	No. of plant infected out of 3 inoculated	Per cent transmission
1.	30	3	100
2.	40	3	100
3.	50	3	100
4.	60	3	100
5.	70	0	0
6.	80	0	0
7.	90	0	0

The results revealed that all the inoculated plants developed symptoms up to 60^{0}C but not at 70^{0}C. Therefore, attempts were made to find out the exact TIP between 60 and 70^{0}C and results are presented in Table 18.

The results of Table 18 showed that up to 60 % transmission was observed at 61^{0}C and no transmission was obtained beyond 61^{0}C. Hence the TIP of the viruswas determined as 61^{0}C.

Table 18. Determination of TIP of the virus

S. No.	Temperature (^{0}C)	No. of plant infected out of 3 inoculated	Per cent transmission
1.	60	5	100
2.	61	3	60
3.	62	0	0
4.	63	0	0
5.	64	0	0
6.	65	0	0

4.9.2. Dilution End point (DEP)

Dilution End point (DEP) was carried out by inoculating the local lesions host (*P. vulgaris* cv. Singtamy) at different dilutions. The results are presented in Table 19.

Table 19. Determination of DEP of the virus

S. No.	Dilution	Average No. of Local lesions per leaf
1.	Standard	87
2.	10^{-1}	23
3.	10^{-2}	7
4.	10^{-3}	0
5.	10^{-4}	0
6.	10^{-5}	0
7.	10^{-6}	0

Results of Table 19 showed that singtamy plants produced 7 local lesions up to dilution of 10^{-2}. The local lesions were 23 at 10^{-1}. However the standard extract produced 87 local. No symptoms were observed at dilution of 10^{-3} and more. Therefore the DEP of the virus was considered as 10^{-2}.

4.9.3. Longevity in vitro (LIV)

To know LIV of the virus test plants of *Phaseolus vulgaris* var. Singtiamey Were inoculated at different time intervals from the inoculum maintained at RT (30^0C). The results are presented in Table 20.

Table 20. Determination of LIV of the virus

S. No.	Time (h)	Average of local lesions
1.	1	11
2.	2	7
3.	3	0
4.	4	0
5.	5	0
6.	6	0
7.	7	0

The result revealed that inocuous sap remains infective at room temperature (30^0C) up to 2 h. No symptoms were observed in plants inoculated after 3 h of sap store. Hence LIV of the virus was considered only 2 h at RT.

4.10. Virus extraction

4.10.1. Efficacy of different buffers on virus extraction

It is evident from the Table 21 and Fig. 16 that out of 5 buffers used for the extraction of the virus preparation in phosphate buffer showed maximum number of virus particles (15.0 particles per 40 μm area of EM grid) and particle loss in the pellet was comparatively less (one particle per 40 μm area). Therefore, phosphate buffer was used in further studies.

Table 21. Efficacy of different buffers for extraction of the virus

S. No.	Buffer	No. of particles in 40 μm* area of EM grid	
		Pellet	Supernatant
1.	Tris-HCl	2.0	6.4
2.	Tris citrate	2.0	6.0
3.	Sodium citrate	2.0	8.0
4.	Sodium borate	2.0	6.8
5.	Sodium phosphate	1.0	15.0

* Average of 5 counts.

4.10.2 Efficacy of molarity of sodium phosphate buffer

The result of Table 22 and Fig 17 showed that phosphate buffer (pH 7.0) at 0.05 M yielded highest number of particles in the supernatant (14.0 particles per 40 μm area) and loss of particles in pellet was also less (1.0 per 40 μm area). Therefore, 0.05 molarity of the phosphate buffer was used in further studies.

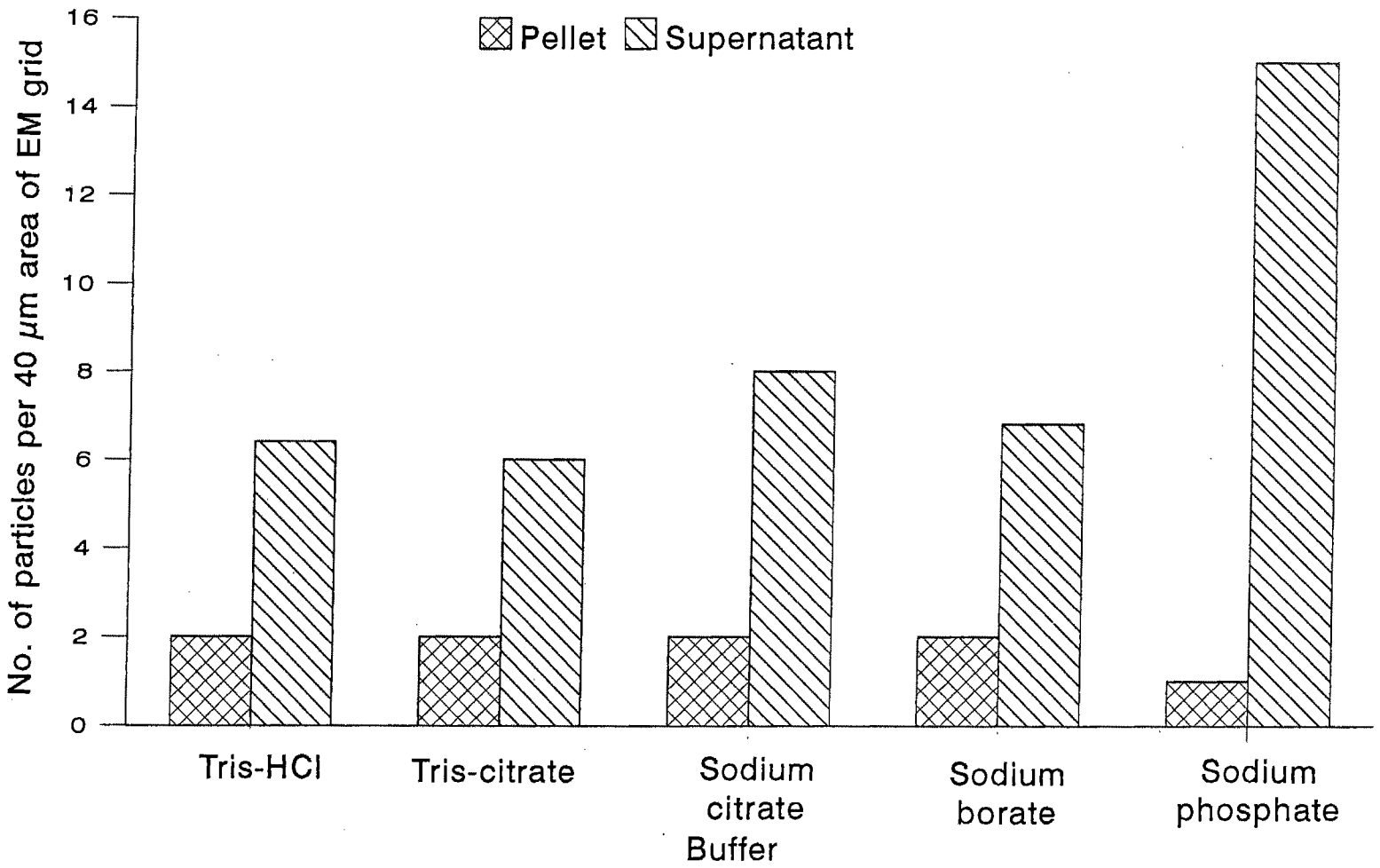


Fig. 16 Efficacy of different buffers for the extraction of the virus

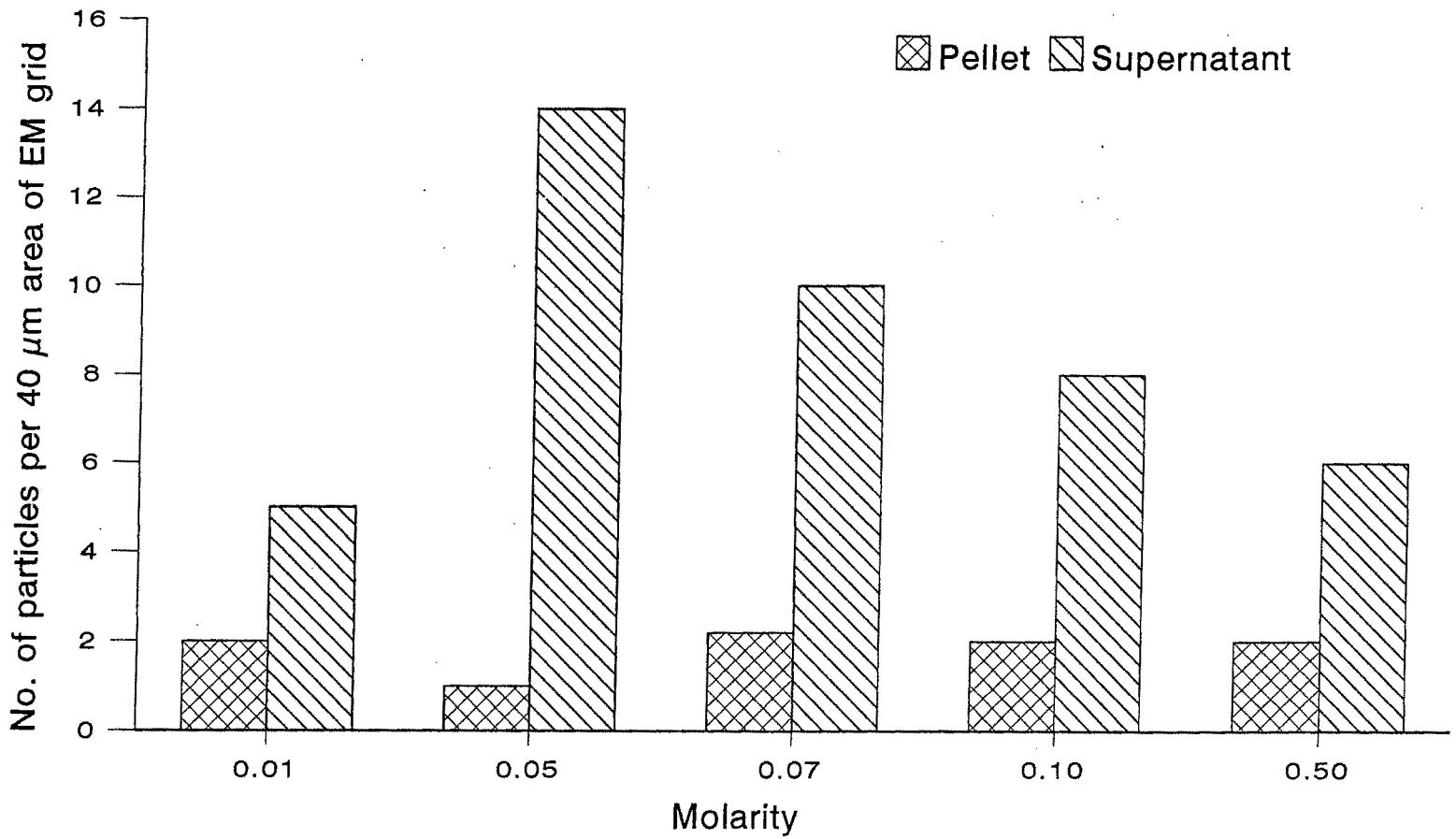


Fig. 17. Efficacy of different molarities of phosphate buffer for extraction of the virus

Table 22. Efficacy of different molarities of phosphate buffer for extraction of the virus

S. No.	Molarity	No. of particles in 40 µm* area of EM grid	
		Pellet	Supernatan
1.	0.01	2.0	5.0
2.	0.05	1.0	14.0
3.	0.07	2.2	10.0
4.	0.10	2.0	8.0
5.	0.50	2.0	6.0

* Average of 5 counts.

4.10.3. Effect of pH of 0.05 M phosphate buffer for the extraction of the virus

Table 23. Efficacy of different pH of phosphate buffer for extraction of the virus

S. No.	pH	No. of particles in 40 µm* area of EM grid	
		Pellet	Supernatan
1.	6.5	2.0	6.8
2.	7.0	2.1	9.0
3.	7.2	1.0	15.0
4.	7.5	2.0	10.4
5.	8.0	2.0	11.0

* Average of 5 counts.

The results of Table 23 and Fig. 18 revealed that maximum number of virus particles were obtained when the extraction of the virus was done at pH 7.2 of 0.05 M, phosphate buffer. The loss of virions in pellet at this pH was comparatively less. Therefore, 0.05 M, phosphate buffer pH 7.2 was used for isolation of the virus.

4.11 Virus purification

Different procedures were used for the purification of the virus and the comparative results of these methods are given in Table 24 and Fig. 19

Table 24. Comparative efficacy of different purification protocols

S. No.	Protocol	No. of particles in 40 µm* area of EM grid	
		Pellet	Supernatan
1.	Byadgi *et al.*, 1993	2.0	20.0
2.	Levy and Gumpf, 1991	2.0	15.0
3.	Bar-Joseph *et al.*, 1985	2.3	15.0
4.	Rustici *et al.*, 2000	1.0	26.0

* Average of 5 counts.

It is clear from Table 24 that the protocol described by Rustici *et al.* (2000) yielded maximum number of virus particle (26 particles) per 40-µm area of the EM grid. The number of virus particles isolated by this method were least aggregated and free from the host contaminates as compared to others protocols.

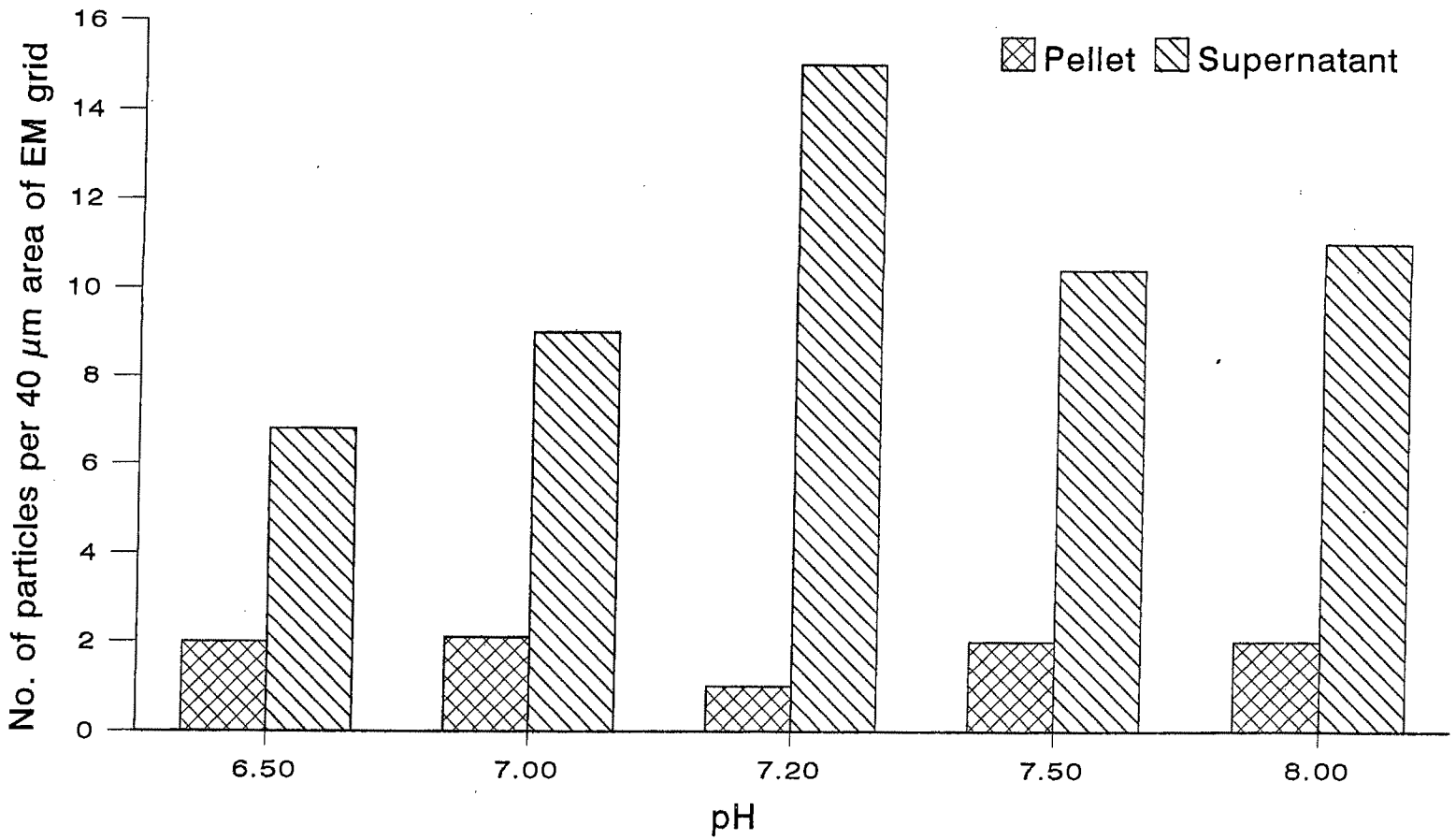


Fig. 18. Efficacy of different pH of phosphate buffer for extraction of the virus

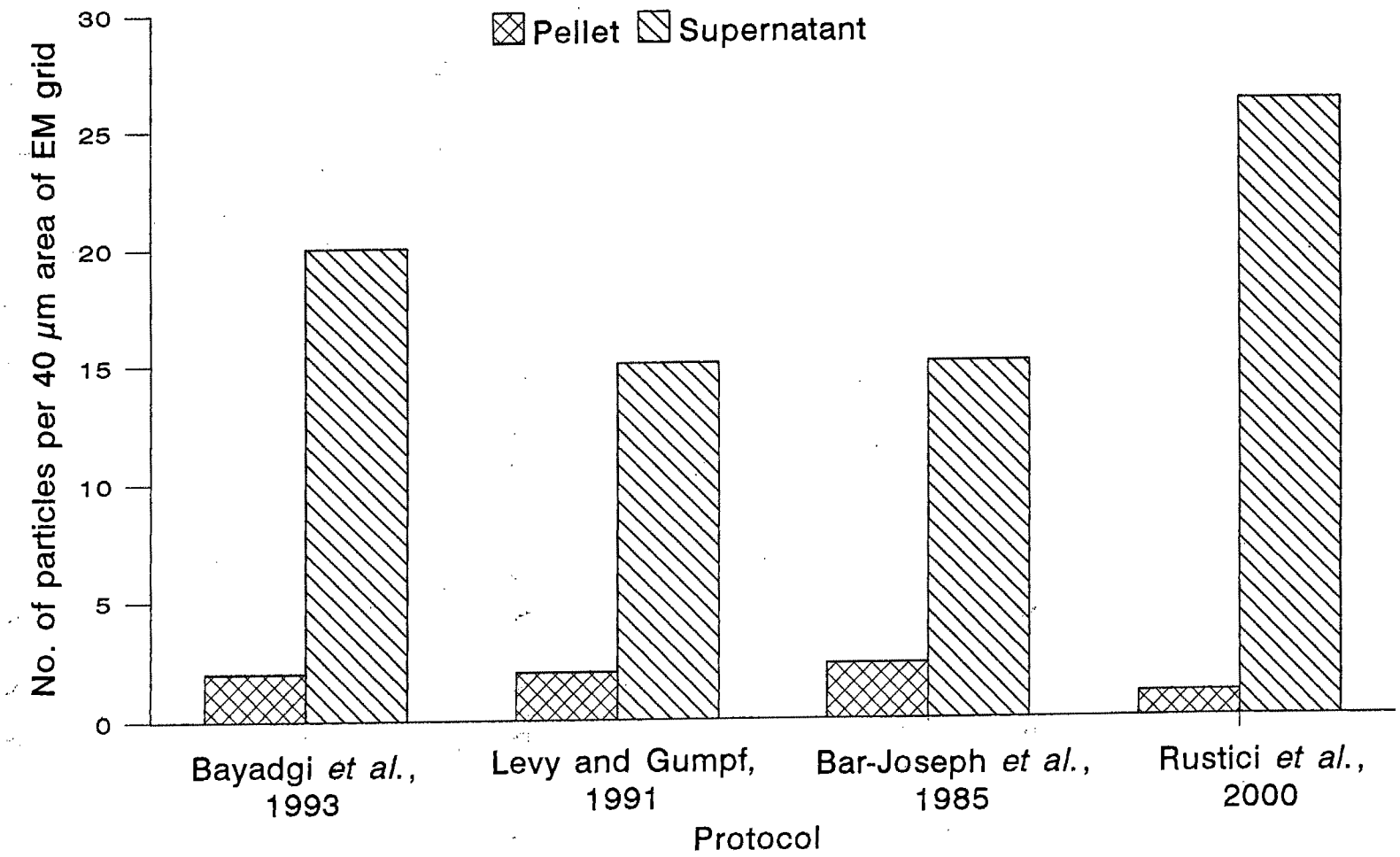


Fig. 19. Comparative efficacy of different purification protocols of the virus

4.11.1. Final purification protocol

The final protocol developed with various experimentations as described earlier is given in a flow chart below:

Flow chart of standardized virus purification protocol

Discard material on this side	1.	Grind 30 g symptomatic leaves of *phaseolus vulgaris* cv. Singtamy collected 9 - 10 days after mechanical inoculation in 10 vol. (w/v) of 0.05 *M* phosphate buffer, pH 7.2 + 5 mM DIECA + 10 mM EDTA + 20 mM sodium sulphate
Debris	2.	Filter through cheese cloth
	3.	Add 10 % (v/v) chloroform and stir for 10 min at room temperature
	4.	Centrifuge at 12,000 rpm for 10 min.
Pellet	5.	Centrifuge supernatant at 45,000 rpm for 2 h.
Supernatant	6.	Pellet dissolved in phosphate buffer 0.05 M, pH 7.2
	7.	Centrifuge at 10,000 rpm for 5 min.
Pellet	8.	Layer supernatant on 10-40 % cesium sulphate density gradient and centrifuge at 38,000 rpm for 3 h.
	9.	Collect virus band through syringe and centrifuge at 70,000 rpm for 30 min.
Supernatant	10.	Pellet dissolved in phosphate buffer 0.05 M, pH 7.2.
	11.	Dialysed in phosphate buffer with three 6 hourly changes.
	12.	Purified preparation, > 100 flexuous virus particles per 40 μm area of EM grid apparently free from host organelles.

4.11.2. UV absorbance profile of purified virus preparation

The purified preparation was diluted 1 : 16 in 0.05 *M* phosphate buffer pH 7.2 for recording UV absorption at 260 nm and 280 nm. The OD value at 260 nm was 0.6442 and at 280 nm was 0.5735 the ratio of $A_{260/A280}$ was 1.123. The results suggested that the preparation in question, was a nucleoprotein.

4.11.3. Electron microscopy

The purified preparations were examined in JEOL - 100 CX - 11 transmission electron microscope. Filamentous virus particles in large number were observed in electron microscope. The model length of these particles was 685 nm in length and 14 nm in width (Fig 20).

4.12. Serology

4.12.1. Production of antiserum

The antiserum of the virus was prepared in albino rabbit as mention in materials and methods. The serological studies were conducted with the stored antiserum.

4.12.2. Purification of immunoglobulin (IgG)

The IgG from antiserum were purified by saturated ammonium sulphate precipitation method and later passing through DEAE sephacel column as described in materials and methods.

All the fractions collected through cellulose column were scanned through spectrophotometer at 280 nm. The absorbance of each fraction has been shown in Table 21 and Fig. 21.

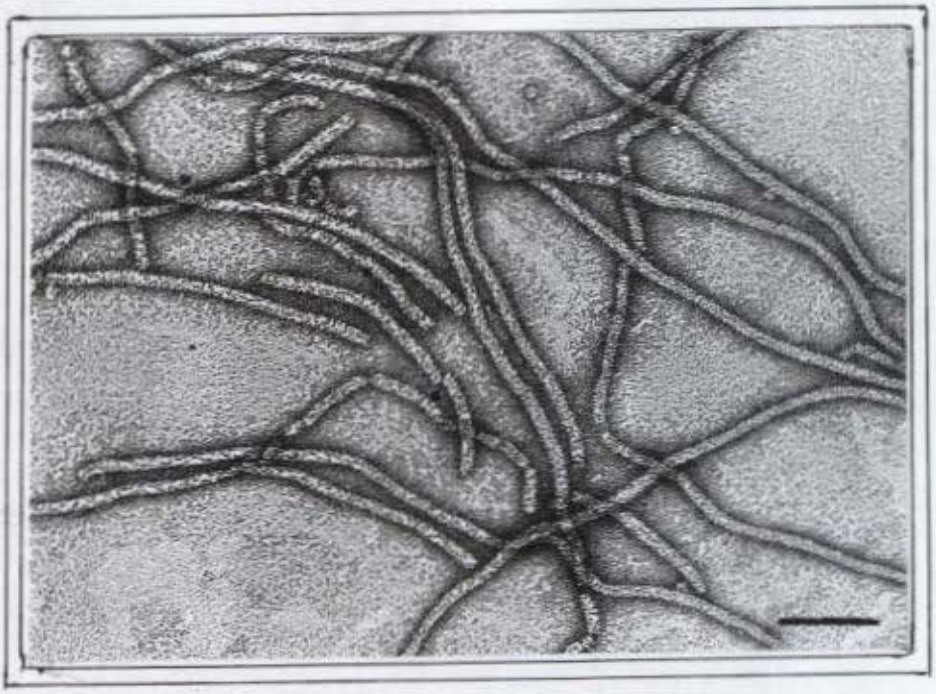


Fig. 20. Electron micrograph of purified preparation of the virus under study showing filamentous particles. Mag. 135,000; Bar = 100 nm

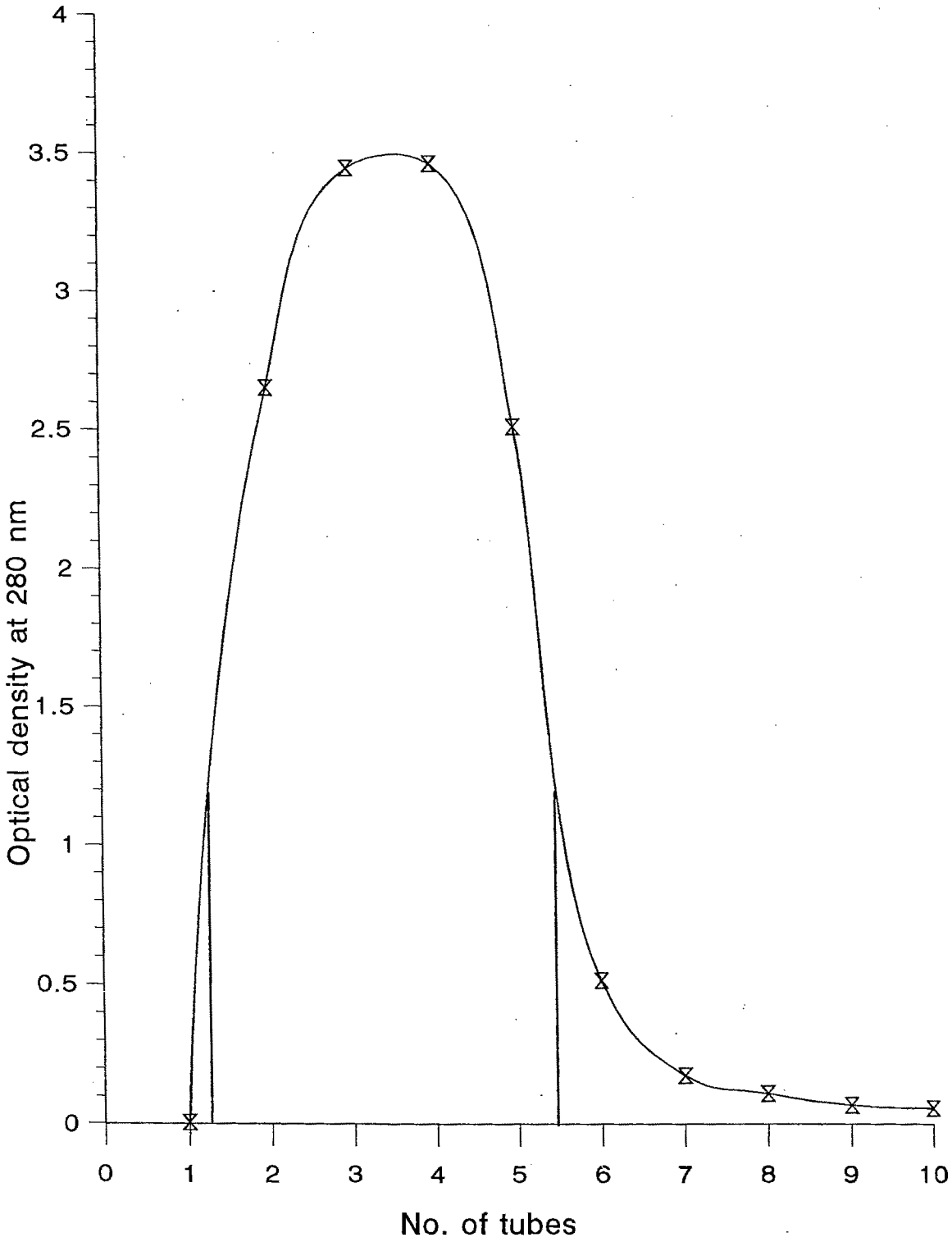


Fig. 21. Elution curve of IgG by DEAE-cellulose column

Table 25. Optical density of various fractions of antiserum at 280 nm

Fraction No.	Optical density
1.	-0.0051
2.	2.6543
3.	3.4498
4.	3.4667
5.	2.5169
6.	0.5169
7.	0.1750
8.	0.1098
9.	0.0681
10.	0.0580

As evident from Table 25, four fraction from 2 to 5 showed OD values over 2.5 or more. These four fractions were pooled and the OD was adjusted to 1.4 by diluting with half strength PBS buffer to get a concentration of 1 mg/ml.

4.12.3. Preparation of antibody-enzyme conjugate for Enzyme-Linked Immunosorbent Assay (ELISA)

The conjugate from homologous antiserum was prepared with alkaline phosphatase by the method of Clark and Adam (1977) as detailed in materials and methods. The homologous conjugate was used for the detection of the virus in double antibody sandwich ELISA (DAS-ELISA).

4.12.4 Double Antibody Sandwich (DAS-ELISA)

The IgG concentration on the performance of DAS-ELISA was evaluated. The plates were coated with IgG concentration of 1 and 2 µg/

ml, the conjugate was used at a dilution of 1: 500, 1 : 1000, 1 : 2000. The antigen dilution were used as 1: 10 and 1 : 20. OD value at 405 nm were recorded after 30 min of addition of the substrate. The results are given in Table 26.

Table 26. Optimum concentration of coating and conjugate antibodies and antigen for detection of the virus in DAS-ELISA

Treatment	Antigen dilution	Mean absorption					
		Coating antibody concentration					
		1 µg/ml			2 µg/ml		
		Conjugate antibody dilution					
		1:500	1:1000	**1:2000**	1:500	1:1000	1:2000
Buffer control		0.22	0.20	**0.19**	0.33	0.25	0.20
Healthy	1:10	0.41	0.35	0.29	0.30	0.32	0.30
Control	**1:20**	0.25	0.30	**0.28**	0.21	0.31	0.29
Diseased	1:10	0.60	0.56	0.69	0.55	0.56	0.50
Control	**1:20**	0.56	0.54	**0.71**	0.50	0.52	0.52

The results of DAS-ELISA indicated that the virus was successfully detected in DAS-ELISA with its homologous conjugate. The results clearly showed that antigen dilution upto 1 : 20, conjugate dilution upto 1 : 2000 and coating antibody 1 µg/ml can successfully detected the virus (Fig. 22).

4.12.5. Direct Antigen Coating ELISA (DAC - ELISA)

DAC-ELISA was performed using virus-specific antibodies (IgG) at various dilutions as first antibody. The conjugate (Goat antirabbit-alkaline

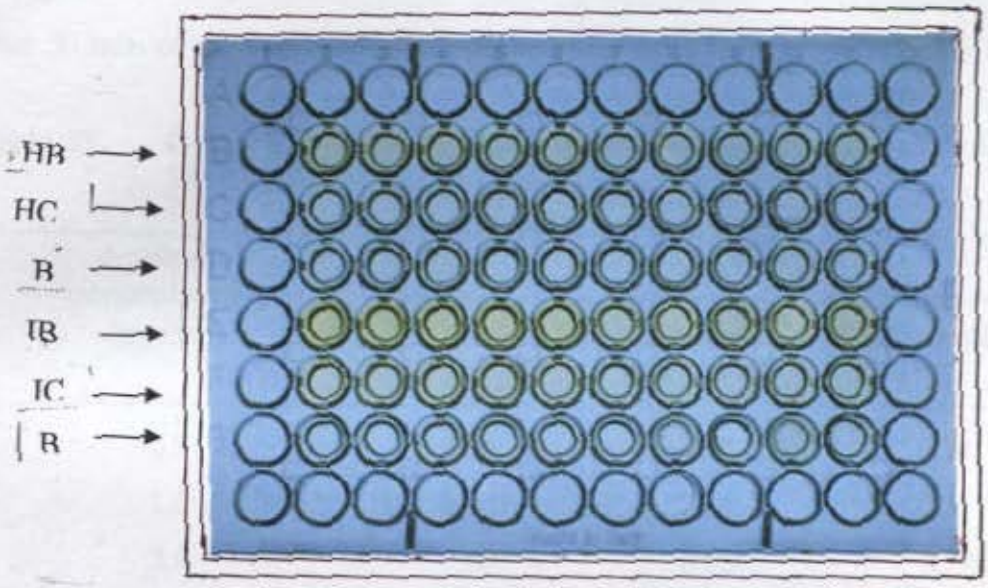


Fig. 22. Photograph of an ELISA plate showing results (yellow colour)of virus detection in DAS – ELISA

HB- Healthy Bean
HC- Healthy Citrus
B- Buffer
IB- Infected Bean
IC- Infected Citrus
B- Buffer

phosphatase, SIGMA Chemical Co.) was used at a constant dilution of 1 : 5000. The antiserum (IgG) was cross absorbed with healthy leaf extract at dilution of 1:50. Healthy and diseased extract were used at 1: 20 dilution alongwith PBS as control. The absorbance value at 405 nm were recorded after 30 min of the addition of substrate. The result are given in Table 27.

Table 27. Optimum antibody dilution for the performance of DAC-ELISA for detection of the virus

Antibody concentration (µg/ml)	Absorbance (405 nm)		
	Buffer	Healthy 1: 20	Diseased 1: 20
0.5	0.00	**0.05**	**0.18**
1.0	0.00	0.06	0.17
2.0	0.00	0.05	0.22

It is evident from Table 27, that the virus can be detected in DAC-ELISA even at a very low concentration of virus specific antibody i.e. 0.5 µg/ml.

The DAC-ELISA and DAS-ELISA were further used to detect the virus in different citrus species maintained in the glasshouse and also from field trees. The results are given in Table 28 and Table 29.

It would be seen from the Table 28 that the virus was detected only in two lemon samples although these trees did not show visible symptoms in the field. The virus was not found in other citrus species / cultivars samples from the field.

Table 28. Detection of the virus from different species of citrus trees in the field by DAS-ELISA

S.No.	Samples	Absorbance (405 nm)	EM test
1.	Buffer	0.14	-
2.	Healthy (negative control)	0.20	-
3.	Positive control	0.80	+
4.	Mosambi sweet orange	0.10	-
5.	Kinnow mandarin	0.13	-
6.	kinnow mandarin	0.11	-
7.	Lemon	0.41	+
8.	Lemon	0.38	+
9.	Pumelo	0.13	-

The results of Table 29 showed that the virus could be successfully detected from different citrus species where the disease was established by transmission tests.

4.12.6. Determination titre of antiserum by immunosorbent electron microscopy (ISEM) for detection of the virus by trapping

Immunosorbent electron microscopy (ISEM) was used for specific detection of the virus by trapping. The dilution of antiserum were used from 1:500 to 1: 10000. The results are given in Table 30.

Table 29. Detection of the virus from different infected citrus species by DAC-ELISA from the glasshouse

S. No.	Treatment	Absorbance
1.	Buffer	0.27
2.	Healthy (negative control)	0.56
3.	Positive control	1.54
4.	Kinnow mandarin	1.50
5.	Marsh grape fruit	0.79
6.	Mosambi	1.54
7.	King mandarin	2.03
8.	Rough lemon	0.73
9.	Rangpur lime	0.72
10.	Sadafal	1.59
11.	Citrumelo	0.62
12.	Kagzi Lime	0.88
13.	Lemon	1.18
14.	Sour orange	0.93
15.	Decumana	0.73
16.	Trifoliate orange	2.03
17.	Pectinifera	1.47

Table 30. Trapping of virus particles in ISEM using various dilution of homologous antiserum

Antiserum dilution	Particles in 40 µm area of EM grid*
1: 500	36
1: 1000	21
1: 2000	15
1: 4000	12
1: 5000	08
1: 10000	-
Control (Healthy)	-
Leaf dip preparation (+ tive control)	05

Table 30 showed that virus particles were trapped up to 1: 5000 dilutions. But the maximum trapping was obtained with a dilution of 1: 500 where virus particles were trapped more than 7 times as compared to normal leaf dip preparation.

4.12.7. Determination titre of antiserum by immunosorbent electron microscopy (ISEM) for detection of the virus by decoration

Immunosorbent electron microscopy (ISEM) was used for specific detection of the virus by decoration. The dilution of antiserum were used from 1:2 to 1/32768. The results are given in Table 31.

It is clear from Table 31 that virus particles were decorated with a dilution up to 1: 16384 of the antiserum. Heavy decoration was observed up to a dilution of 1: 8192 (Fig 23). No decoration was observed when antiserum was diluted to 1:32768.

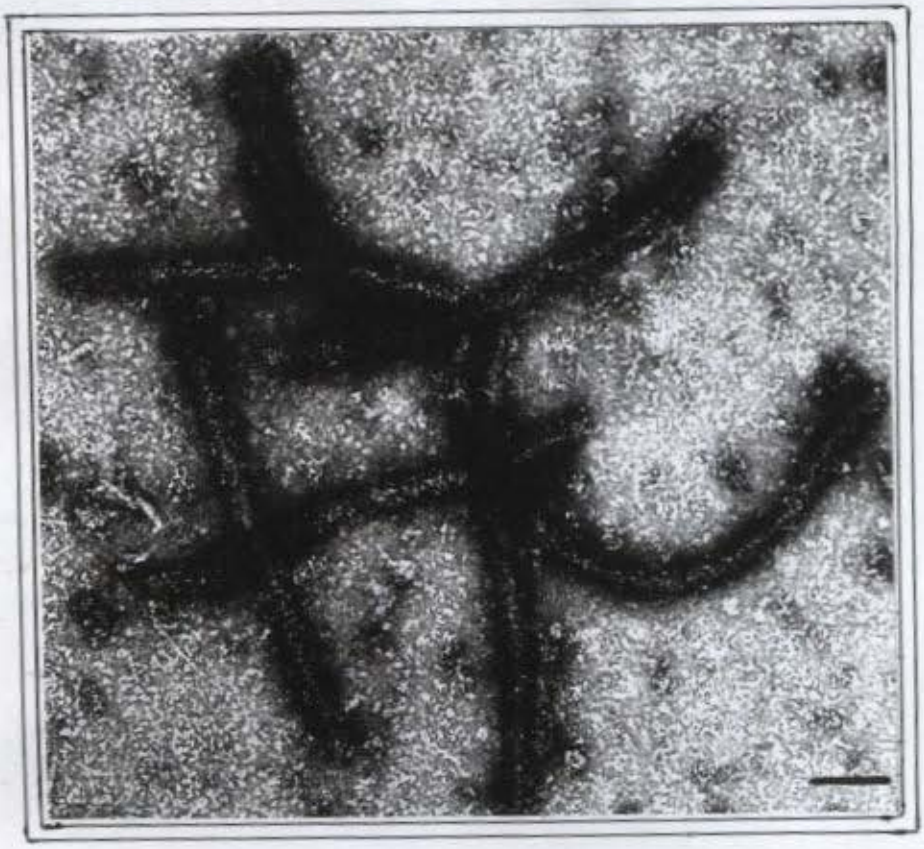


Fig. 23. Electron micrograph of the virus decorated with homologous antiserum
Mag. 108,000, Bar = 100 nm

Table 31. Determination of the titre of the antiserum for decoration of virions in ISEM

Antiserum dilution	Decoration
1/2	+++
1/4	+++
1/8	+++
1/16	+++
1/32	+++
1/64	+++
1/128	+++
1/256	+++
1/512	+++
1/1024	+++
1/2048	+++
1/4096	+++
1/8192	+++
1/16384	++
1/32768	-

+++ = Heavy; ++ = Intermediate ; - = No decoration

4.13. Serological relationship of the virus

To ascertain the serological relationship of the virus with other filamentous viruses, antisera of 9 viruses belonging to 4 groups of viruses were obtained and tested in ISEM tests. The results are presented in Table 32.

Table 32. Serological relationship of the virus with other filamentous viruses

Group	Antiserum	Source	Result in ISEM
Ungrouped	Citrus yellow vein clearing virus (CYVV)	India	+++
Poty	Henbane mosaic virus (HMV)	India	-
Poty	Papaya ringspot virus (PRSV)	India	-
Poty	Potato virus Y (PVY)	India	-
Ungrouped	Indian ringspot virus (ICRSV)	India	-
Potex	Potato virus X (PVX)	India	-
Carla	Garlic latent virus (GLV)	Germany	-
Carla	Shallot latent virus (SLV)	Netherland	-
Carla	Carnation latent virus (CLV)	Netherland	-
Carla	Garlic mosaic virus (GMV)	India	-

The results indicated that none of the nine antisera reacted with the present virus in ISEM. However the virus reacted only with its homologous antiserum.

4.13.1 Determination of molecular weight of coat protein

The purified preparation of the present virus was denatured and electrophoresed on 12% polyacrylamide gel with molecular weight markers as detailed in materials and methods. The results are given in Table 33, Fig (24, 25).

Table 33. Distance migrating of each protein band and tracking dye

Sample	Distance migration (mm)	Relative mobility (Rf)	Molecular weight (KDa)
Purified virus preparation	36	0.51	32.0
Protein markers			
1. β-glactosidase	8	0.11	118.0
2. Bovine serum albumin	13	0.19	79.0
3. Ovalbumin	22	0.31	47.0
4. Carbonic anhydrase	35	0.50	33.0
5. β-lactoglobulin	45	0.64	25.0
6. Lysozyme	58	0.83	19.5

One band related to the coat protein of the virus in quistion was visualised as evident from (Fig.24). The electrophoretic mobility (Rf) of the band was 0.51 (Fig. 25). The estimated molecular weight based on their Rf values was calculated as 32 KDa.

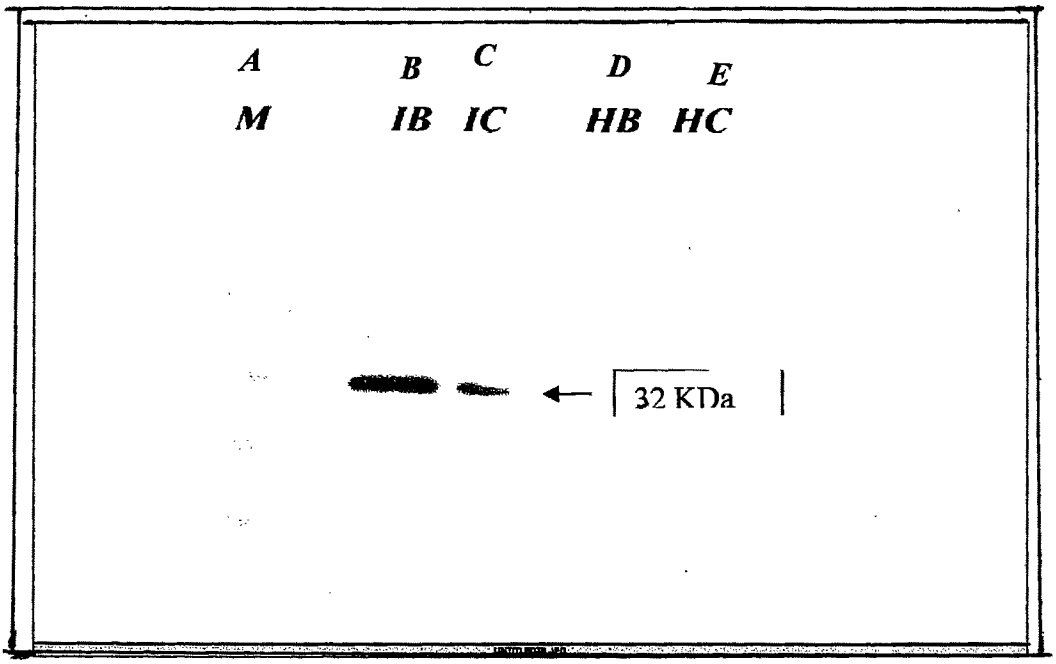


Fig. 24. Electrophoresis of purified preparation of the virus on 12 % polyacrylamide gel

Lane A: Molecular weight markers

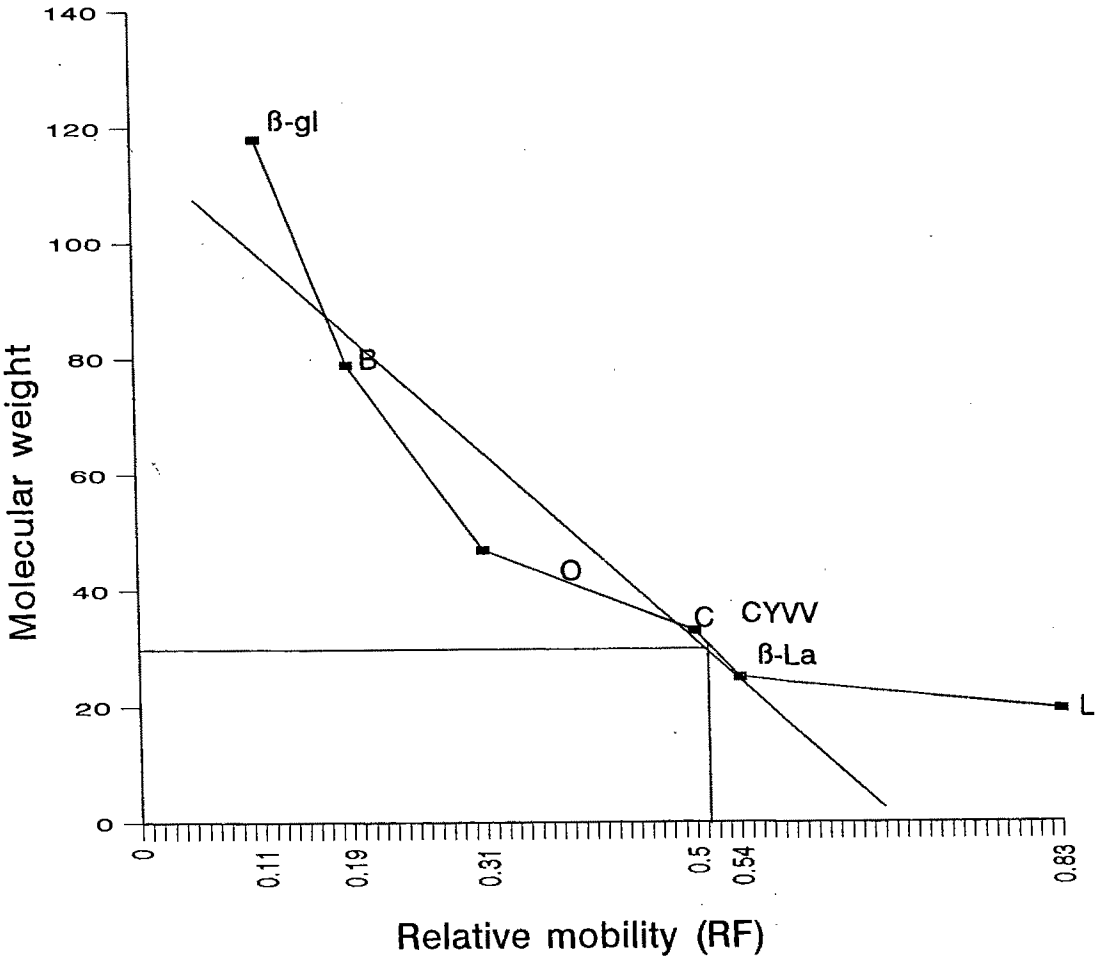
1. β- glactosidase	118.0 KDa
2. Bovine serum albumin	79.0 KDa
3. Ovalbumin	47.0 KDA
4. Carbonic anhydrase	33.0 KDa
5. β – lactoglobulin	25.0 KDa
6. Lysozyme	19.5 KDa

Lane B: One band of 32 KDa observed after electrophoresis of the virus coat protein in infected bean

Lane C: One band of 32 KDa observed after electrophoresis of the virus coat protein in infected citrus

Lane D: No band was observed after electrophoresis of the virus coat protein healthy bean

Lane E: No band was observed after electrophoresis of the virus coat protein healthy citrus



Marker protein : ß-glactosidase (ß-gl); Bovine serum albumin (B); Ovalbumin (O); Carbonic anhydrase (C); ß-lactoglobulin (ß-La); Lysozyme (L)
Citrus yellow vein clearing virus (CYVV)

Fig. 25. Determination of molecular weight of the virus coat protein

4.14. Characterization of the genome of the virus

4.14.1. Viral nucleic acid isolation

Viral nucleic acid was isolated from partially purified particles by proteinase/SDS method. Agarose gel electrophoresis (non-denaturing) of viral nucleic acid showed clearly two bands, one, on par with 1.9 Kb, another on par with 3.5 Kb bands of RNA MW marker (Fig 26).

In order to ascertain exact size of the molecule, 5-µl aliquot of viral nucleic acid was electrophoresed in formaldehyde agarose gel (denaturing). In denaturing agarose gel totally four bands were visible. One on par with 3.8 Kb band of the marker, two seen on par with 0.8 Kb and 0.7 Kb band of the RNA markers. There was another faster moving band ~ 550 Kb length. In both denaturing and non-denaturing agarose gel, no bands were seen with preparation from healthy plants. The viral nucleic acid pattern was also resolved in denaturing 6% polyacrylamide gel electrophoresis. The ethedium-bromide stained PAGE showed separation of viral nucleic acid into 13 bands in non - denaturing acrylamide gel electrophoresis (Fig 27). No such bands were seen in preparation from uninoculated plants. The approximate length of the viral nucleic acid as judged by visual observation in comparison with RNA marker is shown in Fig 27 and Table 34.

In denaturing PAGE, several bands were seen. However, they are not of uniform intensity. The bands on par with 3.3 Kb, 3.2 Kb, 2.8 Kb and 1.9 Kb are bright and visible. Other bands were not that intense indicating the low concentration. Size of the fragments given were approximatly by calculated by comparing the distance between the bands on par with the

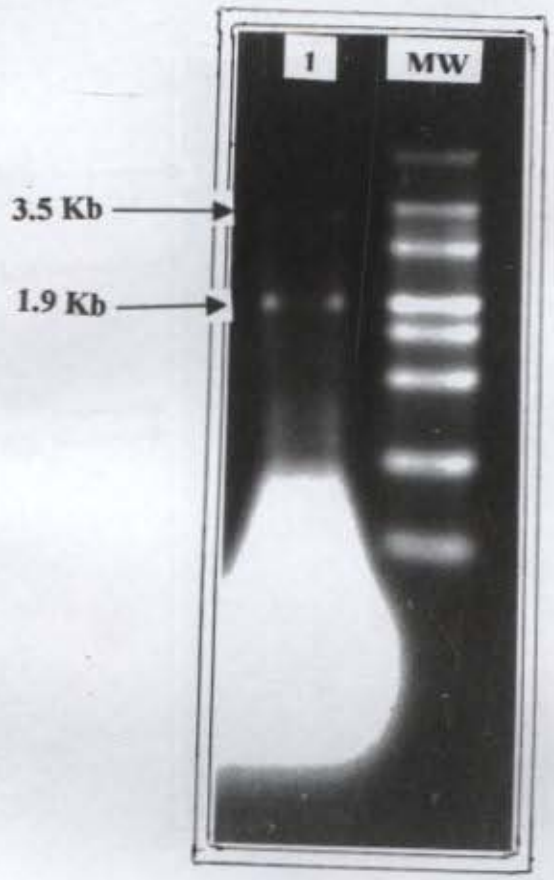


Fig. 26. Non – denaturing agarose gel electrophoresis of viral nucleic acid

Lane MW – RNA molecular weight marker

Lane 1 – Viral nucleic acid isolated from virions

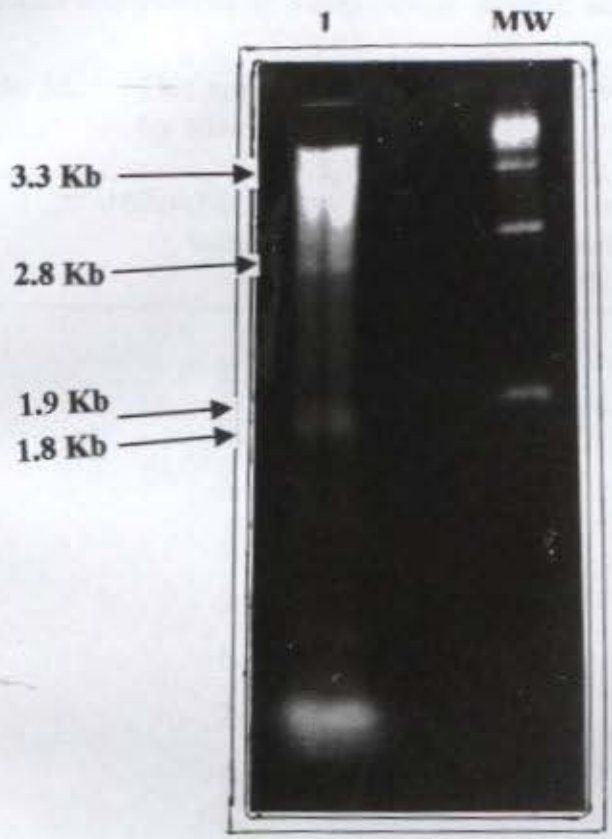


Fig. 27. Denaturing polyacrylamide gel electrophoresis of viral nucleic acid isolated from virion

Lane MW – RNA molecular weight marker

Lane 1 – Viral nucleic acid isolated from virion

marker. It is concluded that the intense bands represent genomic nucleic acid, and the bands on par with 2.6, 2.4, 1.6, 1.4, 1.0 and 0.8 Kb represent different derivatives of subgenomic nucleic acid.

Table 34. The approximate length of the viral nucleic acid as judged by visual observation

Number of the bands	Approximate length of the nucleic acid band, on par with RNA marker (Kb)
1.	3.3
2.	2.9
3.	2.8
4.	2.6
5.	2.4
6.	2.3
7.	1.9
8.	1.8
9.	1.2
10.	1.0
11.	0.8
12.	0.7
13.	0.5

In RNase and DNase treatment, due to extreme low concentration of viral nucleic acid, it was difficult to locate all the bands distinctly (Table 35). The approximate size of the fragment was assessed in comparison with RNA MW marker. In DNase treatment, the nucleic acid band of the virus were retained, and occupied the same position as in the non - denaturing PAGE. Viral RNA disappeared when treated with RNase both

in low salt (0.01 M NaCl) and in high salt buffer (0.3 M) condition, indicating that the nucleic acid is a single stranded RNA molecule (Fig. 28). The separation in to several bands was very clear, not showing sign of any degradation or shearing. Therefore, it is presumed that the viral RNA segment obtained are not products of any degradation or shearing, but representative of encapsidated forms.

Table 35. The approximate length of the viral nucleic acid as judged by visual observation with RNase and DNase treatment

Number of the bands	Approximate length of the nucleic acid band, on par with RNA marker (Kb) in treatment with RNase	Approximate length of the nucleic acid band, on par with RNA marker (Kb) in treatment with DNase
1.	-	2.8
2.	-	1.9

4.15. PCR amplification with total RNA isolated frominfected plant

Agarose gel electrophoresis of PCR products showed that, no characteristic amplification products were seen in RNA isolated from infected plants with ICRSV specific primers. No product was also seen with cDNA initiated with oligo dT or random primers. At the same PCR condition, a clear PCR product of 500 bp was observed with RNA isolated from CRSV infected samples. Absence of any amplification with CRSV specific primers clearly indicated that the viral under study to is a distinct one and different from ICRSV.

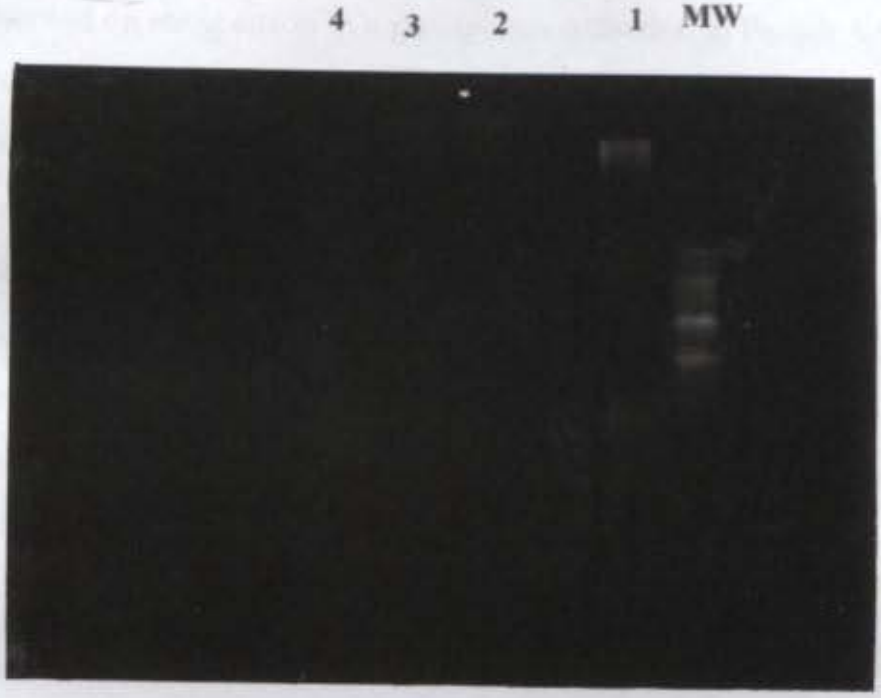


Fig. 28. Denaturing polyacrylamide gel electrophoresis of viral nucleic acid after nuclease treatment

Lane MW - RNA molecular weight marker

Lane 1- 3 – Viral nuclease treated with

1 – DNase

2 – RNase in 0.01 M NaCl

3 – RNase in 0.3 M NaCl

Lane 4 – Viral nucleic acid isolated from virion

DISCUSSION

A disease showing yellowing of veins and veinlet was first observed on etrog citron in a germplasm collection at Punjab Agricultural University Research Station (PAU), Abohar. The same disease was also observed in the germplasm block at PAU, Ludhiana on etrog citron, sour orange, hill lemon and *Citrus pectinifera*. Subsequent surveys showed the distribution of the disease at Anand in Gujarat and Pune in Maharashtra. The symptoms of the disease were clearly visible when the leaves were seen against light. The vein yellowing and vein banding symptoms on the dorsal side and water soaking area along the vein on ventral side of the leaves were the main symptoms of the disease.

The samples collected from the field were transmitted to etrog citron, mosambi and several other citrus hosts by grafting. To ascertain the virus infection in samples from graft inoculated plants in the glasshouse these were examined in the electron microscope. A filamentous virus similar to Indian citrus ringspot virus (ICRSV) was observed in all the samples. Therefore, immunosorbent electron microscopy (ISEM) was done to establish if the virus associated with the disease was that of ICRSV. It was interesting to note that during ISEM tests using ICRSV antibodies decorated about 50 per cent particles and rest remained undecorated suggesting that there was a mixed infection of the filamentous virus in the field samples. It therefore, became essential to develop a pure culture of the virus from the mixed infection. Accordingly, transmission tests were conducted by various methods including mechanical inoculation using various buffers, their varying pH

and molarity. It was found that the virus, in question, was mechanically transmissible to mosambi sweet orange, *Phaseolus vulgaris* and *Chenopodium quinoa.* the plants thus infected were again tested in ISEM with ICRSV antibodies and it was found that no ICRSV particles were present in the mechanically infected plants. The pure culture of the virus was therefore multiplied from the hosts and maintained in the glasshouse for different studies.

For the purpose of present studies, the Abohar isolate was selected. The present virus was transmitted by different methods of grafting such as wedge, bud and petiole grafting and their comparative efficacy was determined. Transmission upto 100 per cent was obtained by wedge grafting and hence this method was used for virus multiplication. The virus was also transmitted by mechanical inoculations to *Phaseolus vulgaris* var. saxa, singtarn and ghieusemi and also *Chenopodium quinoa.* The inoculated plants of *P. vulgaris* cultivars developed local lesions on inoculated leaves followed by systemic infection but in *C. quinoa* only necrotic lesions were developed on inoculated leaves and no systemic symptoms were observed.

The disease could not be transmitted by three aphid species *Aphis gossipii, A. citricola* and *M. persicae, Bemisia tabaci* and *Planococcus citri.* The virus was also not transmitted through dodder (*Cuscuta reflexa*) and also by seed.

The virus was transmitted to 16 citrus species causing variable symptoms. The thermal inactivation point (TIP) of the virus was determined as 61°C, dilution end point (DIP) 10^{-2} and longevity *in vitro* only for 2 h at room temperature.

For isolation of the virus various buffers were tried with different molarity and pH. Sodium phosphate buffer with a molarity of 0.05 and pH 7.2 was found to be the best for isolating the virus from infected tissues. The virus was initially purified by using the protocol of Byadgi *et al.* (1993), Levy and Gupf (1991), Bar Joseph *et al.* (1985) and Rustici *et al.* (2000), but the protocol described by Rustici *et al.* (2000) provided the maximum concentration of purified virions and was used for further purification of the present virus. The A_{260}/A_{280} ratio was determined as 1.123 suggesting that the purified preparation was the nucleoprotein. The virus had the filamentous particles of 685 nm in length and 14 nm in width.

Antibodies for the present virus were developed in rabbit from the purified virus preparations. The IgGs were isolated from the whole serum and used for the detection of the virus in both DAS and DAC-ELISA. The results of DAS ELISA were better than DAC-ELISA and hence it was used for virus detection from various isolates. The optimum concentration of the ragents used in DAS-ELISA were as follows : antigen dilution 1 : 20, conjugate dilution 1 : 2000 and coating antibody 1 µg/ ml. These dilutions detected the virus effectively both from glasshouse and field trees.

To determine the specificity of the antibodies developed against the present virus, ISEM decoration tests were conducted. The antibodies developed during the present studies decorated all the particles from the pure culture and purified preparations as well suggesting the specificity of the antibodies with the virus. Both trapping and decoration tests were used to determine the titre of the homologous antiserum for the purpose

of virus detection. A dilution of 1/500 of the homologous antiserum trapped seven times more particles as compared to normal leaf dip preparation suggesting that this test could be a powerful tool for detection of the virus even at a very low concentration. The homologous antiserum heavily decorated the virions upto a dilution of 1/8192 suggesting the antiserum to be high titred one which is difficult to obtain for viruses affecting citrus trees.

The serological relationship of the present virus was studied in ISEM using antisera of 9 viruses including ICRSV. But the virus in question, did not react to any of these antisera except the homologous one suggesting that the virus was serologically distinct to henbane mosaic virus (HMV), papaya ringspot virus (PRSV), potato virus Y (PVY), Indian citrus ringspot virus (ICRSV), potato virus X (PVX), garlic latent virus (GLV), shallot latent virus (SLV), carnation latent virus (CLV) and garlic mosaic virus (GMV). The studies revealed that this virus has a single stranded RNA as its genome and the molecular weight of the coat protein as determined in 12% polyacrylamide gel was 32 kDa. The virus could not be amplified in PCR using ICRSV specific primers further indicating that the virus under study is distinct from ICRSV.

Symptoms similar to the present disease have been reported on lemon from Pakistan as yellow vein clearing disease (YVCD) by Grimaldi and Catara (1996). They reported long filamentous particles ranging from 530 nm to 1800 nm with a diameter of 13-14 nm with a modal value 670-700 nm associated with the YVCD. However, ultra thin sections of tissues of YVCD affected plants showed the virions in phloem tissues. They thought that the particles represent a closterovirus like virus. The

present disease is distinct from YVCD in its symptomatology such as water soaking veins on the ventral side of leaves. However, the associated virus is also distinct in its morphology from the one reported by Grimaldi and Catara (1996). Mechanical transmission to citrus and herbaceous hosts further differentiate the two viruses. No antibodies were available of the virus associated with yellow vein cleaning disease of lemon as these have not been dedveloped as yet (Catara - personal communication). Therefore, no serological comparison could be made.

A disease with yellow vein symptom was also described from California (Weathers, 1960, 1961) but except symptoms and its graft transmission no other information is available on this disease. The symptoms of water soaking of veins is a characteristic symptom of the present disease which has not been reported from yellow vein disease of California. No other information is available for comparison of the two viruses.

The present disease was transmitted by grafting and also by mechanical inoculations similar to Indian Citrus Ringspot Disease as reported by Byadgi and Ahlawat (1995), Pant and Ahlawat (1997). However, the present disease could not be transmitted through dodder like the Indian Citrus ringspot (Pant and Ahlawat, 1998). Moreover unlike ICRSV the present virus was transmissible mechanically from etrog citron to mosambi.

The virus associated with yellow vein disease was not transmissible by different insects. Fawcett (1938) also failed to transmit prorosis through insect but showed the spread of the disease by bud propagation from infected trees (Fawcett, 1939). Timmer and Gransey (1980) failed

to transmit the CRSV using several species of plant hoppers, leaf hoppers and Aphid (*A. citricola*). Navas Castillo *et al.* (1991) also reported non transmission of CRSV by *A. gossypii and A. citricola.* Pujol and Benatena (1965) suggested involvement of insect vector, propaply sucking insect of an Argentina isolate of psorosis. However, like these viruses, the present virus also could not be transmitted by aphids, whitefly and mealy bug. This disease is perhaps spreading in nature through inadvertant propagation from contaminated trees.

The present virus was also not transmitted through seeds. Pujol and Benatena (1965), Wallace (1978), Garnsey (1975), Timmer and Garnsey(1980) and Byadgi and Ahlawat (1995) also did not get seed transmission when thousands of seedlings were raised from seeds obtained from fruits of infected trees. However, seed transmission of CRSV upto 15-30% has been reported in *Carrizo citrange* from Florida (Bridges *et al.* 1965). Childs and Jonson (1966) reported the seed transmission of Psorosis-A. Campiglia *et al.* (1976) reported one percent seed transmission of ringspot in trifoliate orange in Uruguay.

Hostrange of the present virus was restricted to families of *Rutaceae, Chenopodiaceae* and *leguminoceae.* Variable symptom expressions were observed in different citrus species tested for host range. The present virus produced yellow vein symptoms on *Etrog citron, Sour orange* and *Lemon* with water socked veins on the lower side of the leaves. The virus also produced shock reaction on mosambi and kinnow mandarin followed by leaf fleaking and vein clearing symptoms on young leaves, and chlorotic patches or mottiling symptoms on mature leaves. The variation in symptoms on different hosts with different virus isolates has been

reported by various workers (Fawcett, 1938; Wallace, 1945; Timmer *et al.* 1978; and Navas - Castillo and Moreno, 1992, 1993). All the sixteen citrus cultivars tested under these studies were found susceptible to citrus yellow vein virus (CYVV), and no source of resistance was available.

Four different purification methods were used to purify the virus (Levy and Gumpf, 1991; Byadgi *et al.* 1993; Bar joseph *et al.* 1985; Rustici *et al.* 2000). Among them Rustici *et al.* 2000 was proved to be the best method. Detailed studies were conducted on the requirement of buffer, its molarity and pH which helped in developing a standard purification protocol to obtain highly purified and concentrated virus prepartion.

For extraction of the virus from host tissue 0.05 M sodium phosphate buffer (pH 7.2) was found to be the most suitable buffer than tris-citrate; tris-HCl, sodium-citrate and sodium borate.

Differential centrifugation successfully isolated the virus from the extract obtained by grinding virus infected tissues. The final preparation was further purified by passing through 10-40% cesium sulfate gradient. The gradient when run at 38,000 rpm for 3 h showed only one hand which showed filamentous particles in EM. The model length of the virions was calculated as 685 x 14 nm in size.

The O.D. values of virus preparation at 260 and 280 nm were 0.6442 and 0.5735 respectively. The A_{260}/A_{280} ratio was 1.123. The O.D. value suggested that the preparation contained nucleoproteins. Byadgi and Ahlawat (1995) and Pant and Ahlawat (1998) also obtained the A_{260}/A_{280} ratio as 1.125 and 1.126 respectively for ICRSV.

Analysis of coat protein of filamentous particles of the present virus in 12%. SDS-PAGE revealed one protein band at 32 kDa. Levy and Gumpf (1991) found 29 kDa protein associated with California isolate of psorosis (PS-203m). Derrick *et al.* (1991) in florida; Garcia *et al.* (1991) in Argentina and Navas-Castillo *et al.* (1991) found 48-50 kDa coat protein for citrus ringspot virus. A 29 kDa coat protein of ICRSV has also been reported by Byadgi *et al.* (1993). Pant and Ahlawat (1995) reported 60 and 67 kDa protein associated with thin filamentous particles of Indian citrus ringspot virus. It is evident from the above reports that different viruses throughout the world have shown variable molecular weight of coat protein.

Antiserum of the virus was developed and titre of antiserum was determined for conducting ELISA and ISEM tests for virus detection. In ISEM tests, CYVV antigen reacted with its homologous antiserum at dilution of 1:1000 whereas in ELISA homologous IgG reacted with antigen upto a dilution of 2 µg/ml. The virus antigen did not react to the antiserum of ICRSV further suggesting the differences of the ICRSV from that of the present virus. The antisera of 9 filamentous viruses did not react with the antigen of present virus in ISEM tests suggesting the virus to be serologically distinct from these group of viruses.

For serodiagnostic studies, the IgG were purified from the whole serum according to the procedure followed by Clark and Adams (1977) and homologous antibody-enzyme conjugate was prepared which positively reacted in DAS-ELISA. DAC-ELISA was also done with purified IgG, but DAS-ELISA provided better results. In DAC-ELISA, satisfactory results were obtained when IgG were cross adsorbed with

healthy plant sap. 1 µg/ml concentration of IgG and Goat antirabbit (GAR) were optimum for detection of CYVV in DAC-ELISA. Levy and Gumpf (1991) also reported 1:10 dilution of cross observed antiserum as the optimum condition for the detection of psorosis virus. Since DAS-ELISA proved superior to DAC-ELISA, it was used for indexing of field trees.

The DAS-ELISA technique was applied to analyse glasshouse maintained virus isolate and also samples collected from orchard trees. Out of 6 orchard samples analysed, 2 reacted positively in DAS-ELISA.

ISEM tests were conducted for detection of the virus when no symptoms were visible in samples or poor absorbance values were obtained in ELISA. The homologous antiserum trapped optimum virus particles at an dilution of 1:1000 and decorated virus particles at 1 : 8192 dilutions. Derrick *et al.* (1991) daGarca *et al.* (1991) and Navas-Castillo *et al.* (1991) have also conducted ISEM tests for accurate detection of CRSV isolates.

The studies of virus detection in ELISA and ISEM showed that the homologous antiserum was a good workable reagent and can be employed for indexing against this virus in certification and quarantine programmes.

Due to extremely labile nature of the nucleic acid, it was very difficult to get consistently good preparation but preliminary evidence obtained indicated that the genome of the virtus was a single stranded RNA (ssRNA).

In denaturing polyarcylamide gel several bands ranging in size from 3.3 to 0.5 kb were obtained. The prominent bands could represent the genomic RNA and other bands may represent the secondary structure of the molecule of the same RNA or may represent subgenomic RNA (Hull, 2001).

From gel electrophoresis of samples digested with RNase at low and high ionic strength buffer there were disappearance of bands, suggesting that the virus, in question, has ssRNA as its genome. However, the virus is distinct from capillo, Allexi, Forca and trichovirus as it lacks polyadenylated 3' end as shown by absence of cDNA synthesis using oligo dt primer (Rustici *et al.* 2000).

The primers specific for coat protein gene of Indian citrus ringspot virus (ICRSV) did not show any amplification product with RNA template isolated from citrus yellow vein virus (CYVV) infected leaves. Same evidence was obtained when the antisera of ICRSV did not trap CYVV and vice-versa.

The studies conducted on the disease, in question, and the virus associated with it clearly indicated that the present virus and the disease appears to be the new records. Therefore, the name of the disease is proposed as citrus yellow vein disease and the virus as citrus yellow vein virus.

The following new informations have been generated during the present studies :

1. Yellowing of veins in leaves of citrus spp. was believed to be a reaction of Indian citrus ringspot virus but during the present

studies it has been established that these symptoms are caused by an unidentified virus.

2. Mechanical inoculation of CYVV to mosambi proved to be a good technique to differentiate CYVV from ICRSV.
3. *Phaseolus vulgaris* cvs. Saxa, Suigtami and Gheasimi proved to be the good multiplication host of CYVV which made the purification an easy task.
4. *Chenopodium quineoa* was identified as a good local lesion host for virus assay.
5. The antibodies against CYVV were developed and used for virus diagnosis in ELISA and ISEM.
6. The genome of the virus as ssRNA has been determined.
7. the two virus (CYVV and ICRSV) having the virions of similar morphology could provide a risk of CYVV movement through planting material had it not been investigated and diagnostics developed.
8. The informations generated will be of great significance in quarantine and budwood certification programmes.

Further thrusts

1. To characterise the genome of the virus.
2. Cloning and sequencing of the virus genome.
3. To establish the taxonomic position of CYVV based on the homology of the nucleotids of known viruses.
4. To develop NA-based diagnostics.

SUMMARY

An undescribed virus was observed in a mixed infection with Indian citrus ringspot virus (ICRSV) in ISEM tests using ICRSV antibodies. The virus was separated out from the mixed infection by mechanical inoculations to mosambi sweet orange plants and thus pure culture of the new virus was obtained. The virus was constantly associated with yellow vein disease of citrus spp. From the pure culture the virus was transmitted to *Phaseolus vulgaris* cvs. Saxa, Singtaini and Gheosimi and also to *Chenopodium quinoa.* The cultivars of *P. vulgaris* showed local lesions on inoculated leaves but later the infection became systemic whereas *C. quinoa* showed only local lesions on inoculated leaves.

The virus was also transmitted by wedge, but and petiole grafting but not by 3 aphid species, whitefly and mealybug and also by dodder (*?Cuscuta reflexa*) and through seed. In host range studies the virus infected 16 citrus spp.

The inoculum was multiplied on *P. vulgaris* cv. Saxa and used for virus purification. The virus was purified by differential centrifugation followed by cesium sulphate gradient by the protocol of Rutici *et al.* (2000). The virions were 685 x 14 nm in size showing clear cross banding. The A_{260}/A_{280} ratio was 1.123 suggesting the virus preparation to be a nucleoprotein. The virus had a coat protein of 32 kDa.

The polyclonal antibodies against the virus were developed in rabbit and their titre was determined for virus detection in ELISA and ISEM systems. In ISEM, 7 times more particles were trapped as compared

to normal leaf dip preparations. The optimum conditions for virus detection in DAS and DAC-ELISA were worked out and ELISA has been used for detection of the virus from glasshouse and field affected trees.

The present virus was serologically distinct to 9 viruses belonging to 3 group of filamentous viruses. the genome of the virus is a ssRNA. The studies revealed that the virus is distinct in symptomatology, host-virus interaction, and serological behaviour to Hisquen citrus viruses and hence the virus has been tentatively named as citrus yellow vein virus (CYVV) and the disease as citrus yellow vein disease.

During the present investigations, following new informations have been generated :

1. Yellowing of veins in leaves of citrus spp. was believed to be a reaction of Indian citrus ringspot virus but during the present studies it has been established that these symptoms are caused by an unidentified virus.

2. Mechanical inoculation of CYVV to mosambi proved to be a good technique to differentiate CYVV from ICRSV.

3. *Phaseolus vulgaris* cvs. Saxa, Suigtami and Gheasimi proved to be the good multiplication host of CYVV which made the purification an easy task.

4. *Chenopodium quineoa* was identified as a good local lesion host for virus assay.

5. The antibodies against CYVV were developed and used for virus diagnosis in ELISA and ISEM.

6. The genome of the virus as ssRNA has been determined.

7. the two virus (CYVV and ICRSV) having the virions of similar morphology could provide a risk of CYVV movement through planting material had it not been investigated and diagnostics developed.

8. The informations generated will be of great significance in quarantine and budwood certification programmes.

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APPENDIX I

Buffers for mechanical transmission and purification

Phosphate buffer for 1L, 0.1 M (pH 7.0)

Sol. I Sodium dihydrogen phosphate (NaH_2PO_4)	7.09 g
Sol. II Di-potassium hydrogen orthophosphate (K_2HPO_4)	6.80 g

Mix sol. I with sol. II to adjust pH and make the volume up to 1 L with DW.

Sodium citrate buffer for 1 L, 0.1 M (pH 7.0)

Sodium citrate	29.41 g

Adjust pH with citric acid

Sodium borate buffer for 1 L, 0.1 M (pH 7.0)

Boric acid	38.137 g

Adjust pH with 1 N NaOH solution

Tris - HCl buffer for 1 L, 0.1 M (pH 7.0)

Tris	12.11 g

Adjust pH with HCl

Tris - citrate buffer for 1 L, 0.1 M (pH 7.0)

Tris	12.11 g

Adjust pH with citric acid

Reagents and buffers used for ELISA

Phosphate buffer saline (PBS) pH 7.4 (10X)

NaCl	8.0 g
KH_2PO_4	0.2 g

Na_2HPO_4 .12 H_2O	2.9 g
or anhydrous Na2HPO_4	1.15 g
KCl	0.2 g
Distilled water	1000 ml

Coating buffer (pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 0g
Distilled water	1000 ml

Washing buffer (PBS - T)

1x PBS	1000 ml
Tween - 20	0.5 ml

Antigen extracting buffer For DAS - ELISA

PBS - T containing 0.2 % ovalbumin and 2 % soluble polyvinyl pyrolidone (MW 44,000)

Antigen extracting buffer For DAC - ELISA

Coating buffer containing 2 % soluble polyvinyl pyrolidine (MW 44,000).

Blocking solution

1 % Bovine serum albumin prepared in PBS - T

Conjugate buffer

Same as DAS - ELISA antigen extraction buffer

Substrate buffer (pH - 9.8)

Diethanolamine	9.7 ml
Distilled water	80 ml

NaN_3	0.02 g

Adjusted pH to 9.8 and volume made up to 100 ml with distilled water.

SDS - PAGE Buffer

A. 5 x Tris - glycine electrode buffer pH 8.3 (0.025 M Tris - HCl, 0.25 M Glycogen).

Tris	15.10 g
Glycine	94.00 g
SDS	5.000 g
DW	1000 ml

B. Stock solutions

1. Tris - HCl (pH 6.8) 0.5 M

Tris	60.05 g
DW	100 ml

2. Tris - HCl (pH 8.8) 1.5 M

Tris	18.16 g
DW	100 ml

3. Sodium dodecyl sulphate solution 10 %

SDS	10 g
DW	100 ml

4. Acrylamide / bisacrylamide stock 30 %

Acrylamide	29 g
N. N Bismethylene acrylamide	1 g
DW	100 ml

(Store in amber color bottle at 4^0C)

C. Sample buffer

0.5 M Tris - HCl buffer pH 6.8	1.0 ml
Distilled water	4.0 ml
Glycerol	0.8 ml
10 % (W/V) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
0.05 % (W / V) bromophenol blue	0.2 ml

D. Ammonium persulphate solution

Ammonium persulphate	0.1 g
Distilled water	1.0 ml

(Prepared fresh whenever required)

Reagents and buffers used for ELISA

Phosphate buffer saline (PBS) pH 7.4 (10X)

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_2HPO_4.12H_2O$	2.9 g
or anhydrous Na_2HPO_4	1.15 g
KCl	0.2 g
Distilled water	1000 ml

Coating buffer (pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 0g
Distilled water	1000 ml

Washing buffer (PBS - T)

1x PBS	1000 ml
Tween - 20	0.5 ml

Antigen extracting buffer For DAS - ELISA

PBS - T containing 0.2 % ovalbumin and 2 % soluble polyvinyl pyrolidone (MW 44,000).

Antigen extracting buffer For DAC - ELISA

Coating buffer containing 2 % soluble polyvinyl pyrolidine (MW 44,000).

Blocking solution

1 % Bovine serum albumin prepared in PBS - T

Conjugate buffer

Same as DAS - ELISA antigen extraction buffer

Substrate buffer (pH - 9.8)

Diethanolamine	9.7 ml
Distilled water	80 ml
NaN_3	0.02 g

Adjusted pH to 9.8 and volume made up to 100 ml with distilled water.

3.14.2. Preparation of polyacrylamide gel

10 ml of 30 % polyacrylamide gel was prepared as described below :

30 % acrylamide stock	2.5 ml
10 X TBE	1.0 ml
Urea	3.0 g
10 % APS	0.075 ml
TEMED	0.01 ml
SDW	3.415 ml

10 X gel - running buffer (500 ml)

0.2 M morpholinopropanesulfonic acid (MOPS), pH 7.0	20.6 g
50 mM sodium acetate	2.05 g
10 mM EDTA, pH 8.0	0.04 g

Dissolve 20.6 g of (MOPS) in 400 ml of diethyl pyrocorbonate (DEPC) treated with 50 mM sodium acetate. Adjusted the pH to 7.0 with 2 N NaOH. Added 10 ml of DEPC - treated 0.01 M EDTA (PH 8.0). Adjusted the volume up to 500 ml with DEPC treated water.

10 X RNA dye loading buffer

Glycerol	50 %
EDTA	I mM
Bromophenol blue	0.4 %
Xylene cyanol	0.4 %