MOLECULAR CHARACTERIZATION OF INDIAN CITRUS RINGSPOT VIRUS AND ITS MANAGEMENT BY SHOOT TIP GRAFTING

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MOLECULAR CHARACTERIZATION OF INDIAN CITRUS RINGSPOT VIRUS AND ITS MANAGEMENT BY SHOOT TIP GRAFTING

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CERTIFICATE

This is certify that the thesis entitled:" **Molecular Characterization of Indian Citrus Ringspot Virus and Its Management by Shoot-tip Grafting**", 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 **Nguyen Van Hoa** embodies the results of the ***bona-fide*** research work carried out by him under my guidance and supervisions. 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.

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Abstract

Indian citrus ringspot virus (ICRSV) becomes one of the most destructive viruses infecting citrus plantations in India. The incidence of the disease ranged from 5 to 83.8% and the yield loss was estimated upto 98.38%. During the present investigation, four isolates of Indian citrus ringspot virus, ICRSV–Dl, ICRSV–Ab, ICRSV–Ah and ICRSV–Pu, have been studied for their molecular characterization. Polyclonal antiserum against ICRSV–Ab isolate has been produced and used effectively for the detection of ICRSV in DAS -, DAC - ELISA and DIBA systems. The virus was also detected in RT-PCR and NASH. One set of primer (ICRSV-1096/-1420). identified all the four ICRSV isolates, but two other sets of primer (ICRSV-518/-951 and ICRSV-518/-1420) identified only ICRSV-Dl and ICRSV-Ab isolates. Probe made from 350 bp amplicon of primer ICRSV – 1096/-1420 was also detected all the four isolates in NASH system. 1283 nucleotides of Coat protein of the four ICRSV isolates was cloned in pGEM-T easy vector and sequenced. Nucleotide and deduced amino acid sequences have been analyzed showing variation at N terminus and conserved in the core region. Sequence analysis and phylogenic tree of both nucleotide and amino acid sequences showed the close relationship of the two isolates ICRSV-Ab and ICRSV-Dl with ICRSV-D and formed one cluster, while the two isolates ICRSV - Ah and ICRSV-Pu were distantly related to first cluster and formed another cluster and hence they were considered as different strains of the same virus. The restriction maps were generated and confirmed the sites of restriction by cutting with the common enzymes like Sac I, Pst I and Cla I. For management of the disease, protocol for STG has been standardized in case of Mosambi and Kinnow and virus-free (CTV, ICRSV, CVV, CYMV) plants of Kinnow and Mosambi were produced, multiplied and are being maintained in the containment glasshouse as virus-free nucleus material. French bean plants were transformed with truncated coat protein gene of ICRSV–Ab and proved by PCR and Southern hybridization, and coat protein gene expression in transformed plants was confirmed by DIBA.

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INTRODUCTION

INTRODUCTION

Among major fruit producing countries of the world, China produces 13.9% of total production followed by India (9.7%), Brazil (7.1%), USA (7.0%). (Indian Horticulture Database, 2002). *Citrus* spp is a major world food crop in terms of nutrient, generation of income, foreign exchange, and employment (Piestun *et al.*, 2000). In all over the world, more than 50 countries are growing citrus in different agro-climatic conditions with the total production per annum of 93.75 m tones (Singh and Naqvi, 2001).

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 in the year 2000 - 2001 was 4.96 lakh ha with a production of 43.995 lakh tones.

Citrus is the collective generic term comprising of a number of species, varieties grown all over the world. The word 'citrus' probably has been originated from Greek word 'Kedros', meaning 'Fruit of the Godly tree'. The Greek word was latinized to 'Cedrus', which later became popular as 'Citrus' (Bhattacharya and Baruah, 1998).

Citrus belongs to family *Rutaceace* and has approximately 150 genera and 1600 species, which have been found in tropical, temperate and arid regions of the world. The *Rutaceace* is divided into seven subfamilies comprising 11 tribes and 93 genera (Engler, 1931). Of these, *Aurantioideae*, the subfamily of orange, is classified into two tribes *Clauseneae* and *Citrineae* and six subtribes, which contain over 215 species and 65 varieties. The genus citrus and its close relatives such as *Fortunella, Eremocitrus, Clymenia,*

Poncirus and *Microcitrus* are the "True Citrus Fruit Trees" in-group of the subtribe *Citrineae* (tribe *Citreae*). Out of them, only *Fortunella* (kumquats), *Poncirus* (trifoliate orange) and *Citrus* (eight species) are commercially important (Chapot, 1975).

The following eight citrus species are commercially cultivated:

Citrus reticulata Blanco. (Mandarin),

C. grandis (L.) Osb. (shaddok or pummelo),

C. paradisi Macf. (Grape fruit),

C. aurantium (L.) (sour orange),

C. sinensis (L.) Osb. (Sweet orange),

C. aurantifolia (Christm) Swing (acid lime),

C. limon (L.) Burmf. (lemon),

C. medica (L.) (citron).

The southern slope of Himalayan region is believed to be the origin of citrus. Citrus cultivation is done throughout the tropics and subtropics roughly between 40°N and 40°S latitudes in more than 89 countries. The total global area on citrus accounts to about 1.5 million hectare (Samson, 1980), and the annual production is about 85 million metric tones (Timmer *et al.*, 2000), and USA being the world's largest producer of citrus fruits.

In India, citriculture is the third largest component of the fruit industry after mango and banana in respect to cultivated area and production and ranks sixth among top citrus producing countries in the world (Bhattacharya and Baruah, 1998). The major citrus growing states in India are Uttar Pradesh, Andhra Pradesh, Maharashtra, Punjab, Himachal Pradesh, Tamil Nadu, Rajasthan, West Bengal, Sikkim and Northeastern states. Among the citrus fruits, mandarins occupy the largest area (1.689

lakh ha) followed by limes and lemons (1.642 lakh ha), and sweet orange (0.806 lakh ha)(Indian Horticulture Database, 2002), and the production is 14.14 lakh tones, 13.77 lakh tones and 11.60 lakh tones respectively).

Four agro - climatic zones have been identified in India for the purpose of citrus cultivation.

1. **North - western zone**: This zone comprises of Punjab, Haryana, Rajasthan and part of Gujarat. Kinnow mandarin, Blood red, Malta, Pineapple, Jaffa, Hamlin and Valencia late sweet orange, Kagzi lime and lemon cultivars such as Galgal, Italian lemon and Baramasi are cultivated here.
2. **North - eastern zone:** Assam, Meghalaya, Manipur, Tripura, Nagaland, part of Orissa and West Bengal belong to this region. In this zone, 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. **Southern zone:** Part of Andhra Pradesh, Karnataka, Tamil Nadu and Kerala form part of this region. Sathgudi and Batavian are the main sweet orange grown here, Coorg mandarin and Acid lime is also grown in this zone.

A large number of virus and virus like diseases have been reported in citrus from different parts of the world. These are citrus tristeza (Vasudeva and Capoor, 1958; Chakraborty *et al.*, 1993; Roy, 2000), Cachexia and psorosis A (Childs, 1950; Nagpal, 1959), bud union crease (Grimm *et al.*, 1955), greening (Oberholzer, 1947, Fraser *et al.*1966; Patil and Warke, 1968;

Bove *et. al.*, 1993), mosaic from Japan (Ishigai and Jinno, 1958), mosaic/yellow mosaic from India (Dakshinamurty and Reddy, 1975; Ahlawat *et al.*, 1996), crinkly leaf (Fawcett and Lee, 1926, Ahlawat and Sardar, 1976), leaf curl (Salibe, 1959; Mali *et al.*, 1976a), infectious variegation (Fawcett and Klotz, 1939; Yora *et al.*, 1977), concave gum (Fawcett and Lee, 1926; Mali, 1979), vein enation and woody gall (Wallace and Drake, 1953; Mali *et al.*, 1976b), impietratura (Ruggieri, 1961; Ahlawat *et al.*, 1984), multiple sprouting (Searle, 1969; Ahlawat and Chenulu, 1985), Citrus ringspot (Byadgi and Ahlawat, 1995), Citrus yellow vein clearing (Al-Shami, 2002) and yellow vein corky vein (Fadoos, 1999)

In India, Ahlawat (1989) reported a disease similar to Psorosis-A. The ringspot symptoms were observed on leaves of some Mosambi trees in the state of Delhi, Maharashtra, Punjab and Andhra Pradesh. Later, surveys revealed that the disease with ringspot symptoms was widely distributed in commercial citrus cultivars especially in mandarins and sweet oranges (Byadgi *et. al.*, 1993). The affected cultivars were Malta, Mosambi, and Satgudi sweet oranges [*Citrus sinensis* (L) Pers.], Nagpur orange (*C. reticulata* Blanco), Kinnow mandarin, a hybrid (Willow x king mandarin), and Kagzi lime and Kagzi kalan [*C. aurantifolia* (Christen) Swingle] in different citrus orchards at Delhi, Haryana, Punjab, Karnataka and Andhra Pradesh with an incidence ranging from 5 to 83.8%. The yield loss was estimated from 20.5 to 98.38% (Byadgi and Ahlawat, 1995). Recent estimation showed a decrease in fruit weight, size, granulation, TSS, TSS/acid ratio, vitamin C and reducing sugar in fruits of citrus ringspot virus (CRSV) affected plants (Thind *et al.*, 1998).

The disease, similar to ringspot described from India, was found distinct from ringspot diseases reported elsewhere (Derrick, 1988, Garsney

and Timmer, 1970). Based on recent studies, the disease has been named as Indian citrus ringspot disease (Byadgi and Ahlawat, 1995) and the causal virus has been named as Indian citrus ringspot virus (ICRSV) (Pant and Ahlawat, 1998). The main symptoms of citrus ringspot in India are vein flecking of young leaves and chlorotic rings on mature leaves. The mode of natural spread of ICRSV appears to be through propagation of contaminated bud wood (Ahlawat, 1997). The causal virus is sap transmissible to French bean and *Chenopodium quinoa* (Pant *et. al.*, 2000).

Indian citrus ringspot virus showed two types of virion measuring 640 x 15 nm and 690 x 9 nm along with some tubule-like structure (Byadgi and Ahlawat, 1995). This virus had been tentatively identified as a member of Capillovirus group (Pant and Ahlawat, 1998), but its taxonomical position is yet to be ascertained. Therefore, it is essential that the molecular characterization of ICRSV is done and diagnostic reagents for its detection are developed. ICRSV has so far been reported only from India and its incidence is more than 80% in Kinnow mandarin and Mosambi and Malta sweet oranges, which demands its management quickly to avoid its further spread in the country. In view of this, studies were planned with the following objectives:

1. Molecular characterization of Indian Citrus Ringspot Virus (ICRSV)
2. To develop diagnostic reagents and standardization of techniques for indexing of ICRSV.
3. To develop virus-free nucleus material through shoot tip grafting.

REVIEW OF LITURATURE

2. REVIEW OF LITERATURE

Two terms have been used in this review, (i) Citrus ringspot virus (CRSV): This is a virus infecting citrus in several countries other than India, (ii) Indian citrus ringspot virus (ICRSV): this virus infects citrus only in India.

Citrus ringspot disease, reported by Wallace and Drake (1968), is a distinct disease than Indian citrus ringspot. The causal viruses of the two diseases are undoubtedly different. Indian citrus ringspot virus has been worked out after it was first report by Ahlawat (1989). An isolate of ICRSV from Kinnow Mandarin - isolate K1 from Delhi was studied by Rustici *et. al.*(2000) from Italy, and reported the virus different from citrus ringspot virus reported in other parts of the world based on morphology of its virion and serological reactivity (Byadgi *et. al.*, 1993; Byadgi and Ahlawat, 1995; Ahlawat, 1997), and molecular differences (Rustici *et al.*, 2000).

***Geographic distribution of Indian Citrus Ringspot Virus Disease*:**

In India, citrus Ringspot disease was first observed by Ahlawat (1989) and was reported as Psorosis-A based on its symptoms on inoculated Mosambi sweet orange plants. Later, the disease was recorded in citrus plantations in Delhi, Punjab, Haryana, Rajasthan, Uttar Pradesh, Andhra Pradesh and Karnataka (Pant and Ahlawat 1998).

History of the ringspot diseases:

A. Citrus ringspot disease found in countries other than India:

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.

1938 Fawcett: Transmitted Psorosis by bud, described leaf and bark symptoms and provided first evidence of a transmissible virus disease of citrus.

1939 Doige: Failed to observe leaf symptoms on trees with bark lesions. Conducted one of the first eradication programmes and emphasized the need of rapid indexing method.

1942 Fawcett and Cochram: First used bark lesion inoculum for quicker transmission of Psorosis B and reported a long incubation period of 12 to 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 to 10 weeks in inoculated sweet orange plants.

1947 Wallace: Transmitted Psorosis by leaf 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.

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 separated virus diseases.

1967 Corbett and Price: Reported failure of Psorosis to protect against citrus variegation virus.

1968 Wallace and Drake: Reported citrus ringspot 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 bark 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 *Chenopodium quinoa*.

1974 Passos et al.: Reported 57% disease in 10-year-old nucellar selection and 100% in paranucellar propagation. They 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 to 10% and also found 20% of nucellar 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 special mandarin and Tangelo under warm conditions.

1980 Roistacher et al.: Reported mechanical transmission of Psorosis isolate from citron to citron by knife cut.

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 *Chenopodium quinoa.* They also reported strong association of bark scaling Psorosis and CRSV, they believed CRSV might be synonymous to Psorosis B.

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

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), *Chenopodium 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 *Chenopodium quinoa* and *Gomphrena globosa.*

1988 Derrick et al.: Reported spiral shaped virus particles of CRSV, They produced antiserium against CRSV – 4 isolate.

1989 Manjunath: Transimtted Psorosis virus through dodder.

1991 Levy and Gumpf: Purified Psorosis isolate (p-203-m) and found association of 660 – 665 nm flexuous virus particle tentatively identified as Carlavirus and produced antiserum. They also reported 29 KDa coat protein. They 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.: They proposed new name to the virus associated with ringspot disease as "spirovirus"

1992 da Graca et al.: Used immunoblot technique for detection of citrus Psorosis virus using CRSV – 4 antiserum.

1992 da Graca et al.: Suggested that viruses associated with citrus Psorosis and ringspot diseases belong to new group of virus.

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

1992 Roistacher: Reviewed Psorosis virus complex and considered CRSV similar to Psorosis.

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 components contained 48

KDa coat protein. However, they also reported 38 KDa coat protein in their initial experiment and established that this protein was the degradation product of 48 KDa protein.

1994 Garcia et al.: Reported two types of particles 300 - 500 nm and 1500 - 2000 nm x 10 nm as top and bottom components of CRSV. They suggested that CRSV represents a new genus (possibly family) related to Tenuiviruses. But they 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 (CCDW) 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 orange for indexing of citrus ringspot virus complex and overcome the temperature effect.

1995 Navas-Castillo and Moreno: Used ISEM with CRSV antiserum for detection of several ringspot isolate, Psorosis A and Psorosis B showing filamentous flexuous particles. All ringspot isolates 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 types of protein were serologically related in western blot. They suggested that a common virus with different strains might be involved in Psorosis A, Psorosis B and ringspot disease.

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

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.

2000 Legarreta et al.: Used bud grafting for the successful transmission of CRSV.

2000 Sambade et al.: Used fast one - step RT-PCR to amplify citrus Tristeza and citrus Psorosis virus.

B. Ringspot disease found in India:

Indian citrus ringspot disease as Psorosis-A was first reported by ***Ahlawat (1989)*** infecting Mosambi sweet orange trees in India.

1990 Ahlawat and Chakraborty: Reported the association of a flexuous virus with the disease in India.

1993 Byadgi et al.: Reported three types of particles associated with CRSV in India. A virus of Carpillovirus group measuring 640 x 15 nm long, flexuous particles 690 x 9 nm long and tubules 225 x 40 nm long were identified as associated components of ringspot disease.

1995 Byadgi and Ahlawat: Reported 29 KDa coat protein in an Indian isolate of CRSV.

1997 Pant and Ahlawat: Transmitted ICRSV to *Phaseolus vulgaris* and *C. quinoa* by sap inoculations. The virus was purified from citrus and bean and polyclonal antibodies were produced and used for detection of the virus by ELISA and ISEM.

1998 Thind et al.: Transmitted citrus ringspot virus through budwood but not by seed and insects (*Diphoriana citri* and *Dialeurodes citri*).

2000 Rustici et al.: Studied the genome of the virus and reported that the virus has 34 KDa coat protein and a single ssRNA of about 7.5 Kb. The derived amino acid sequence of CP contained some short motifs similar to those of Potex-, Fovea-, Carla-, and Allexiviruses, but otherwise no similarly to any of these groups. They concluded that this virus belongs to a new group of plant viruses.

2002 Rustici et al.: The sequence of the single-stranded RNA genome of ICRSV consists of 7560 nucleotides. It contains six ORFs which encode putative proteins of 187.3, 25, 12, 6.4, 34 and 23 kDa respectively. ORF1 encodes a polypeptide that contains all the elements of a replicase; ORF 2, 3 and 4 compose a triple - gene block; ORF 5 encodes the capsid protein; the function of ORF 6 is unknown. Phylogenetic analysis of the complete genome and each ORF separately, and database searches indicate that ICRSV, though showing some similarities to Potexviruses, is significantly different, as in the presence of ORF 6, the genome and CP sizes, and particle morphology. These differences favour its inclusion in a new virus genus.

Symptomatology:

Citrus ringspot disease as originally described by Wallace and Drake (1968) on the basis of symptoms induced on inoculated seedlings of several citrus species. These symptoms consisted of chlorotic flecking and spotting of young leaves which later developed into yellow blotches, vein banding or distinct rings in mature leaves. Symptoms in young leaves were similar to those described for Psorosis (Fawcett, 1938). Inoculated seedlings of certain citrus varieties also showed a shock effect that resulted in leaf drop and necrosis of first flush after inoculation. Many field trees infected with citrus ringspot did not showed any specific symptoms, while others had Psorosis symptoms (Wallace and Drake, 1968).

Table 1: *Symptoms induced by citrus ringspot isolates on several citrus hosts incubated in cool (18 - 26°C) glasshouse.*

	Duncan grape-fruit	Mexican lime	Etrog citron	Dweet tangor	Rough lemon	Pineaple Sweet orange
RS-ALC	S, D, BF, NE, DF, SS	F	A,D,NE	F,B,D,OL, BF,NF,DF	S, F, B, D,B, F, NE, DF,RF	S,F,B,D,BF, DF
RS-INV	S, BF, NE, DF SS	S,B,BF,NE, DF, SS	F,B,D,R,FE, NE, DF, SS	S,F,B,D,BF, NE, DF, SS, St	S,B,D,R,BF, NE, DF,RF,SS	S,F,B,D,BF, NE,DF,RF,P B,SS, St
RS-CV	S,D,BF,NE,DF, SS	S,D,BF,DF	S,B,D,R,NE	A,F,B,D,OL, BF,DF, RF	F,B,D,BF,N E,DF,RF	S,F,B,BF,RF
RS-SOR	S, BF, NE, DF SS	S, B, D, BF NE, DF	A, F, B, D, BF NE, DF, F, B BF, NE	S, F, B, D OL, BF, NE DF, RF, SS	S, F, B, D BF, NE DF, RF, SS	S, F, B, OL, BF, NE, DF, RF, St
RS-GR	BF, NE, DF	F, B, BF	F, B, BF, NE	F, B, OL, BF DF, PB, St	F, B, D, BF NE, DF, RF	S, F, BF, NE, DF, RF, PB
RS-ALM	BF	F, BF	-	F, B, BF, NE DF	BF	F, BF, DF
RS-BUR	-	-	-	F, OL, BF DF, RF	-	BF, RF
RS-SR	S, B, D, BF, NE DF, SS	S, F, BF NE, DF, RF	S, F, B, D, BF NE, DF, RF	A, F, B, D OL, BF, NE DF, RF	S, F, B, D R, BF, NE DF, SS	S, F, B, D, BF, NE, DF, SS, St
P-121	S, B, D, R, BF NE, DF	S, BF, DF	S, F, B, D, BF NE, DF	A, F, B, D OL, R, BF RF, NE, DF SS, St	S, F, B, D BF, NE DF, SS	S, F, B, D, BF, NE, DF, St
CONTROL	-	-	-	-	-	-

RS-ALC: Ringspot isolate from Alcanar (Tarragona); **RS-ALM**: from Almussafes (Vaencia), **RS-SOR**: from a sour orange rootstock I Rafelguaraf (Valencia); **RS-GR**: from grapefruit in Rafelguaraf (Valencia); **RS-BUR**: from Burjassot (Valencia); **RS-INV:** an isolate from Navel orange in Vila - real (Castellon); **RS-CV**: from clementine in Vila - real (Castellon); **RS-SR**: from Star Ruby (Alicante); **P-121**: Psorosis isolate 121.

A: Leaf abscission in the primary flush after inoculation; **B**: Chlorotic blotches in young leaves; **BF**: Chlorotic blotches in fully expanded leaves; **D**: Deformation in young leaves; **DF**: Deformation in fully expanded leaves, **F**: Chlorotic flecking in young leaves; **NE**: Necrotic etching in fully expanded leaves; **OL**: Oak-leaf pattern in young leaves; **PB**: Psorosis B lesion in adult leaves; **R**: Chlorotic rings in young leaves; **RF**: Chlorotic rings in fully expanded leaves; **S**: Shock reaction with leaf shedding and necrosis in the primary shoot after inoculation; **SS**: Shock reaction in secondary shoots; **St**: Stunting.

Navas - Castillo and Moreno (1993) had studied the biological diversity of eight citrus ringspot isolates in Spain by inoculated on different citrus hosts, the results showed in Table 1. Most inoculated plants of sweet orange and Dweet tangor had chlorotic flecking in young leaves, whereas none of the grapefruit plants showed this symptom. Sweet orange and Dweet tangor were only hosts showed stunting and Psorosis B symptoms in adult leaves. Grape fruit, lime, rough lemon and citron did not react with the isolates RS-BUR, and citron did not react either with the isolate RS-ALM. RS-BUR and RS-ALM were the only isolates that failed to induce shock in all the indicators. In addition, RS-GR produced stunting in Dweet tangor plants, RS-SOR and RS-SR stunted sweet orange.

In India, the symptomatology as reported by Pant and Ahlawat (1998) showed that the Indian Citrus Ringspot virus caused variable symptoms in different citrus hosts.

Table 2: ***Symptoms induced by Indian citrus ringspot virus on several citrus hosts incubated in glasshouse***

Host	Symptom	Host	Symptom
Citrus sinensis cvs		*Citrus reticulata* cvs.	
Mosambi	VC, LF, RS, OLP	Darjeling orange	CS
Sathgudi	CS, RS, LF	Napur orange	LF, NS, RS, WS
Malta	LF, CS, RS	Kinnow	CS, NS, RS
C. aurantium		*C. paradise*	
Sour orange	VC, LF, WS, LC	Grapefruit	LF, CS
C. limettiodes	CS, VC	*C. jambhiri*	
Sweet lime		*Rough lemon*	CS, VC
C. decumana	CS	*C. limon cv.* Galgal	VC
C.aurantifolia cvs.		*C. medica* Etrog citron	VC, WS
Kagzi lime	CS, LF		
Kagzi kalan	CS, RS		

VC:	Vein clearing	CS:	Chlorotic spot
OLP:	Oak - leaf pattern	RS:	Ring spot
LF:	Leaf flecking	NS:	Necrotic spot
WS:	Water soaking	LC:	Leaf curling.

The first visible symptoms developed upon inoculation were vein clearing and flecking of young leaves followed by necrotic and chlorotic rings on mature leaves (Pant and Ahlawat 1998).

Host ranges:

The host range of the both CRSV and ICRSV studies showed that it is restricted to family *Rutaceae* (Catara and Grasso, 1968; Timmer and Garnsey, 1979; Byadgi *et al.* 1993 and Pant, 1995). The inoculated plants showed shock reaction followed by leaf flecking and vein clearing symptoms in young leaves and ringspots of various diameters on mature leaves (Pant and Ahlawat, 1998) (Table 2).

Some of the herbaceous hosts, such as *Capsicum anuum, C. fructescens, Catharanthus rosesus, Chenopodium album, C. quinoa, C. amaranticolor, Crotolaria spectubilis, Cucumis melo, C. sativus, Cucubita pelo* cvs. Richgreen, small sugar pumkin, *Helianthus annus, Nicotiana glandiflora, Physalis floridana, Zinnia elegans, Gomphrena globosa, Sesamum indicum, Solanum nigrum, Petunia hybrida, Pisum sativum* and *Vigna unguiculata,* are reported to be hosts of citrus ringspot virus. (Garnsey, 1975; Garnsey *et al.*, 1976; Timmer *et al.*, 1978; Levy and Gumpf, 1991 and da Graca *et al.*, 1991). But it did not infect to *Lycopersicon esculentum, Chenopodium murale, Citrullus lanatus, Nicotiana tabaccum* cv. *KY 57, Cucumis pepo* cv. *Zucchini* (Garnsey *et al.*, 1976; Timmer *et al.*, 1978). ICRSV was transmitted to *Phaseolus vulgaris* var. *singtamy* and *saxa, C. quinoa,* Cowpea, soybean (Pant *et al.*, 2000; Rustici *et al.*, 2000), but not to *Brassica oleracea* var. *botrytis,* var. *capitata, B. juncea, Citruleus vulgaris. Vigna mungo, V. sinensis, V. ungiculata, Caranthus roseus, Cucumis melo, C. sativus, Gomphrena globosa* and *Pisum sativum* (Pant, 1995)

When leaves of *Chenopodium quinoa* were mechanically inoculated with a preparation of infected citrus leaves, necrotic local lesions developed in about 10 days. Similarly, the virus also infected on *C. amaranticolor,*

Glycine max (Soy bean) and *Vigna unguiculata* (cowpea) showing local lesions, while in case of *Phaseolus vulgaris* var *singtamy, gheusemi, alapatri* and *saxa,* the inoculated plants showed first local lesions followed by systemic symptoms like vein banding, veinal necrosis and mosaic mottlings (Pant *et al.,* 2000; Rustici *et al.,* 2000). Optimum conditions for symptom expression under Phytotron were observed to be 28°C day temperature and 18°C night temperature with a relative humidity of 75%. Among the herbaceous host *P. vulgaris* cv *singtamy* local had comparatively higher concentration of the virus and therefore used for multiplication of the virus (Pant *et al.,* 2000).

Transmission:

Portillo and Bentena (1989) reported the transmission of Psorosis by aphids namely *Toxoptera citricidus, T. aurantii* and *A. citricola.,* but other workers reported that the virus spread through natural means (Timmer and Garsney, 1980; Roistacher, 1992; Navas - Castillo *et al.,* 1991) and it is well known that viruses pass through pollen and seeds of the infected plants but only few of them are seed transmitted, Childs and Johnson (1966) reported seed transmission of Psorosis was 15-30% in Carrizo citrange at Florida, Campiglia *et al.* (1976) observed 1% seed transmission of Psorosis in trifoliate orange (*Poncirus trifoliate*) seedlings at Salto region Uruguay. 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.

The disease caused by ICRSV is transmissible by grafting and through dodder from citrus to citrus such as Mosambi, Sathgudi, Kinnow mandarin, Kagzi kalan (Byadgi *et al.,* 1993; Pant and Ahlawat, 1998; Pant *et*

al., 2000). Indian citrus ringspot virus was neither insect nor seed transmitted (Pant, 1995). The virus was successfully transmitted to several cultivars of *Phaseolus vulgaris* (Pant *et al.*, 2000; Rustici *et al.*, 2000), but not to *Citrus* spp. (Byadgi *et al.*, 1993; Pant and Ahlawat, 1998). *Chenopodium quinoa* was identified as a local lesion host of the virus (Byadgi *et al.*, 1993; Rustici *et al.*, 2000).

Causal agent:

Citrus ringspot virus at California belongs to a genus Carlavirus as reported by Levy and Gumpf (1991), but later it was proposed to belong to new genus namely Spirovirus by Derrick *et al.*, (1992), and Ophiovirus by Garcia *et al.*(1994). Both of them reported that the virus has two types of particles 300 - 500 and 1500 - 2000 nm in length and 10 nm in diameter. However, the ICRSV also has two types of particles measuring 640 x 15 nm and 690 x 9 nm (Byadgi *et al.*, 1993), which was confirmed by Pant and Ahlawat (1998). They suggested that the virus belongs to genus Capillovirus, and latter the virus was studied up to molecular level by Rustici *et al.* (2000), suggested that the virus apparently belongs to a new virus group.

Purification:

Citrus Psorosis A was first purified by Bouhida (1984) and observed that the virus had flexuous particles. Ahlawat and Chakraborty (1990) used phosphate buffer 0.05 M (pH 7.6) containing thioglycolic acid for extraction of ICRSV from infected leaves of citrus, while Derrick *et al.* (1991), Garcia *et al.* (1991) and Navas-Castillo *et al.*, (1991) used Tris-HCl 0.05 M (pH 8.0) containing 0.1% ascorbic acid, 0.1% cystin and 0.5% of 2 - mercaptoethanol (TACM) as extraction buffer, and Levy and Gumpf (1991) used borate

buffer 0.5 M (pH 8.3) containing 0.1% sodium sulphite and 0.5% 2 - mercaptoethanol as extraction buffer.

For clarification of the extracted sap, Ahlawat and Chakraborty (1990), Navas - Castillo *et al.*(1993) used 6 % n - butanol, Byadgi *et. al.*(1993) used 10% n - butanol, Levy and Gumpf (1991) used 50% carbon tetrachloride and chloroform, Derrick *et al.* (1991) used 8% carbon tetrachloride for clarification of the extracted sap. Rustici *et al.* (2000) used 10% chloroform for extraction of ICRSV from French bean.

The partially purified virus was precipitated by polyethylene glycol (MW - 600) by Levy and Gumpf (1991) and Byadgi *et al.*(1993). Final concentration of CRSV was done by density gradient centrifugation using 10 to 40% sucrose gradient in TACM buffer (Derrick *et al.*, 1988; Navas-Castillo *et al.*, 1991). Levy and Gumpf (1991) used cesium chloride density gradient. Byadgi *et al.* (1993) used 0 - 30% cesium sulphate gradient. Rustici *et al.* (2000) used 10 - 40% cesium sulphate density gradient centrifugation for further purification of ICRSV.

Electron microscopy:

In electron microscopic studies, leaf dip preparation, are used for detection of virus particles from infected tissue and determination of their panicle properties such as length, modal length, morphology, etc. The CRSV associated with flexuous particles measuring 640 nm in length (Bouhida, 1984), Levy and Gumpf (1991) found filamentous particle measuring 660 - 665 x 12 nm with Psorosis isolate (p-203-m). Ahlawat and Chakraborty (1990), Navas-Castillo *et. al.* (1993) also found filamentous particles from Psorosis - A infected citrus leaves. Garcia *et. al.* (1994) observed two types of particles 300 - 500 and 1500 - 2000 nm in length and 10 nm in diameter. Byadgi *et, al.* (1993), Pant and Ahlawat (1998) also

found association of two types of particles measuring 640 x 15 nm and 690 x 9 nm with citrus ringspot disease in India.

Coat protein:

In electrophoresis of the CRSV coat protein in sodium dodecyl sulfate - polyacrylamide gels (SDS-PAGE), Levy and Gumpf (1991) reported the presence of a single polypeptide of 29 KDa in a isolate of Psorosis. 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, Garcia *et al.* (1994) also reported 48 and 50 KDa coat protein in CRSV. Navas-Castillo *et al.* (1993) reported 38 KDa and 48 KDa protein associated with capsid protein of CRSV. Byadgi *et al.* (1993) reported 29 KDa coat protein in an Indian isolate of ICRSV. One isolate of Psorosis and one isolate of ringspot had 46 and 48 KDa protein respectively (Navas-Castillo and Moreno, 1995). Pant and Ahlawat reported 60 and 67 KDa coat protein associated with thin flexuous particles of ICRSV. Rustici *et al.* (2000) reported 34 KDa coat protein associated with an isolate of ICRSV.

Serology:

Polyclonal antibodies specific for CRSV and ICRSV had been produced and used CRSV antiserum for detection in indirect Enzyme-linked immuno-sorbent assay from citron and sweet orange plants inoculated with isolate p-203-m (Levy and Gumpf, 1991), Byadgi *et al.* (1993) used ELISA for detection of ICRSV. ISEM technique was used for detection of CRSV in diseased trees by Derrick *et al.* (1991); Navas-Castillo *et al.* (1991); da Graca *et al.* (1991), Garcia *et al.* (1994) and Ahlawat *et al.* (1995). Derrick *et al.* (1991) and da Graca *et al.* (1991, 1992) reported western blotting technique for detection of 48 KDa coat protein of CRSV in infected

plants. Byadgi *et al.* (1993) detected 29 KDa coat protein from isolate of ICRSV in western blotting.

Genome organization:

Citrus ringspot virus 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). Derrick *et al.* (1991) estimated that the migration of bottom component ss RNA was 3.0 to 4.0 x 106. Garcia *et al.* (1991) observed total loss of infectivity of citrus ringspot when it was incubated with RNase and reported CRSV as a ss RNA virus.

Sanchez *et al.*(1998) reported that the citrus ringspot virus (isolate CtRSV-4), contains at least 3 genomic RNAs, two of them (RNA 2 and RNA 3) in the top component, and the largest (RNA 1) in the bottom component.

ICRSV-D, an isolate on Kinnow mandarin from Delhi has been studied, has single - stranded RNA genome consisting of 7560 nucleotides. It contains six open reading frames (ORFs) which encode putative proteins of 187.3, 25, 12, 6.4, 34 and 23 kDa respectively. OFR1 encodes a polypeptide that contains all the elements of a replicase; ORF 2, 3, and 4 compose a triple - gene block; ORF 5 encodes the capsid protein; the function of ORF 6 is unknown. (Rustici *et al.*, 2002).

Management of the disease:

Cheema *et. al.*(1999) had tried to control the disease by exposing the bud-sticks to moist-hot-air. They found that the buds exposed at 50°C for 30 min or more and at 45°C for 120 minutes were most effective *in vivo* and in vitro for eliminating the virus from the infected bud-sticks. However, the virus was not reported to be transmissible through any vector, so other methods of management should be generated like virus - free nucleus

materials, which can be used for disease free certification programme or transgenic approaches must be used.

In citrus, shoot-tip grafting (STG) *in vitro* has been extensively used to developed disease-free plants from commercial citrus varieties (Navarro *et al.*, 1975). Observations made during the routine indexing of the recovered plants indicated that in some instances they still carried infectious. But the potential value of shoot-tip grafting as tool for separating several virus and virus - like agents was suggested (Navarro, 1981). The protocols for STG have been modified with the purpose of increasing the percentage of successful grafting and reducing the virus infection. Increasing the size of shoot-tip could increase the percentage of success graft (Juarez *et al.*, 1990). MT medium was standardized for citrus by Murashige and Tucker (1969), which contained MS medium plus 100mg l^{-1} Myo inositol, 5% sucrose, 10mg l^{-1} thiamine HCl, 10mg l^{-1} pyridoxine HCl, Nicotinic acid 5mg l^{-1} and glycine 2mg l^{-1}. Rapid oxidation of shoot tips reduces the success of STG. The effect of plant growth regulators pre-treatments on the shoot-tip were studied by Edriss and Burger (1984) showed that dipping the shoot tip in 2,4 - D and Kinetin before grafting doubled the percentage of successful grafts. Later, treatment of 2,4 - D (10 mg/l), zeatin (1ppm) and DIECA (100ppm) alone or incombination for improvement of success rate were studied by Mishra *et al.* (2000). The technique of STG *in vitro* has been found effective in recovering citrus cvs. Free of tristeza, Psorosis-A, Psorosis-B, concave gum, infectious variegation, vein enation, dweet mottle, yellow vein, cachexia, sturborn and exocortis. Beside STG, ovule culture was also used for both citrus breeding and permits a high rate of disease free material multiplication (Ollitrault, 1991). The factors effecting in vitro embryogenesis from undeveloped ovules of mature *Citrus* fruit was studied by Moore (1985). The effect of Cytokinin on

the embryogenic callus formation on orange has been studied by Starrantino and Caponnetto (1990). Later the effect of plant growth regulators on embryoids formation and plantlet regeneration were studied by Vijay Kumari and Singh (2000b).

Although, the STG plants serves as a good source of virus - free materials, but no information is available for ICRSV. The another strategy emerging rapidly is pathogen derived resistance infers that pathogen genes can be used to interfere with the normal pathogenic process by causing the host to express pathogen gene products at the wrong time, in the wrong amount, or in counter functional form (Sandford and Johnson, 1985). Either native or altered form of genes might prove useful for interfering with various stages in the viral life cycle such as uncoating, translation, replication, cell to cell or long distance movement or vector mediated transmission.

The genetic engineering strategies that have been employed to date to develop resistance to virus plants include the gene encoding for viral coat protein (Powell-Abel *et al.*, 1986; Golles *et al.*, 1997a - Nepovirus, Gutierrez and Moore, 1997; Moore, 2000 - CTV), truncated coat protein (Golles *et al.*, 1997b; 2000 - grapevine fanleaf virus), viral replicase (Golemboski *et al.*, 1990 - PVY, Anderson *et al.*, 1992 - CMV, Rubino & Russo, 1995 - CymRSV, and Piestun *et al.*, 2000 - CTV), Helper component (Vardi *et al.*, 1993.), 3′ NCR (Zaccomer *et al.*, 1993), movement protein (Lapidot *et al.*, 1993).

Beside these, other strategies like satellite RNAs (Gerlach *et al.*, 1987), antisense RNAs (Cuozzo *et al.*, 1988), plantibodies (Tavladoraki *et al.*, 1993) and pokeweed proteins (Lodge *et al.*, 1993), have been shown to meet with limited amount of success.

Three main methods have been used to produce transgenic plants of *Citrus* spp.: *Agrobacterium* - mediated DNA transfer to plant cells (Hidaka *et al.*, 1990) or protoplasts (Vardi *et al.*, 1990); and biolistic or particle gun introduction of DNA into plant cells (Yao *et al.*, 1996). All these methods have been tried, or are currently being tested in an attempt to transform citrus. One group used *Agrobacterium* - mediated transformation (AMT) of embryogenic nucellar calli (Hidaka *et al.*, 1990). However, seedling internodal segments have been more successful as explant for AMT (Pena *et al.*, 1995.; Piestun *et al.*, 1998, 2000).

Coat Protein mediated resistance (CPMR)

A variety of terms have been used to describe the resistance conferred to transgenic plants by expression of genes encoding virus capsid protein. They are, genetically engineered resistance; virus protection; coat protein protection; coat protein mediated protection or resistance; genetically engineered cross protection.

Coat protein mediated resistance (CPMR) is used to refer to the resistance caused by the expression a virus coat protein (CP) gene in transgenic plants. Accumulation of the CP confers resistance to infection and/or disease development by related viruses. This concept has been described by Sanford and Johnson (1985). The first transgenic plant possessing virus gene-derived, genetically - engineered resistance was the tobacco plant expressing capsid protein (CP) gene of tobacco mosaic virus (TMV)(Powell - Abel *et al.*, 1986). Soon after and since then many other reports came in where CP and other viral genes from other plant RNA viruses successfully protected (or cross protected) the transgenic plant against infection by that (homologous) virus (homologous resistance) as well as against infection by heterologous virus(es) (heterologous

resistance)(Mandahar, 1999). Capsid protein gene, generally singly but sometimes in multiple copies, has been mostly employed for developing resistance. This gene, from at least 41 viruses of 19 different viral groups (Papu *et al.*, 1995), gives fairly good control of diseases of annual plants at experimental level.

In coat protein mediated resistance (CPMR), resistance is manifested by several features:

1. *The number of sites where infection occurs in inoculated plants may be reduced (Nelson et al., 1987), they reported 95-98% fewer necrotic local lesions caused by TMV infection on CP (+) than on CP (-) tobacco plants.*

2. *Resistance may be a delay or absence or systemic disease development (Hemmenway et al., 1988) throughout the CP (+) plants (Powell-Abel et al., 1986).*

3. *Resistance may be a reduction in virus accumulation in infected CP(+) plants compared to infected CP (-) plants (Nelson et al., 1988; Hemenway et al., 1988, Cuozzo et al., 1988; Lawson et al., 1990).*

4. *It may also be a reduction in severity of disease symptoms in plants that become infected.*

All the different manifestations of resistance can usually, but not always be overcome by inoculating with increasing concentration of virus (Powell-Abel *et al.*, 1986).

Mechanism of coat protein mediated resistance

Viral CP is a multifunctional protein. It is needed for viral assembly, RNA binding, and for protecting the viral genome. It is also the primary site of and is involved in several other viral functions in different plant

viruses. It is involved in movement of some viruses within the host plants, is essential for genome activation, infection and replication of some plants viruses, is directly or indirectly involved in accumulation of viral plus - strand RNA of some viruses, plays a crucial role in virus transmission by insect vectors since it is the recognition site of the vectors, is involved in recognition by host resistance functions (as TMV in *Nicotiana* species with N genotype), and has often been used as a transgene for imparting resistance to transgenic plants against virus diseases. Consequently, CP - mediated transgenic plants can pose certain grave risks because they, after infection with a second virus, are likely the plants naturally infected with two viruses and can be expected to show heteroencapsidation and recombination (Mandahar, 1999).

The possible mechanisms of CPMR, have been discussed extensively (Beachy *et al.*, 1990). It includes:

1. *Prevention of uncoating of the incoming virus.*
2. *Interference with viral translation and/or replication and*
3. *Interference with cell to cell and/or long distance movement.*

In each of the above said mechanism, there is a potential interference point for the coat protein with the incoming virus, and good evidence to indicate that the mechanism of protection is not same in every virus - coat protein - host combination (Beachy *et al.*, 1990).

In several cases, the CPMR is overcome when plants are inoculated with viral RNA rather than whole virions, eg. TMV (Register and Beachy, 1988). Thus it has been proposed that the CP interferes with initial uncoating of the virus. For other viruses, such as PVX (Hemenway *et al.*, 1988), PVS (Mackensize and Tremanie, 1990), the CPMR is effective against

viral RNA as well as whole virions, suggesting that some step other than, or in addition to, uncoating is being affected.

Other mechanism show inference with systemic spread, Hammon and Kamo (1993) observed that although transgenic plants expressing BYMC CP were initially infected, leaves that developed later were either symptomless or reduced or altered symptoms.

Another feature of CPMR that may be reflective of mechanism is the relationship to the level of CP expression. In several experiments the amount of protection observed was directly correlated to the amount of CP presence, eg. TMV (Powell-Abel *et al.*, 1986), PVX (Hemenway *et al.*, 1988). In other viruses, the level of protection was not correlated with the level of CP expression, eg., SMv (Stark and Beachy, 1989), PVY (Lawson *et al.*, 1990), etc. The explanations for the lack of correlation may be cell specific accumulation or differential sub-cellular localization of the CP. It is also possible that the RNA, rather than CP, is responsible for conferring protection.

Consistent with these observations, Vandun *et al.* (1988) and Powell-Abel *et al* (1986) showed that it was CP molecule and not the CP mRNA that confers resistance against AlMV and TMV respectively. Transgenic plants expressing frameshift mutation of the CP gene, or a CP gene lacking the translation initiation codon were not protected against the corresponding virus.

In most of the cases, the transgenic plants were tested for resistance against the virus strain from which the CP gene was derived, i.e. homologous virus. To assess the breadth of protection, many studies also tested for resistance against additional virus strains and/or related, homologous virus.

In general, the highest level of resistance was conferred against the homologous virus. There are many examples where a given CP gene conferred protection against other viral strains and other related viruses.

3. MATERIALS & METHODS

3.1. BIOLOGICAL CHARACTERIZATION

3.1.1. Collection and Maintenance of Indian Citrus Ringspot Virus (ICRSV) culture

Bud sticks from *Citrus spp.* showing ringspot symptoms were collected from citrus orchards at Abohar (ICRSV-Ab), Delhi (ICRSV-Dl), Abmehabad (ICRSV-Ah) and Pune (ICRSV-Pu). These bud-woods were grafted on 3 months old healthy seedlings of Mosambi and Malta sweet oranges, and Kinnow mandarin. These plants were maintained in the glasshouse of the Advanced Center for Plant Virology, Plant Pathology Division, IARI, New Delhi - 110012, and constantly observed for appearance of symptoms.

3.1.2. Multiplication of virus culture

All the four ICRSV isolates were maintained in the glasshouse. However, these isolates were tested for the presence of purity of ICRSV by Immuno sorbent electron microscopy (ISEM) by the method of Pant and Ahlawat (1998) and only pure cultures were multiplied and used in other studies.

3.1.3. Transmission

3.1.3.1. Wedge Grafting

Transmission of all the four isolates was done by wedge grafting on 3-4 month old seedlings of Kinnow and King mandarin, Mosambi and

Malta sweet orange and Rough lemon. Ten plants of each cultivar were inoculated from each isolate.

3.1.3.2. Mechanical transmission

Healthy seedlings of 11 plant species belonging to four families were raised in 4 inches polyethylene pots in an insect-proof glasshouse. These were *Chenopodium amaranticolor, C. quinoa, Cucumis sativus* L., *Cucurbita mosschata* (Dutch), *Nicotiana glutinosa, N. tabaccum., Vicia faba major L. , Glycine max* L., *Phasilus vulgaris* var *singtamy, P. v.* var. *saxa,* and *Vigna sinensis.* Five plants of each species were inoculated by method of Walky, 1983 with suitable control. The inoculated seedlings were observed daily for 4-5 weeks and finally tested for the presence of virus in electron microscope by leaf-dip method.

3.1.4. Physical properties

The dilution end point (DEP), thermal inactivation point (TIP) and longevity in vitro (LIV) at room temperature were worked out following the method of Ahlawat and Chelulu (1984). The virus culture used for these studies was taken from ICRSV inculated *P. vuigaris* var. *singtamy* plants and test plants of the same variety were used.

3.2. SEORLOGICAL CHARACTERIZATION

3.2.1. Virus Purification

Two methods of purification of ICRSV were tried (Byadgi *et al.,* 1993 and Rustici *et al.,* 2000) with the samples from *P.v* var *singtamy* inoculated plants and their comparative efficacy was determined by counting the number of particles in 40 μm area in the Electron microscope.

The symptomatic leaves of *Phaseolus vulgaris* var. *singtamy*, at 15-20 days after inoculation were harvested for this purpose.

3.2.2. Ultra - violet absorbance Profile

Twenty microlitres of purified preparation was diluted to 25 times with phosphate buffer (0.05 M, pH 7.2) to make the volumes 500 µl. The quartz cuvette was filled with phosphate buffer and calibrated to zero absorbance. After calibration, the cuvette was taken out and filled with 500µl of the virus preparation. UV absorbance was taken at 260 nm and 280 nm in Kontron - UV KON- 93 spectrophotometer.

3.2.3. Electron microscopy (EM)

For electron microscopy, 10µl of purified virus preparation was put on carbon coated copper grid 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 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 nm 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:

$$\text{Size of virus particles (nm)} = \frac{\text{Particles size in mm}}{\text{Magnification}} \times 1{,}000{,}000$$

3.2.4. Production of Antiserum

For preparation of polyclonal antibodies (PAbs), freshly purified virus preparations were used. The virus suspension (700 µl per injection) emulsified with Fruend's incomplete adjuvant (1:1 v/v) was injected intramuscularly to New Zealand white albino rabbit. Four injections were given at weekly interval and the rabbit was bled two weeks after the last injection and about 15 ml blood was collected. The blood was allowed to coagulate at room temperature and then kept at 4°C overnight. The clean antiserum was decanted and clarified by low speed centrifugation at 6,600 g for 5 minutes. The antiserum (supernatant) was mixed with equal volume of glycerol and stored at 4°C in small aliquots of 500 µl. A very small amount of sodium azide was added to each vial to prevent infection by bacteria and fungi. The antiserum was also used for preparing reagents for detection of the virus in ELISA system as given below.

3.2.4.1. Purification of immunoglobulin (IgG)

Immunoglobulin (IgG) was purified from polyclonal antiserum as described by Clark and Adams (1977).

1. *To 1.0 ml antiserum in glass tube added 9 ml distilled water*
2. *Added 10ml saturated neutralized ammonium sulfate solution*
3. *Left 30-60 min at room temperature*
4. *Centrifuged at 12000 rpm for 10 min at 4°C to collect precipitate*
5. *Dissolved precipitate in 2ml half strength phosphate buffer saline*
6. *Dialyzed 3 times against 500 ml half strength PBS*
7. *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 10ml of DEAE - cellulose (DEAE - sephacel. Sigma chemicals) slurry was poured down from the sides of column to avoid air bubbles and allowed the cellulose to settle. The column was calibrated by washing with half strength PBS (pH 7.4) till the pH of ingoing buffer equals with the pH of effluent. The liquid level was never allowed to drain at 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 precalibrated 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 fraction to obtain elution curve from purified IgG.

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

One - step glutaraldehyde method (Clark and Adam, 1977) was used for preparation of antibody-alkaline phosphatase as homologous conjugate (virus IgG AP). 2 ml of purified IgG (1 mg/ml) were mixed with 200µl (= 2500 units) of enzyme, alkaline phosphatase (Sigma Chemical) and final concentration was made with 0.05% fresh EM grade glutaraldehyde. The mixture was incubated at room temperature for 4 hrs

till a faint brown colour was developed. The mixture was dialysed against three changes of 500 ml half strength PBS at 8 hrs interval. After dialysis, Bovine serum albumin was added to a concentration of 5 mg/ml and stored at 4 °C in a refrigerator.

3.2.4.3. Titre of antiserum in Enzyme-Linked Immunosorbent Assay (ELISA)

3.2.4.3.1. *Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA)*

To determine the optimum concentration of antigen, IgG and homologous conjugate for detection of ICRSV in citrus plants by DAS-ELISA, method of Clark and Adams (1977) was followed. The test was performed as detailed in the Flow chart: The composition of various buffers and reagents used in this experiment are given in Appendix I.

1. *Added 200 µl purified γ-Globolin (IgG) in Coating buffer (1µg/ml and 2µg/ml) to each well in the micro – titre plate (Borosil).*
2. *Incubated plates at 37°C for 2 hrs.*
3. *Washed with PBS – T (3 times, 3min/time).*
4. *Added 200 µl test sample. (Grind 1 part of tissue in 10 – 20 parts of phosphate buffer, pH 7.8. Extract of healthy leaves served as negative control. Partially purified virus: buffer (1:100) as positive control).*
5. *Incubated plates at 4°C overnight.*
6. *Washed with PBS – T (3 times, 3min/time).*
7. *Added 200 µl Enzyme-labelled γ-globulins diluted in conjugate buffer to appropriate concentrations. (1:1000, 1:2000, 1:3000 and 1:4000).*
8. *Incubated at 37°C for 2-3 hrs.*
9. *Washed with PBS – T (3 times, 3min/time).*
10. *Added 200 µl p-nitrophenyl phosphate 0.6mg/ml in substrate buffer.*
11. *Incubated at room temperature for 30 min to 2 h for colour development*

12. *Stopped reaction by adding 50 µl of 3 M NaOH to each well.*
13. *Measured the absorbance of each well at 405 nm in a micro-plate reader (TECAN A – 5082, Sun Rise, Austria).*

3.2.4.3.2. *Direct Antigen Coating – ELISA (DAC – ELISA)*

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

1. *Coated the each well of the plate with 200µl of prepared samples of antigen. The samples were prepared by grinding tissue in PBS buffer containing 2% PVP (MW 44,000) at 1:20 (w/v). The homogenate was centrifuged at 5000 g for 10 min. and the supernatant was used for coating the plate directly. Control samples were also prepared in the same way.*
2. *Incubated the plate over night at 4°C.*
3. *Washed the plate with PBS-T as described for DAS – ELISA.*
4. *Added 200µl/well of the blocking solution and incubated at 37°C for one h.*
5. *Washed as described earlier.*
6. *Added 200µl/well of virus–specific antibody (IgG) at dilution of 0.5, 1, 2, 5µg/ml and incubated at 37°C for 2 hrs.*
7. *Washed as described earlier.*
8. *Added enzyme conjugate 200µl/well at 1:10,000 dilution (anti rabbit IgG – Alkaline phosphatase (Sigma chemical co,).*
9. *Incubated at 37°C for 1 h.*
10. *Washed as described earlier.*
11. *Added 200µl/well of the substrate (P-nitrophenyl phosphate, 0.6 mg/ml) and allowed for colour development at room temperature.*
12. *Intensity of colour was measured at 405 nm using a micro plate reader (TECAN A-5082, Sun Rise, Austria).*

The optimum conditions of DAC-ELISA were used for virus detection from field trees.

3.2.4.4. *Dot Immuno Binding Assay (DIBA):*

One experiment was planned if all the four isolates of the ICRSV could also be detected by this method. The following protocol was used.

1. *Extracted tissue 1:10 (w/v) in antigen extraction buffer and filtered through muslin cloth.*
2. *Took 0.8 ml of filtrate into a microfuge tube and added 0.4 ml chloroform, vortexed and centrifuged at 12000 g for 2 min.*
3. *Took 200 µl of the clarified sap into 800 µl of antigen extraction buffer and vortexed.*
4. *Cut a desired size of nitrocellulose membrane (NCM) and made squares of 1 x 1 cm with a pencil (Handle NCM with gloves/forceps).*
5. *Wet NCM by floating it on TBS and then air dried. Spot the test/control samples (5-10 µl) by hand.*
6. *Allowed the NCM to air dry and immersed in blocking solution with gentle shaking for 1 h at room temperature.*
7. *Rinsed once in TBS for 10 min.*
8. *Incubated the NCM with primary antibody diluted TBS-SDM.*
9. *Washed the NCM three times (10 min each) with TBS.*
10. *Transferred the NCM into second antibody (enzyme labeled anti-rabbit IgG alkaline phosphatase).*
11. *Washed the NCM as earlier.*
12. *Incubated the NCM in substrate solution at room temperature and watched for colour development (good colour development take 5-10 min).*
13. *Rinsed the NCM twice (10 min each) in fixing solution and then air dried*
14. *Photographed NCM (dry or wet) and stored in dark.*

For comparative efficacy, the samples tested in DAC-ELISA were also tested in DIBA.

3.2.4.5. Estimation of optimum antiserum titre for detection of ICRSV by Immunosorbent Electron Microscopy (ISEM)

The titre of ICRSV antiserum was estimated by decoration method of ISEM (Derrick, 1973). A constant dilution of leaf extract as 1:10 from infected leaves was used. Carbon coated grids (film side down) were placed on a drop of this extract for 15 minutes at room temperature. The grids were rinsed with 20 drops of distilled water. Virus coated grids were separately floated on drops of diluted antiserum (1:2 to 1: 8192) and incubated for 15 minutes at room temperature. After incubation, grids were washed with distilled water followed by staining with 2 % uranyl acetate, and examined in electron microscope.

3.3. MOLECULAR CHARACTERIZATION

3.3.1. Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

3.3.1.1. Isolation of RNA

The total RNA was isolated from ICRSV infected citrus leaves following manufacturer's instruction (QIAGEN). Initially 100 mg of tissue were taken for isolating the RNA. The samples were ground with the help of liquid nitrogen, powdered samples were immediately soaked with 450 µl lyses buffer (RLT buffer), vortexed for 10 sec. and incubated at 56°C for 3 min in water bath. Clear lysate was then passed through another spin column and centrifuged at 8,000 g for 2 min in a bench top centrifuge at room temperature. The flow-through was transferred to a new eppendorf and half volume (225µl) of 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 700µl 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 extracted from non-infected tissue used as control. RNA was isolated from ICRSV-Ab, ICRSV-Ah, ICRSV-Dl and ICRSV-Pu isolates using this protocol. Further protocol was followed as described by Sambrook *et al.* (1989) and Sambrook and Russel (2001). The compositions of various buffers or reagents used are given in Appendix II.

3.3.1.2. Primers

Four different primers have been synthesized from "Genset Oligos: a division of Genset". Of them, two were forward primers, and two were reversed primers, from them four sets of primers were tried for amplification of the coat protein gene of ICRSV.

- ICRSV- 518: 5′ - CAACCTACTCAACATCAACC - 3′ (FW)
- ICRSV-1096: 5′ - CGACCCCTTTCAACACTTAA - 3′ (FW)
- ICRSV-951: 5′ – GTTCCAGAGGGACATAGAGG - 3′ (RW)
- ICRSV-1420: 5′ - GTCAATGACCTAATCGGTCC- 3 ′ (RW)

However, these primers amplify only fragments from the coat protein of the virus. Therefore, another set of primers was designed for amplification of the putative coat protein gene. They are CP-FW (5'-cccgggatccATGAGCTTTGACTACACA-3') containing the putative ATG start codon and an adaptor arm with *BamH* I restriction site and CP-R-Eco (5'-cacccgggaattc TTAAGTGTTGAAAGGGG-3') containing the putative stop codon and an adaptor arm with *EcoR* I restriction site.

3.3.3.3. cDNA synthesis

The RNA isolated from infected leaves of citrus using Rneasy Kit (Appendix II) was used as template to perform the following reaction for cDNA synthesis:

Template RNA	10µl
RT buffer 10 X	2µl
dNTPs 10mM	2µl
Complementary primer (ICRSV - 1420, 100ng/µl)	2µl
Reverse transcriptase (M-MuLV, 20 units/µl)	1µl
RNase-free water	2µl
RNAase inhibitor	1µl
	20µl

Template RNA was incubated at 70 °C for 5 - 10 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°C for 45 min.

3.3.3.4. PCR Amplification

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

SDW	26.50µl
10 X buffer	5.00µl
2mM dNTPs	1.00µl
25mM $MgCl_2$	2.00µl
Forward primer (100ng/µl)	2.00µl
Reverse primer (100ng/µl)	3.00µl
2.5 Unit Taq polymerase	0.50µl
RNA template (cDNA)	10.00µl
	50.00µl

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

94 °C	4 min	
94 °C	30 sec.	
55-60 °C	30 sec.	35 cycles
72 °C	3min.	
72 °C	30min	

3.3.3.5. Agarose Gel Electrophoresis

The PCR amplified product was resolved on a 1% agarose gel as follows. 0.4mg of agarose was added to 40ml 1X TAE (prepared in sterile distilled water) and boiled, cooled up to 50°C and 1µl of Ethidium Bromide (10µg/100ml stock) mixed with 6 X loading dye (Orange G or Bromophenol Blue) was added and loaded. Electrophoresis was run at 50-60 Volts for 1 to 1.5 hr. Result was evaluated on a UV-transilluminator light.

3.3.4. Cloning of Viral Coat Protein Gene

PCR product was purified by running the product in 1% agarose gel. The desired band was cut from the gel and was purified using gel extraction kit (Appendix II) following their protocol. The ICRSV CP fragments of ≈1kb and ≈ 350 bp were cloned in pGEM-T easy vector (Appendix II) to get the final overlap product of 1280 bp of CP gene. The viral DNA was restricted with restriction enzymes *EcoR* I.

3.3.4.1. *Ligation*

The purified DNA getting after gel extraction was used for ligation, the composition as following:

Viral DNA	5µl
pGEM-T easy vector	1µl
10X ligase buffer	3µl
T4 DNA ligase (0.2 U/µl)	1µl
	10µl

The ligation mixtures were incubated at 4°C for 18 h. Both the ligation mixtures were then ready for transformation.

3.3.4.2. *Preparation of Competent cells*

The competent cells were prepared by $CaCl_2$ method and transformed as described by Mendel and Higa (1970)

1. *50ml Luria broth (LB) was inoculated with 500µl overnight grown culture of DH5α strain of Escherichia coli (strategene) and incubated at 37°C for 90 min. with constant shaking at 200 rpm in a shaker incubator till the bacterial growth as measured by optical density reached 0.3 OD at 600nm.*

2. *The culture was then aseptically transferred to 40ml sterile screw capped tubes and kept on ice for 10 min.*
3. *The cells were centrifuged at 500rpm for 10 min. at 4°C in a Sorvall SS-34 rotor to pelletize the cells.*
4. *The cells were resuspended gently in 10 ml ice cold 0.1 M $MgCl_2$ solution and kept on ice for 1 h.*
5. *The pellet was resuspended in 10 ml ice cold 0.1 M $CaCl_2$ solution and kept on ice for 1 h.*
6. *The cells were recovered by centrifuging at 5000 rpm for 10 min. at 4°C and the pellets were resuspended in 1 ml of chilled 0.1 M $CaCl_2$ and used for transformation after keeping on ice for 1 h.*

3.3.4.3. *Transformation of Competent cells*

1. *200µl competent cells were added to 10 µl of the ligation mixture in a sterile microfuge tube, gently mixed and kept on ice for 1 h.*
2. *The bacterial cell-DNA mixture was given a heat sock at 42°C for 2 min. 1ml of LB medium was then added and the transformants were allowed to grow at 37°C for 1 h in shaker incubator at 200 rpm.*
3. *200µl of cell suspension was aseptically plated either as such or after concentration on Luria Agar (LA) plate containing 100µg/ml of ampicillin, 200µg/ml of X-gal, and 20µl of 0.1 M IPTG for 100ml LA).*
4. *The plates were incubated overnight at 37 °C.*

3.3.4.4. *Selection of Transformants*

The transformants were selected on the basis of blue/white colony colour. The white colonies were selected and subsequently plated on IPTG – X-gal – Ampicillin plates. The plate having individual transformants in grid served as master plate.

3.3.4.5. *Rapid Screening for the Recombinant Clones having insert*

White recombinant bacterial colonies may be formed either due to insertion of viral genome or due to even a small deletion in the lac Z operon of vector itself. To know which white colonies have the insert a rapid screening method was used which was as follows:

1. *A small amount of overnight grown bacterial colonies were picked from master grid individually with the help of sterile tooth pick and mixed with 50µl of 10 mM EDTA (pH 8.0) in sterile microfuge tubes. One or two blue colonies of pGEM-T transformed bacterial cells were also taken for control.*

2. *50µl of fresh lysis solution (2N NaOH, 0.5% SDS, 20% sucrose) was added in each tube and vortexing was done for 30 seconds, which caused rapid disruption of bacterial cell wall.*

3. *The mixture was then incubated at 70°C for 5 min. and was cooled down to room temperature.*

4. *1.5µl of 4 M KCl and 0.5µl of 0.4 per cent of bromophenol blue were added to each tube and vortex was done for 30 seconds.*

5. *The mixture was then incubated for 5 min. on ice and centrifugation was done at 10,000 rpm for 30 min. at 4°C in bench top centrifuge.*

6. *Bacterial cell debris was removed and 30 µl of supernatant from each tube along with the control were electrophoresed in 0.8 per cent agarose gel.*

7. *The lanes, which showed higher plasmid band than the control was considered as recombinant plasmid and the respective colonies were taken for isolation of recombinant plasmid DNA.*

3.3.4.6. *Isolation of Recombinant Plasmid DNA by Miniprep Method*

Isolation of recombinant plasmid DNA was done by modified alkaline lysis method (Birnboim and Doly, 1979).

1. *Selected white colonies, which were presumed to contain viral insert in rapid screening were individually inoculated in 2 ml of LB medium containing ampicillin (50μg/ml) in sterile capped culture tubes.*
2. *Tubes were then incubated overnight at 37°C at 200 rpm in a shaker incubator.*
3. *The overnight grown bacterial cells were then transferred to 1.5 ml sterile eppendorf tube and cells were harvested by centrifuging in a bench top centrifuge for 1 min. Care was taken to remove the medium adhering to the cell pellet.*
4. *The pellet was resuspended in 100 μl of solution I and mixed vigorously by vortexing.*
5. *Then 200μl of freshly prepared lysis solution i.e. solution II was added and mixed gently.*
6. *150 μl of ice cold solution III was then added and mixed gently with lysed cell suspension and the mixture was kept was kept on ice for 15 min.*
7. *The chromosomal DNA and the bacterial cell debris were removed by centrifuging at 15,000 rpm for 15 min. at 4°C in a bench top centrifuge.*
8. *The supernatant was again centrifuged for another 15 min. at 15,000 rpm at 4°C to pelleted any unwanted bacterial debris.*
9. *The supernatant was taken and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. It was vortexed well, centrifuged in a bench top centrifuge for 15 min. at room temperature.*
10. *The clear aqueous phase was transferred to fresh eppendorf tube.*

11. *The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol, and kept on ice for 10 min.*
12. *The mixture was then centrifuged at 15,000 rpm for 20 min. at 4°C*
13. *To the pellet 200µl of 70% ethanol was added. The tube was rotated well so that the pellet from the wall gets suspended in 70% alcohol. This ensures removal of adhering salts by 70% alcohol. DNA was then pelletized by centrifuging at 15,000 rpm for 15 min.*
14. *The pellet was finally suspended in 30µl sterile distilled water.*

3.3.4.7. *Large scale Plasmid DNA Preparation*

Large scale plasmid DNA preparation of selected ICRSV - CPG clones were done following QIAGEN midi plasmid purification protocol (Appendix II). The procedure was followed as in manual.

3.3.4.8. *PCR Bacterial Colony*

To confirm the ICRSV Coat Protein gene clone in white recombinant colonies obtained, PCR procedure was used. A single colony was picked up from master plate and dissolved in 20µl sterile distilled water and other PCR mixtures were added as before to make up the volume 50µl, then the mixture was transferred to PCR tube and amplified as in section 3.3.3.4.

3.3.5. Restriction Analysis

For analysis of the restriction sites of the CP gene, the sequences of the ICRSV isolate were analysized using clone map software (version 2.11), the common restriction sites were chosen and shown on the map

along with those common for all isolates, and the restriction maps were confirmed by using common restriction enzymes *Pst* I, *Sac* I and *Cla* I to digest the PCR products of primers CP-FW/CP-R-Eco.

3.3.6. Nucleotide sequencing

Selected recombination clone with an insert of ≈ 350 and ≈ 1000 bp ICRSV and purified PCR product of 4 isolates were sequenced at Department of Biochemistry, South Campus, University of Delhi. Sequences were aligned, translated and compared with the sequences had been published (ICRSV-D) using the MacVector program (BLAST) and Clustal W (version 1.82) multiple sequence alignment. Cluster dendograms with pairwise distance matrices were also generated.

3.3.7. Diagnostics for Indian Citrus Ringspot Virus

3.3.7.1. Reverse Transcriptase - Polymerase chain reaction (RT-PCR)

Three sets of primers were used for detection of ICRSV, ICRSV-1096/ICRSV-1420, ICRSV-518/ICRSV-951 and ICRSV-518/ICRSV-1420 primers, which can amplify the products of ≈ 350, 450 and 900 bp respectively.

The total RNA was isolated from leaves of infected plants from four isolates by following manufacturer's protocol in RNeasy kit as described earlier. The product of 50 µl of total RNA extraction of each isolate was incubated at 70°C for 10 min, chilled on ice for 3-5 min and went for RT-PCR as following:

For amplification, the following reactives were added

10 X PCR buffer	10.0µl
20 mM Dithiotheritol (DTT)	10.0µl
5X Q solution	20.0µl
5 X dNTPs (100 µM)	3.0µl
Forward primer (100ng/µl)	1.5µl
Reverse primer (100ng/µl)	1.5µl
RNase inhibitor (40U/µl)	0.5µl
2.5 Unit Taq (5U/µl)	1.0µl
Reverse transcriptase (M-MuLV, 20units/µl)	1.0µl
Template (Total RNA)	51.5µl
	100.0µl

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

42 °C	45 min	
94 °C	2 min	
94 °C	30 sec.	
57 °C	2 min.	35 cycles
72 °C	3 min.	
72 °C	30 min	

The amplified product was resolved on a 1% agarose gel as described earlier. Electrophoresis was run at 60 volts for 1-1.5 hour. Results were evaluated on a UV – trans-illiminator lamp.

3.3.7.2. Nucleic Acid Spot Hybridization (NASH)

Similar to dot blot immunoassay for detection of protein, NASH was used for the detection of nucleic acid. The procedure described by Dijkstra and de Jager (1998) was followed.

3.3.7.2.1. Blotting

1. *Put on disposable gloves and cut a piece of membrane.*
2. *Drew a lattice of 1 cm square with a soft lead pencil and cut one edge to indicate orientation.*
3. *Wet the membrane in 20 X SSC for 30 min and placed it on a sheet of Whatman – 3 mm filter paper.*
4. *Ground the leaf material of 4 isolates separately in TE buffer (1:1 w/v). Also included healthy control. Pressed through muslin cloth.*
5. *Took 200µl of this extract and denatured DNA by adding 0.5 N NaOH for 10 min.*
6. *Added 25µl 3 M sodium acetate (pH 5.0) and made dilution of 1:10 and 1:100 in TE.*
7. *Pipetted carefully 2.5µl of each of the samples in each square of the membrane.*
8. *Let the spots dried at room temperature and baked the membrane in vacuum oven for 2 hrs at 80°C*

3.3.7.2.2. Probe production by random labeling method

In these experiments, the PCR products of ICRSV-1096/ICRSV-1420 primers and ICRSV-518/ICRSV-951 primers from ICRSV-Ab isolate were used for making probe.

1. *DNA fragments obtained from PCR amplification were separated in low melting point (LMP) agarose gel (0.7%) in 1 X TAE buffer at 40V and directly purified by commercial gel extraction kit (Appendix II).*

2. *Three µl of gel mix was taken into a sterile eppendorf tube and 1µl of random hexanucleotide primer was added and boiled for 1 min. at boiling temperature.*

3. *Reaction mixture for probe was prepared by mixing the following reagents using Random Primer Labeling Kit (Appendix II) and radiolabelling was done with ($\alpha^{32}P$) dCTP by random priming method (Feinberg and Vogelstein, 1984)*

3.3.7.2.3. Reaction mixture

Denatured DNA (≅ 30ng)	3.0 µl
Primers (random hexa nucleotide 100µg/ml)	1.0 µl
10 X labeling buffer	2.5 µl
dNTPs (2.5mM)(excluding dCTP)	2.0 µl
Dithiothreitol (DTT)(20mM)	2.5 µl
(α^{32}P) dCTP (4000 Ci m/mol)	3.0 µl
Klenow (DNA Pol. I) (3U/µl)	1.0 µl
Sterile double distilled water	35.0 µl
	50.0 µl

The reaction mixture was then incubated at 37 °C for 2 h

3.3.7.2.4. Prehybridization

1. *The baked NCM blots were kept in hybridization cylinders*
2. *Prehybridization solution was added at the rate of 0.2 ml/sq cm.*
3. *The cylinders were then incubated at 65°C for 4 h in hybridization oven with gentle rotation.*

3.3.7.2.5. Hybridization

1. *The double stranded ^{32}P-labelled DNA probe was denatured in a boiling water bath for 5 min.*
2. *The denatured probe was added to prehybridization solution at 0.5 X 10^6 cpm/ml concentration.*
3. *It was allowed to hybridize for ON at 65°C in hybridization oven with gentle rotation.*

3.3.7.2.6. Autoradiography

1. *The hybridization solution was discarded and the membrane was washed 3 times with 2 X SSC, 0.1 percent SDS at 65°C, each time with duration of 15 min.*

2. *The washed membrane was dried on a paper tower, then placed within folds of cling film, placed in lead cassette and exposed to X-ray film (Kodak) for ON at - 70 °C.*

3. *Autoradiogragh was developed by washing in appropriate developer and fixer and water. The signals were evaluated on a light by their intensity.*

3.4. PRODUCTION OF VIRUS - FREE NUCLEUS PLANTING MATERIALS

3.4.1. SHOOT TIP GRAFTING (STG)

3.4.1.1. Preparation of rootstock seedlings

Seeds of Trifoliate orange (*Pocirus trifoliate* (L.) Raft.), Mosambi and Malta sweet orange (*Citrus sinensis* Osbeck), Rough lemon (*Citrus jambhiri* Lusk), and Kinnow mandarin were used to grow seedlings of the rootstock used in various experiments. Seeds were peeled, disinfested by immersing in a 0.1% Mercury chloride solution for 5 min, and rinsed 3 times with autoclaved distilled water. The seeds were sown in Murashige and Skoog (1962) medium, solidified with 1% agar. The pH of the medium was set initially at 5.7. Each medium was sterilized by autoclaving at 121°C for 15 min and distributed in 25 ml aliquots in 25 x 150mm culture tubes and the 80 x 80 x 100 mm boxes. One seed per tube and nine seeds per box were sown. The cultures were put at constant temperature of 27°C. Seedlings raised in constant darkness were compared with those obtained under 16 hrs daily illumination with 1000 lux light. Different ages of seedlings (10, 15, 20, 25 and 30 days old seedlings) were used for STG.

3.4.1.2. Preparation of scions

3.4.1.2.1. Source of excised shoot tips

Different size of budwood from ICRSV infected field trees and glasshouse plants of Kinnow mandarin and Mosambi sweet orange were taken for preparing shoot tips in various experiments. Sterilization of tissues with $HgCl_2$ was evaluated using various concentrations.

Actively growing shoots of 3 to 5 cm were used in order to avoid shoot tips that were abscising or otherwise degenerating. Terminals 1 cm long were pinched off, stripped of larger leaves, surface sterilized by soaking in 0.1% mercury chloride for 3 min. The disinfested tissues were rinsed 3 times with autoclaved distilled water, and their shoot tips were excised and used as scions.

In another experiment, budwood containing lateral buds were first sterilized in 0.1% mercury chloride solution, and wash with sterilized water for 3 times and then placed in nutrient tubes. The medium used as described above for growing seedlings. The cultures were maintained under 16 hrs daily exposures to 1000 lux light at a constant temperature 27°C. The shoots that arose in these cultures were utilized as sources of shoot tips.

To determine the effect of antioxidants and plant growth regulators on percentage of successful grafts, 2,4 - D, kinetin, DIECA, zeatin, BAP were used at different concentrations for pre-treatment of budwood for 10 minutes before excising shoot tip.

3.4.1.2.2. Size of shoot tip

The size of shoot tip was of plus 2, 4 or 6 leaf primordia or the meristem dome alone also tested.

3.4.1.2.3. The grafting procedure

The procedure as initially used was as follows:

- *A 2-week old rootstock seedling in a culture tube was decapitated, leaving 1 to 1.5 cm of the epicotyl. A cut was given to epicotyl for insertion of shoot tip.*
- *The root was shortened to 4 to 6 cm and the cotyledons and their auxillary buds were removed.*
- *A shoot tip composed of the apical meristem and adjacent tissue was isolated from the desired source, using a razor blade silver attached to a Beaver surgical handle as a scalpel, and transferred to the cut of the decapitated epicotyl.*
- *The steps of shoot tip excision and transfer to rootstock were carried out aseptically with the aid of a dissecting microscope.*
- *Three methods of grafting were used: Invert T incision, triangular incision and window cut method.*

3.4.1.2.4. The nutrient medium

The STG plants were placed on Murashige and Skooge medium (1962), which was supplemented with nutrients and vitamins, myo-inositol (100mg/l), nicotinic acid (vitamin B 3)(5mg/l), pyridoxin HCl (Vit. B6)(10mg/l), thiamin HCl (Vit. B1)(10mg/l), glysine (2mg/l), Sucrose (5%). 25 ml medium was placed in tubes with 1% agar and without agar, which was supported by a folded 9 cm circle Whatman filter paper No. 1.

The grafted plants were kept upright in dark for 5 days with a constant temperature of 27°C, and were observed periodically using a dissecting microscope. The adventitious shoots arising from rootstocks

were removed ascetically with a forcep. After 5 days the light intensity was increased to 10,000 lux.

3.4.1.3. Transfer of the STG plants to soil

Plants showing 2-4 expanded leaves in the shoot tip after 5 to 6 weeks of grafting were transplanted directly to soil in 10 cm pots containing a soil mixture and full MS medium, or directly grafted on the Mosambi seedlings in the glasshouse. The pots were covered with polyethylene bags to minimize moisture loss and shaded with brown paper for a week, and then the plants were allowed to grow under glasshouse.

3.4.1.4. Indexing for viruses

After hardening, all STG plants raised from field sources were tested for the presence of viruses by Direct Antigen Coating Enzyme Linked Immonosorbent Assay (Clark and Bar – Joseph, 1984), with specific polyclonal antibodies of ICRSV, CTV, CVCV, CYMV. ICRSV in plants raised from glasshouse was tested by DAC-ELISA and was further confirmed by dot blot hybridization with virus specific probe. Virus - free STG plants were multiplied and maintained in the glasshouse.

3.4.2. OVULE CULTURE

3.4.2.1. Source of embryo

Fertilized and unfertilized ovules of Kinnow and Mosambi were collected from IARI field at different stages. The ovules were excised and

placed on solidified medium in 12 cm Tarson Petri disks. The medium used was either Murashige & Tucker (1969) alone or with benzyladenine (BA) and kinetin (KT) with a concentration from 0 to 10mg/lit, containing 5% sucrose. The pH of the medium was adjusted to 5.7 and added 1% (W/V) agar and then autoclaved. The disks were kept in tissue culture room at 27°C with 16 hrs light.

The callus produced from the ovule cultures was sub-cultured on the MT medium with 1 mg/lit of NAA or 2mg/lit of NAA plus 2 mg/lit of kinetin or with BA and Gibberellic acid (GA) from 1 to 2mg/l and 5% sucrose. All the cultures were maintained at 27 °C with 16 hrs light.

3.4.2.2. The embryo grafting technique

Small *in-vitro* cotyledonary embryos and plantlets were selected and then grafted on four months old Mosambi seedlings by inverted T grafting for embryo and mini wedge grafting for plantlets. The grafted plants were enclosed in transparent plastic bags in order to conserve high hygrometry and maintained at 26 °C with 16 hrs of dim light daily. After 3 weeks, the grafted plants were placed in a glasshouse.

3.4.2.3. Indexing for free of ICRSV

ELISA and Nucleic Acid hybridization were used to test for ICRSV in regenerated plants.

3.5. TRANSFORMATION OF TRUNCATED COAT PROTEIN GENE OF ICRSV-Ab ISOLATE INTO FRENCH BEAN AND CITRUS

3.5.1. Selection of CP-gene of ICRSV for construction

The PCR product of CP-FW/CP-R-Eco primers of ICRSV - Ab isolate was cloned in E. coli DH5α pGEM-T easy vector. The sequence was analyzed as shown in 4.3.3 portion and used for construction.

3.5.2. Cloning of coat protein gene into plant transformation vector

The *Agrobacterium tumefaciens* Ti plasmid derived binary vector pBI 121 (Fig. pBI121), was used for making constructs containing CP gene. In the present study the CP gene of ICRSV - Ab isolate was used. From the original vector, β- glucaronidase gene (gus) was removed leaving intact CaMV 35S promoter and NOS-terminater by restricting the vector with double digestion using *BamH* I and *Sac* I. *Sac* I was used in place of *Sst* I since the sequence recognition is same for both the enzymes (*Isozysomers).* Corresponding *BamH* I enzyme site was generated by oligonucleotide-directed mutagenesis in the forward primer and starting codon ATG were incprporated as well in a few nucleotides upstream to the N terminal end of CP gene. In the C terminal site, the *Sac* I site used was the *Sac* I site of pGEM-T easy vector.

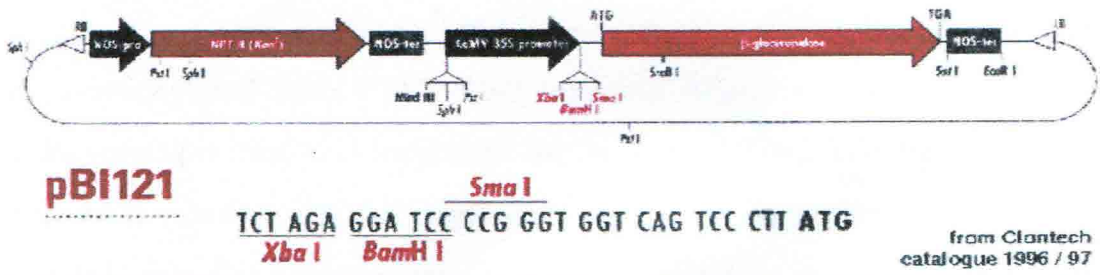


Fig. pBI 121: The map of plant transformation vector pBI 121

3.5.3. Preparation of vector and inserted fragment DNA

Both the ICRSV-CP gene and pPBI 121 was prepared by extraction the plasmid from recombinant *E.coli*, and were restricted with *BamH* I and *Sac* I and checked on 1% agarose gel with 1xTAE buffer and the required products were purified by gel extraction kit (described earlier), and then used for ligation.

3.5.4. Transforming bacterial cells with ligation mix

The competent cells *E. coli* DH 5α for transformation were prepared by Calcium chloride method (Sambrook et al., 1989). The eluted product was ligated with linearised pBI 121 as indicated below. This resulted in the ligation of the CP gene in the sense direction to create CpS (coat protein sense). Later it was renamed as CpSTR (coat protein sense truncation).

Recipe for ligation mix

Reagents	Volume required (µl per reaction)
Linearised pBI 121 vector	10
Insert	15
T_4 DNA ligase	2
10 X ligase buffer	13
	40

Ligation mix was kept at 16°C water bath overnight. Before ligating coat protein gene, the vector DNA restricted ends were dephophorylated with CIAP (Calf Intestine Alkaline Phosphatase @ 2 units/reaction mix) and incubated for 30 min. at 37°C. The enzyme was then heat killed at 65°C for 2 hrs. After ligation, 200 µl competent cells of *E. coli* (strain DH 5α) were transformed with ligation mix. The cells were plated on to LA plates containing Kanamycin (50 µg/ml) and incubated at 37°C.

The insert was confirmed by colony PCR from the transformed colonies with CP-FW/CP-R-Eco primers and later with CP-FW/ICRSV-951 primers. On the other hand, DNA was isolated from the transformed colonies and restricted with same enzymes used for cloning and analyzed in 1% agarose gel in 1xTAE buffer and further confirmed by Southern hybridization with the probe made from PCR product of ICRSV-518/-951 primers, the protocol followed is given below.

Southern hybridization

The same colony PCR product analysed in 1% agarose gel electrophoresis, was used for further confirmation by Southern hybridization. A ^{32}P-labelled probe prepared from CP gene of ICRSV-Ab (Described earlier), was used for confirmation. Standard Procedure for Southern hybridization (Sambrook and Russell, 2001) was followed:

1. *Agarose gel after visualizing on the UV transilluminator (UVP Gel Documentation system) was cut at the position of the wells at the top and the RNAs at the bottom and measured accurately.*
2. *The top right corners were cut to fix the correct orientation of the gel.*
3. *The DNA in the gel was alkali denatured by soaking the gel for 45 min. in several volumes of denaturation solution (1.5 M NaCl. 0.5 N NaOH) (Appendix I) with constant shaking.*
4. *The gel was then neutralized by soaking it in several volumes of neutralization solution (1M Tris-HCl, pH 7.4; 1.5 M NaCl) (Appendix I) for 45 min. with constant shaking.*
5. *Finally, the gel was set up for capillary transfer of DNA onto nitrocellulose membrane (NCM). NCM was of the same size as the gel and was also cut at the top right corner to fix the correct orientation vis-à-vis of the gel.*

6. *20 X SSC (Sodium Chloride Sodium Citrate buffer) (Appendix I) was used as transfer buffer.*
7. *The capillary transfer of DNA was allowed to proceed for 24hrs.*
8. *The membrane was baked at 80°C for 2hrs in vacuum.*

The protocol for pre-hybridization, probe production, hybridization and autoradiography was the same as described in NASH earlier. The film was thereafter processed and the results recorded from the autoradiogram.

3.5.4. Introduction of plasmid into *Agrobacterium* (Triparental Mating)

CP gene construct in pBI121 binary vector (CpSTR) (maintained in *E. coli* strain DH 5α) (Donor strain) was mobilized into the disarmed *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema *et al.*, 1983) by triparental mating procedure (Ditta *et al.*, 1980), using the helper plasmid pRK 2013 (Comoi *et al.*, 1983) in *E. coli* / HB 101 (Helper strain).

In 50 ml LB medium containing kanamycin (50 µg/ml) (Appendix III), single colony of donor strain and helper strain (pRK 2013) in *E. coli* were inoculated for over night growth separately at 37 °C with continuous shaking at 200 rpm. *A. tumefaciens* strain LBA 4404 was inoculated in 50 ml LB medium having Rifampicin (50µg/ml) at 28°C with continuous shaking at 200 rpm, this step has been done one day before growing donor and helper. Next day, one ml each of donor and helper, and 2 ml of recipient cell cultures were added in a sterile culture tube and mixed the three cultures together. From this 100 µl mixed cell suspension was placed on to the sterile nylon membrane (1x1 cm square) positioned in the center of a LA plate without any antibiotics. The plates were incubated at 28°C overnight for conjugation. After 16-18hrs, the nylon membrane with

grown cells was placed in a beaker with 5ml sterile distilled water. The cells were removed from nylon membrane by vigorous shaking and 100 µl cell suspension was spread on LA plates containing Rifampicin (50 µg/ml) and Kanamycin (50 µg/ml) and incubated for 2 days at 28°C for appearance of single colonies of transconjugants. Later, well isolated single transconjugant colony was picked up and transferred to fresh LA plates having rifampicin and kanamycin antibiotics and maintained at 28°C.

3.5.5. Confirmation of gene construct in *Agrobacterium*

Successful mobilization of gene construct in to *Agrobacterium tumefaciens* strain LBA4404 was confirmed by colony PCR using primers CP-FW/ICRSV-951.

Colony-PCR

A single transconjugant colony of *A. tumefaciens* was picked up and rinsed with 20 µl of sterile distilled water in an eppendorf tube. In order to break the bacterial cells and release the plasmid construct, the rinsed bacterial suspension was alternatively immersed in liquid nitrogen and boiling water. The suspension was then pulse centrifuged to remove debris and supernatant solution (containing plasmid) was used as template aliquot for PCR reaction.

Procedure followed there after was same as DNA PCR described earlier. The colony PCR product was analysed in 1% agarose gel electrophoresis.

3.5.6. French bean and citrus transformation with coat protein gene

3.5.6.1. Source of explant

Seeds of French bean (*Phaseolus vulgaris* var. *saxa*) were sown in Murashige and Skoog (1962) medium, solidified with 1% agar. The pH of

the medium was set at 5.7. Four to five seeds per majenta box were sown one to two weeks before transformation. While in case of citrus, Kinnow mandarin seeds were sown one month before (as described earlier), and another set of experiment was done with the citrus plants grown from ovule culture.

The hypocotyls of young seedlings were excised to segment of 1-2 cm, each segment was cut with three open wound and used as explants for co-cultivation with *Agrobacterium* cells having coat protein gene construct.

3.5.6.2. *Preparation of A. tumefaciens bacterial suspension for transformation*

Single colony of *A. tumefaciens* containing the construct was transferred to 50 ml LB medium with rifampicin (50µl/ml) and kanamycin (50µg/ml) and incubated at 28°C for 36 h with shaking at 200 rpm. Bacterial cells without vector were also inoculated to serve as control. Well grown bacterial cells were pelletized by centrifugation at 5000 rpm for 10 min and were suspended in 10 ml half-strength modified MS medium without hormone.

3.5.6.3. Co-cultivation

Co-cultivation was carried out as described by Horsch *et al.*, (1985), which involved three day co-cultivation of the explants with *Agrobacterium* having coat protein gene construct and kanamycin resistant selectable marker. The stem segments from the explants were submerged in *A. tumefaciens* culture for 10 min by gentle shaking to ensure bacteria to attach to all cut edges. Removed the segments and blotted dry them on

sterile filter paper before, transferring to the co-cultivation medium (CM) (Appendix III) and incubated in culture room with temperature 26°C for 2 days. The explants submerged in half-strength MS nutrient solution and co-cultivated with wild *Agrobacterium* (without construct), which served as control. The segment explants co-cultivated with *A. tumefaciens* containing plasmid construct were simultaneously transferred to selection medium (SM) (Appendix III). The control explants were transferred to regeneration medium/co-cultivation medium (CM), and SM. In each plate 15-20 co-cultivated leaf explants were transferred and the plates were kept under ambient growth conditions.

3.5.7. Growth of transformed material

The shoot buds regenerated from transformed cut end of segments and control explants were excised out and transferred to majenta box. After 2-3 weeks when the shoot buds of French bean elongated into plantlets, they were transferred into rooting medium (RM) (Appendix III). When profused roots were produced in the rooting medium, plants were carefully pulled out of medium and adhering agar pieces were removed and they were acclimatized by keeping in MS salts devoid of sucrose for one week and in sterile distilled water for another week.

While in citrus, the regenerants were obtained from apical cut in regeneration medium, which contained MT organics (Murashige and Tucker, 1969), 5% sucrose, kinetin and IAA at 1mg/lit. A selective medium of kanamycin was utilized at 200µg/ml. The regeneration medium contained 300µg/ml cefotaxime to prevent excessive growth of the *Agrobacterium*. When meristem started to emerge from the explant

media containing antibitotic against *Agrobacteria* (Cef 300 μg/ml), and kept for 2 weeks.

The plates containing the explants during pre-culture, co-cultivation, and 3 weeks under selection were maintained in the dark at 26°C. During the last week under kanamycin selection, plates were changed to a light/dark regime of 16/8 hrs with low light intensity.

3.5.8. Genome DNA analysis for the transgene integration

The leaves of transformed plants were used for DNA extraction (DNAeasy mini kit)(Appendix II), the protocol as following:

1. *Ground 100 mg of bean leaf under liquid nitrogen to a fine powder using a mortar and pestle, transferred the tissue powder in to eppendorf.*
2. *Added 400μl of buffer AP1 and 4μl of RNase A stock solution (100mg/ml) to a maximum of 100 mg of ground (wet weight) and vortexed vigorously.*
3. *Incubated the mixture for 10 min at 65°C, mixed 2-3 times during incubation by inverting tube.*
4. *Added 130μl of buffer AP2 to the lysate, mixed and incubated for 5 min on ice.*
5. *Applied the lysate to the QIA shredder spin column sitting in a 2 ml collection tube and centrifuged for 2 min at maximum speed.*
6. *Transferred flow-through fraction from step 5to a new tube without disturbing the cell debris pellet.*
7. *Added 1.5 volume of buffer AP3 to the cleared lysate and mix by pipetting.*
8. *Apply 650 μl o the mixture from step 7, including any precipitate which may have formed, to the DNeasy mini spin column sitting in a*

2 ml collection tube. Centrifuged for 1 min at 8000 rpm and discarded flow-through.

9. *Placed DNeasy column in a new 2 ml collection tube, added 500 µl buffer AW to the DNeasy column and centrifuged for 1 min at 8000 rpm. Discarded flow-through and reused the collection tube in next step.*
10. *Added 500µl buffer AW to the DNeasy column and centrifuged for 2 min at maximum speed to dry the membrane.*
11. *Transferred the DNeasy column to a 1.5 ml or 2 ml microcentrifuged tube and pipetted 100µl of preheated (65°C) buffer AE directly onto the DNeasy membrane. Incubated for 5 min at room temperature and then centrifuged for 1 min at 8000rpm to elute. It was ready for use in PCR.*

The PCR was done with the same primer for checking the presence of transgene, then analysed in 1% agarose gel in 1xTAE buffer and further confirmed by southern hybridization with the probe made from PCR product of ICRSV-Ab isolate using CP-FW/ICRSV-951 primers, the protocol followed as described earlier.

3.5.9. Expression of transformed coat protein gene

To check the expression of coat protein gene presence in the plant system, total protein from expected transformed plants was extracted by using PST buffer and further tested by DIBA using homologous antiserum as per the protocol described earlier.

RESULTS

4. RESULTS

4.1. BIOLOGICAL CHARACTERIZATION

4.1.1. Collection and maintenance of Indian Citrus Ringspot Virus (ICRSV) culture

During the studies, samples from trees showing ringspot symptoms on Kinnow mandarin and Mosambi sweet orange and necrotic spot symptoms on mandarin (Fig. 1) were collected from commercial citrus orchards in Delhi, Abohar, Ahmedabad, and Pune. These were grafted on healthy Kinnow mandarin, Mosambi and Malta sweet orange in the glasshouse and tentatively named from the place of collection (Fig. 2) and Table 3). All these isolates upon grafting showed similar symptoms as were observed in the field.

Table 3: ***Isolates of Indian Citrus Ring Spot Virus (ICRSV) collected from different locations in India.***

Place of collection	State	Host	Name of isolate
Abohar,	Punjab	Kinnow	ICRSV-Ab
Ahmedabad,	Gujarat	Rough lemon	ICRSV-Ah
IARI, New Delhi	Delhi	Kinnow	ICRSV-Dl
IARI Regional Station, Pune	Maharashtra	Rough lemon	ICRSV-Pu

4.1.2. Establishment of pure culture

The four isolates of ICRSV were tested by Immunosorbent Electron Microscopy (ISEM) using ICRSV antiserum available at Advanced Centre for Plant Virology, IARI, New Delhi. All the four isolates showed

filamentous particles and all the particles were decorated with ICRSV antiserum (Fig. 3).

4.1.3. Transmission

4.1.3.1. Wedge grafting:

All the four isolates were successfully transmitted to Kinnow and King mandarin, Mosambi and Malta sweet orange and rough lemon. The symptoms were appeared after 30 to 40 days after grafting. The symptoms varied in different cultivars as shown in Table 4.

Table 4: ***Reactions of different citrus cultivars upon grafting with four ICRSV isolates.***

ICRSV isolates	Symptoms on inoculated citrus cultivars				
	Kinnow	Mosambi	Malta	Rough lemon	King mandarin
ICRSV - Ab	CS, RS, VC	VC, RS	CS, RS	CS,VC	CS,NS, VC
ICRSV - Ah	CS, RS, VC	VC, RS, VF	CS, RS	CS, VC	NS, VC
ICRSV - Dl	CS,RS,VC	VC, VF, RS	CS, RS,	CS,VC	NS, VC
ICRSV - Pu	CS, RS, VC	VC, RS	CS, RS	CS, VC	NS, VC

VF: Vein flecking, **CS:** Chlorotic spot, **RS:** Ringspot,
NS: Necrotic spot, **VC:** Vein clearing,

4.1.3.2. Mechanical transmission

The virus was mechanically transmissible to *Chenopodium amaranticolor, C. quinoa* showing necrotic local lesions on inoculated leaves after 5 days of inoculation. Inoculated plants of French bean (*Phaseolus vulgaris* var. *saxa, singtamy*); soybean, broad bean and cowpea showed systemic infection. In French bean, however, local lesions were also developed on inoculated leaves followed by systemic symptoms as browning and necrosis of leaf veins (Fig. 4 and Table 5).

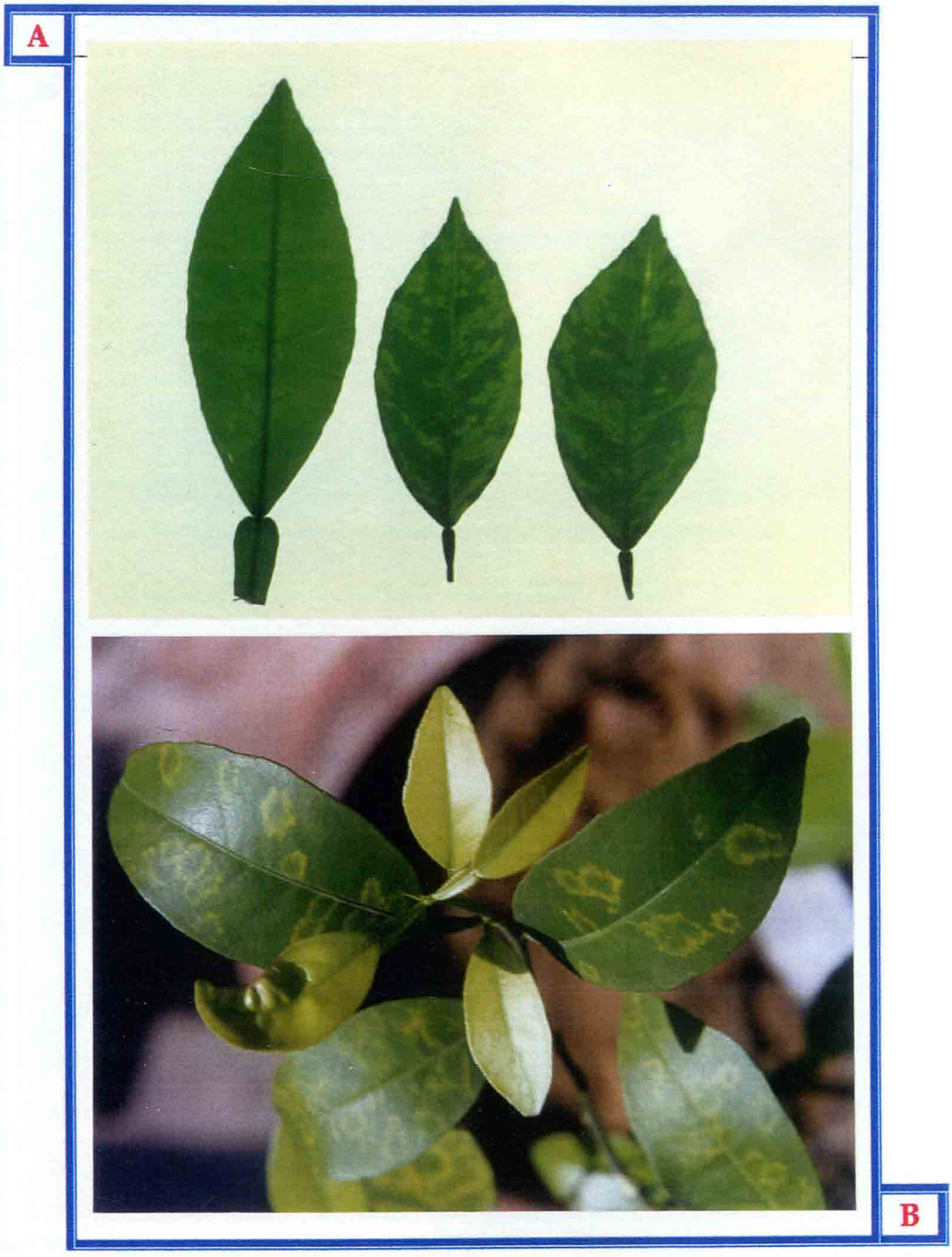


Fig. 1: Symptoms of ICRSV on Citrus:
(**A**)Vein clearing and (**B**) Ringspot on Kinnow mandarin

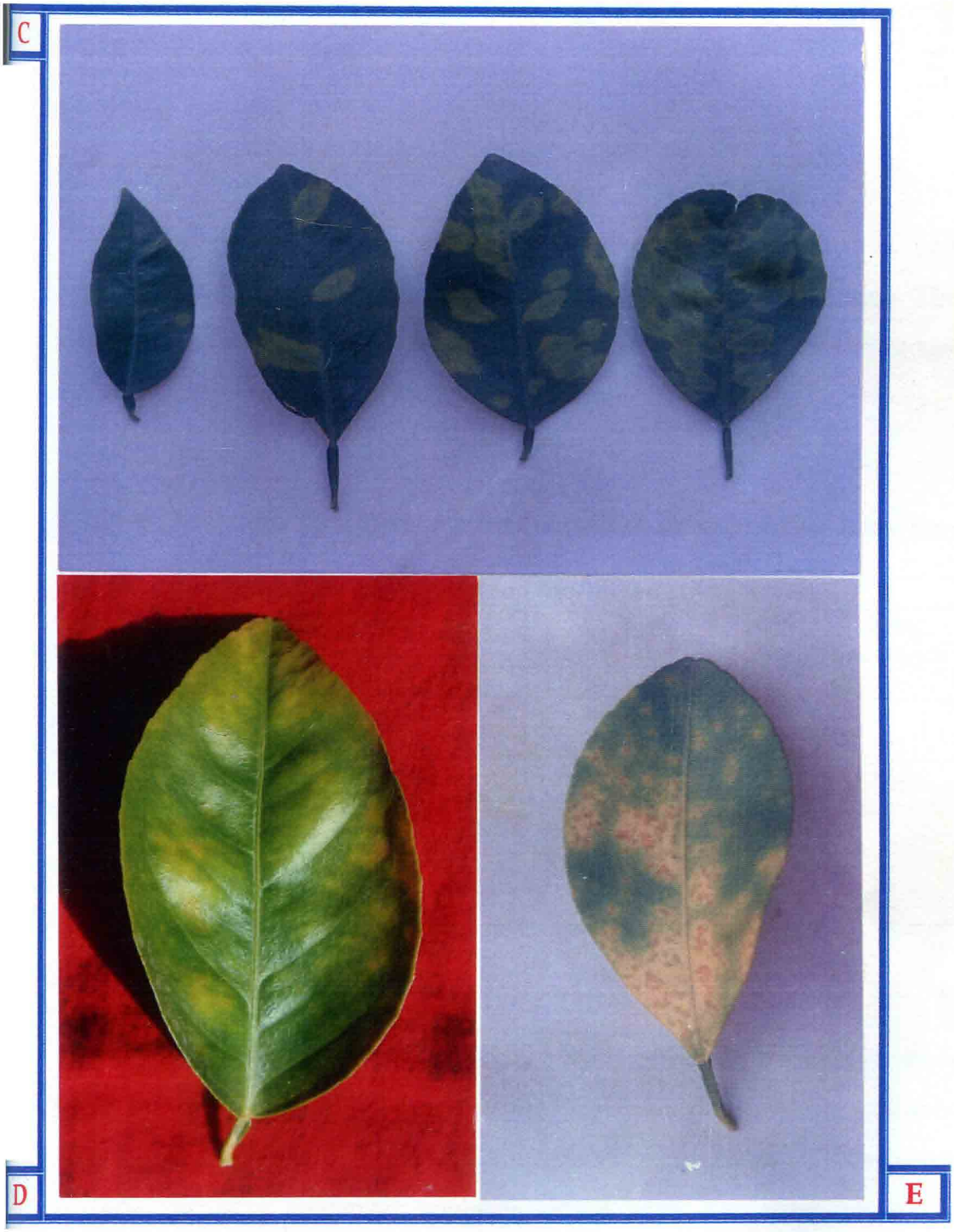


Fig. 1: Symptoms of ICRSV on Citrus (Cont.):
(C) Ringspot on Mosambi sweet orange, (D) Yellow patches on Rough lemon, (E): Necrotic spots on mandarin

Table 5: ***Result of sap inoculation to herbaceous hosts.***

Test plant	No. of plants infected out of 5 inoculated	Symptoms
Legumminosae		
+ *Phaseolus vulgaris* var.		
- *Singtamy*	5	LL, VN, SL, B
- *Saxa*	5	LL, VN, SL, B
+ *Glycine max*	3	LL, VN, SL
+ *Vigna sinensis*	4	VN, M, SL
+ *Vicia faba major* L.	5	VN, B
Chenopodiaceae		
+ *C. amaranticolor*	3	LL
+ *C. quinoa*	4	LL
Cucurbitaceae		
+ *Cucumis sativus* L.	0	-
+ *Cucurbita mosschata* (Dutch)	0	-
Solanaceae		
+ *Nicotiana glutinosa*	0	-
+ *N. tabaccum*	0	-

LL: Local lesion; VN: Vein necrosis, SL: Smalling of leaves,
M: Mosaic, B:Blight

4.1.6. Physical properties

4.1.6.1. Dilution end point (DEP):

The results of DEP are shown in Table 6, which revealed that DEP of the virus was between 10^{-4} and 10^{-5}.

Table 6: ***Determination of DEP of ICRSV.***

Dilution of inoculum	Average No. of Local lesions per leaf (6 replications)
Standard inoculum	227.2
10^{-1}	205.8
10^{-2}	37.2
10^{-3}	7.5
10^{-4}	0.2
10^{-5}	0.0
10^{-6}	0.0
Water control	0.0

Fig. 2: ICRSV isolates: (left to right)
ICRSV-Dl, ICRSV-Ab, ICRSV-Pu and ICRSV-Ah

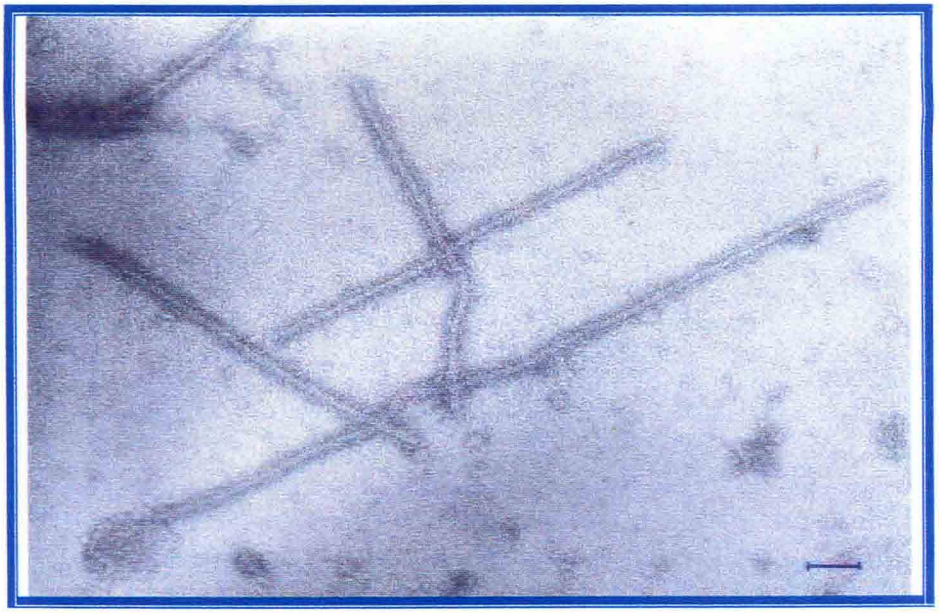


Fig. 3: Electron micrograph of the virus decorated with homologous antiserum
Mag. 108 000, Bar = 100nm

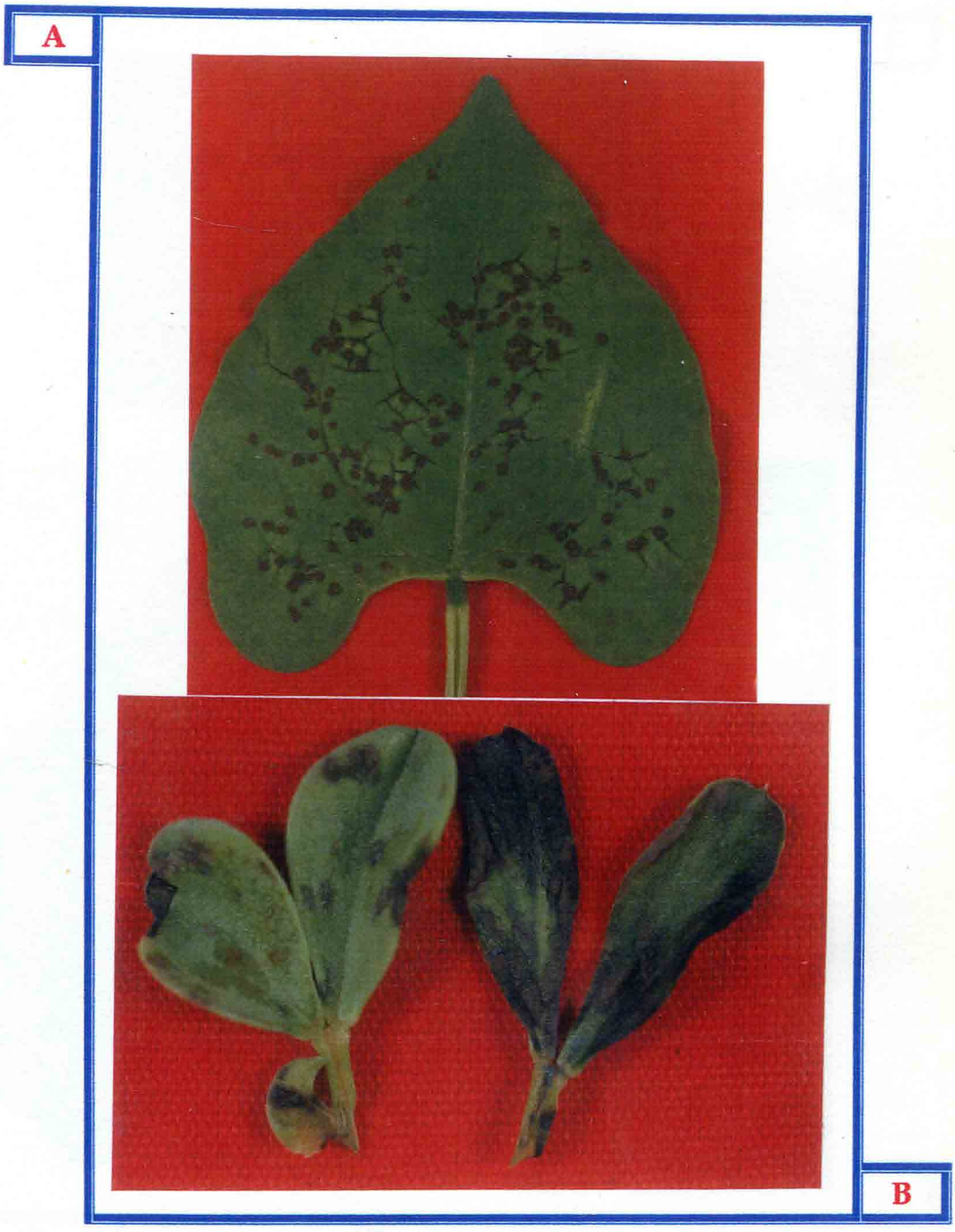


Fig. 4: Symptoms of ICRSV on herbaceous hosts:
(**A**) Local lesion symptoms on *Phaseolus vulgaris* var. *saxa,*
(**B**) Systemic symptoms on *Vicia faba major* L.

4.1.6.2. Longevity In-Vitro (LIV)

The Longevity *In-vitro* (LIV) was only 8 hours at room temperature as shown in Table 7.

Table 7: ***Determination of LIV of ICRSV***

S. No.	Time (hr)	Average No. of Local lesions per leaf. (5 replications)
1.	0	221.2
2.	2	187.2
3.	4	95.4
4.	6	23.0
5.	8	2.0
6.	10	0.0

4.1.6.3. Thermal Inactivation Point (TIP)

The results of TIP in Table 8 revealed that the number of local lesions reduced when the temperature increased beyond 55°C and there were no lesions after 65°C. Therefore, the TIP of the virus was determined between 65 °C and 70 °C.

Table 8: ***Determination of TIP of ICRSV (6 replications)***

S. No.	Temperature (°C)	Average No. of local lesions */leaf	Per cent transmission
1.	30	161.7	(6/6) 100.0
2.	40	137.5	(6/6) 100.0
3.	50	78.7	(6/6) 100.0
4.	55	90.3	(6/6) 100.0
5.	60	29.7	(5/6) 83.3
6.	65	20.3	(3/6) 50.0
7.	70	00.0	(0/6) 00.0
8.	80	00.0	(0/6) 00.0

(*): No. of leaves: 6

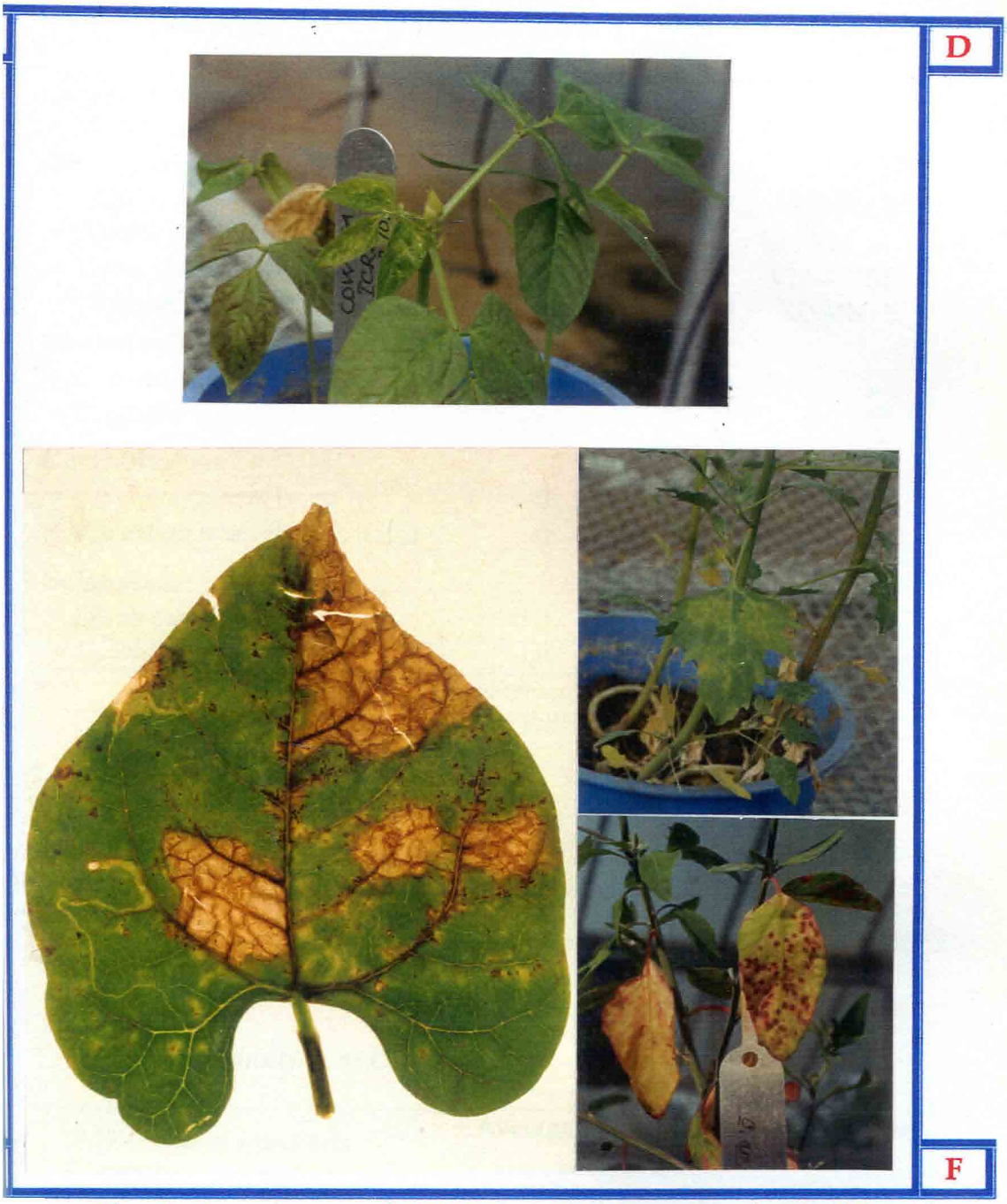


Fig. 4: Symptoms of ICRSV on herbaceous hosts (Cont.)

(**C**) Systemic symptoms on *Vigna sinensis* ,

(**D**) Local lesion symptoms on *Chenopodium quinoa,*

(**E**) Systemic symptoms on *Phaseolus vulgaris* var. *saxa,*

(**F**) Local lesion symptoms on *C. amaranticolor*

4.2. SEROLOGICAL CHARACTERIZATION

4.2.1. Virus Purification

The results of purification of ICRSV by two methods are shown in Table 9.

Table 9: ***Comparative efficacy of two purification protocols***

S.No.	Protocol	No. of particles in 40 µm area of EM grid	
		Pellet	Supernatant
1	Byadgi *et. al.*, 1993	2.0	20.0
2	Rustici *et. al.*, 2000	2.0	30.0

The data showed that the protocols described by Rustici *et al.*, (2000) yielded maximum number of virus particles (30 particles per 40 µm area of the EM grid). The virus particles isolated by this method were least aggregated and free from the host contaminates as compared to the protocol described by Byadgi *et al.* (1993). Repeated purification was done with the following protocol:

1. *Homogenize 50 g ICRSV infected symptomatic leaves in sterilized pestle and mortar in 10 volumes of extraction buffer (0.05 M phosphate buffer containing 0.005M DIECA, 0.01M EDTA and 0.02 M sodium sulphite).*
2. *Filter the extract through double layers of muslin cloth.*
3. *Add 10%chloroform of the total volume, stir for 5 min at room temperature.*
4. *Centrifuge the chloroform extract mixture at 12,000 rpm for 10 min in a SRC - 5 rotor at 4°C.*
5. *Centrifuge aqueous phase at 38,000 rpm for 3 hrs in a Beckman 55.2 Ti rotor at 4°C.*
6. *Re-suspend the pellet in 2 ml of extraction buffer.*
7. *Centrifuge at 1000 g for 2 min.*
8. *Retain the supernatant and re-suspend the pellet in 1 ml extraction buffer.*

9. *Centrifuge at 1000 g for 2 min.*
10. *Layer supernatant on cesium sulfate density gradient (10-40%, prepared in extraction buffer).*
11. *Centrifuge at 41,000 rpm for 2.5 hrs in a Beckman SW 41 rotor.*
12. *Collect the single virus-rich band and dilute in extraction buffer.*
13. *Centrifuge at 70,000 rpm for 30 min in a Beckman R 70 rotor.*
14. *Re-suspend the pellet in 200 µl of 0.01 M phosphate buffer, pH 7.2 and used as final preparation.*

4.2.2. Ultra - violet absorbance Profile

The purified preparation was diluted in 0.05 M phosphate buffer pH 7.2 as described earlier for recording UV absorption at 260 nm and 280 nm. The results were recorded at four different preparations as shown in Table 10.

Table 10: ***UV absorbance value of purified virus preparation of ICRSV***

S. No.	UV absorption at		Ratio 260/280
	260 nm	280 nm	
1.	0.838	0.750	1.12
2.	0.760	0.694	1.10
3.	1.450	1.370	1.06
4.	1.570	1.358	1.16

The A260/A280 ratio for purified virus was from 1.06 to 1.16 when determined at four intervals suggesting that the preparations are nucleoproteins.

4.2.3. Electron Microscopy (EM)

The purified virus preparations were examined under JEOL - 100 CX - 11 transmission electron microscope. The final purified preparation

showed filamentous virus particles in large number without contamination (Fig.5). The model length based on measurement of 100 particles was 650nm in length and 15 nm in width.

4.2.4. Production of antiserum

The polyclonal antiserum of the virus was prepared in rabbit as mentioned in materials and methods. The serological studies were carried out with the stored antiserum. The titre of the antiserum was checked by ISEM, it was 1: 512.

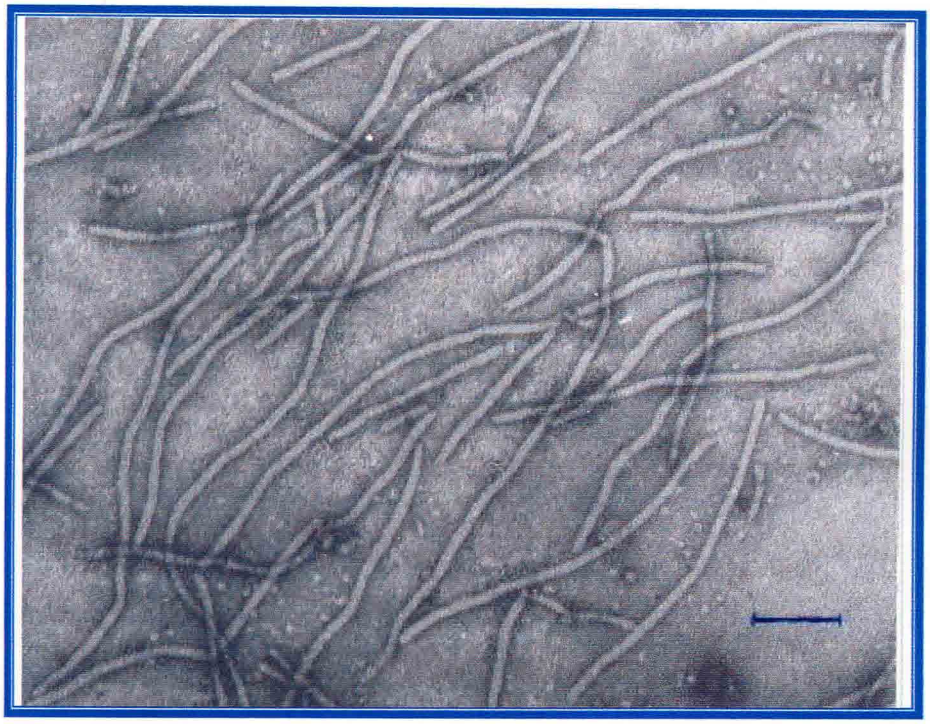
4.2.5. Purification of immunoglobulin (IgG)

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. The OD values of different fractions are given in Table 11.

Table 11: ***Optical density of various fractions of antiserum at 280 nm***

Fraction No.	Optical density
1.	1.032
2.	2.683
3.	3.456
4.	3.437
5.	3.152
6.	2.631
7.	1.325
8.	0.586
9.	0.231
10.	0.108
11.	0.052
12.	0.001

As evidence from Table 11, five fractions from 2 to 6 showed OD values more than 2.6. These five fractions were pooled and the OD was adjusted to 1.4 by adding with half strength PBS buffer to get a concentration of 1mg/ml. (Fig.6)



: Electron micrograph of purified preparation of ICRSV
Mag. 108,000; Bar = 100 nm

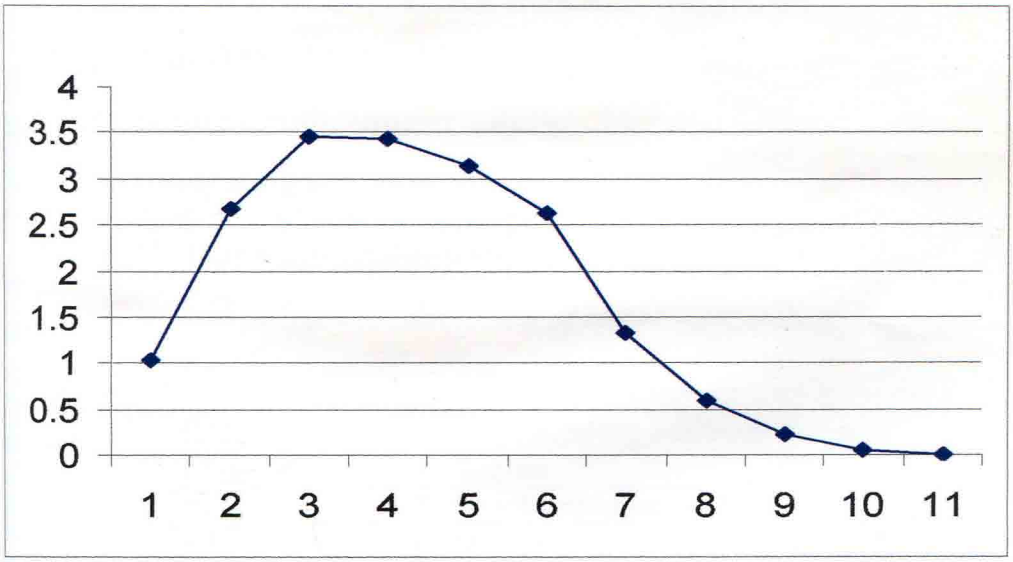


Fig. 6: Elution curve of IgG by DEAE – Cellulose column

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

The conjugate from homologous antiserum was prepared with alkaline phosphatase by the methods 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).

4.2.6.1. Double Antibody Sandwich ELISA (DAS-ELISA)

The IgG concentration on the performance of DAS-ELISA was evaluated. The plates were coated with IgG concentration of 1 and 2μg/ml, the conjugate was used at a dilution of 1:1000, 1:2000, 1:3000 and 1:4000 ml. The antigen dilution was used as 1:10 and 1:20. OD values at 405 nm were recorded after 30 min of addition of the substrate. The results are given in Table 12.

The result of DAS-ELISA indicated that the virus was successfully detected in DAS-ELISA with its homologous conjugate. The best results were obtained with antigen dilution of 1:10, conjugate dilution up to 1:2000 and coating antibody 1μg/ml.

Table 12: ***Optimum concentration of coating and conjugate antibodies and antigen for detection of the ICRSV in DAS-ELISA.***

Treatment	Antigen	Mean absorption (OD 405 nm)							
		Coating antibody dilution							
		1μg/ml				2μg/ml			
		Conjugate antibody dilution							
		1:1000	1:2000	1:3000	1:4000	1:1000	1:2000	1:3000	1:4000
Buffer		0.157	0.169	0.141	0.156	0.167	0.174	0.142	0.154
Healthy	1:10	0.217	0.235	0.230	0.238	0.252	0.236	0.243	0.234
	1:20	0.228	0.236	0.236	0.227	0.230	0.221	0.226	0.212
Infected	**1:10**	0.408	**0.487**	0.392	0.285	0.415	0.328	0.322	0.296
	1:20	0.354	0.244	0.263	0.244	0.322	0.283	0.284	0.259

4.2.6.2. 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 phosphatase, Sigma Chemical Co.) was used at a constant dilution of 1: 10,000. The antiserum (IgG) was cross absorbed with healthy leaf extract at dilution of 1:50. Healthy and diseased extract were used at 1:10 dilution along with PBS as control. The absorbance value at 405 nm was recorded after 30 min of the addition of substrate. The results are given in Table 13.

Table 13: ***Optimum antibody dilution for the performance of DAC-ELISA for detection of the ICRSV.***

Antibody concentration (µg/ml)	Absorbance (405 nm) (1:10 dilution)		
	Buffer	Healthy	Diseased
0.5	0.129	0.213	0.361
1.0	0.135	**0.232**	**0.540**
2.0	0.138	0.241	0.550
5.0	0.132	0.239	0.611

The results of Table 13 showed that the virus could be detected in DAC-ELISA even at very low concentration of virus specific antibody (1µg/ml).

4.2.6.3. Detection of ICRSV from Field samples by DAC- ELISA

The DAC - ELISA was further used to detect the virus in different citrus species from the field trees. The results are given in Table 14.

It would be seen from Table 14 that the virus was detected in one sample of Mosambi sweet orange, one of kinnow mandarin, and two of rough lemon although the symptoms of the disease were not observed on

trees while collecting samples. The presence of ICRSV in these samples was further confirmed in EM.

Table 14: Detection of ICRSV from different citrus species trees in the field by DAC - ELISA.

S. No	Field Samples	Absorbance (405 nm)	EM test
1.	Buffer control	0.161	
2.	Healthy control	0.197	-
3.	Inoculated Bean (Positive control)	0.703	+
4.	Mosambi sweet orange sample 1	0.204	-
5.	Mosambi sweet orange sample 2	0.185	-
6.	Mosambi sweet orange sample 3	0.238	-
7.	Mosambi sweet orange sample 4	0.312	+
8.	Kinnow mandarin sample 1	0.210	-
9.	Kinnow mandarin sample 2	0.295	+
10.	Kinnow mandarin sample 3	0.197	-
11.	Rough lemon sample 1	0.312	+
12.	Rough lemon sample 2	0.345	+
13.	Rough lemon sample 3	0.189	-

4.2.7. Dot Immuno Binding Assay (DIBA):

Samples of all the four isolates were tested by DIBA. The results showed that all the four isolates could be detected in DIBA using homologous antibodies. The detection could be done up to a dilution of 1:20 of the antigen (Fig. 7)

The samples which were positive in DAC - ELISA were also tested in DIBA. The results are showed in Fig. 8. Interestingly, the trees which were ICRSV positive in DAC-ELISA were also positive in DIBA.

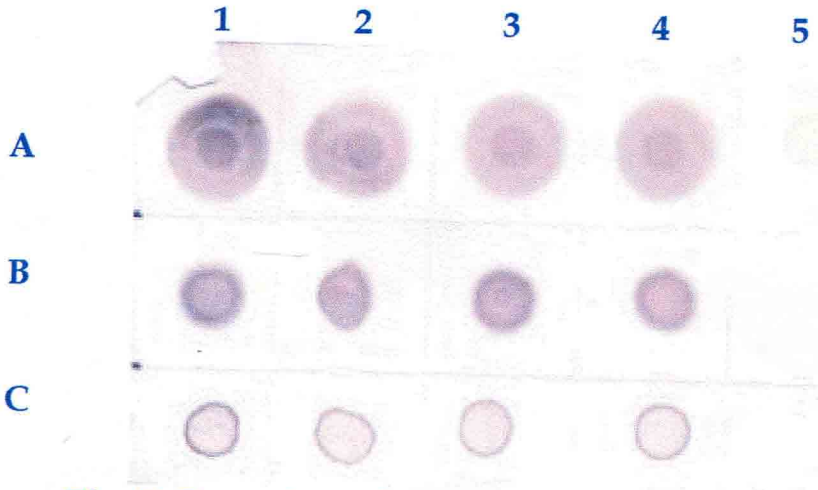


Fig. 7. Detection of ICRSV in DIBA

Samples: 1: ICRSV – Ab
2: ICRSV – DI
3: ICRSV - Ah
4: ICRSV - Pu
5: Healthy

Dilutions: A: 1:1,
B: 1:10,
C: 1:20

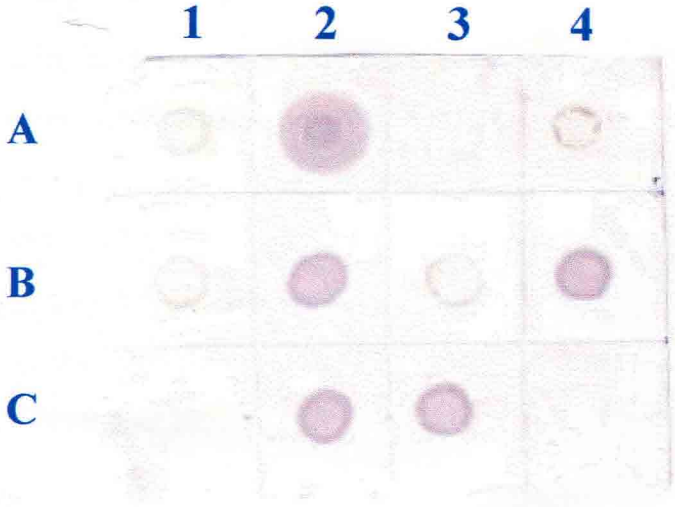


Fig. 8 : Testing of field samples in DIBA

Healthy control – A_1 Infected bean (Positive control) A_2

Field samples:

- Mosambi sweet orange (4 samples): **1(A_3), 2 (A_4), 3 (B_1)** and **4 (B_2)**
- Kinnow mandarin (3 samples): **1(B_3); 2 (B_4), 3 (C_1)**
- Rough lemon (3 samples): **1(C_2), 2 (C_3) and 3 (C_4)**

4.3. MOLECULAR CHARACTERIZATION

4.3.1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

4.3.1.1. Isolation of RNA

Total RNA was extracted from all the four isolates by RNase easy kit (Appendix II). The final product was tested by agarose gel electrophoresis containing formaldehyde. The product of desired size appeared as a single band and hence this preparation was used for subsequent reactions.

4.3.1.2. cDNA synthesis

Complementary DNA was synthesized from total RNA samples using reverse transcriptase (M-MuLV) and reverse primer (ICRSV-951 or ICRSV-1420) in a one step reaction.

4.3.1.3. PCR amplification

Primers for PCR were prepared from stock to a suitable concentration and used in different combinations to get products of different sizes. At first, five sets of primers were used to standardize the annealing temperature and all the concentrations of different components PCR set from ICRSV-DI isolate. All the products were resolved on 1% agarose gel electrophoresis at 60 volts for 1 hour. The results showed different sizes of products when they were run on gel and seen under UV-light as in Fig. 9 and 10.

The results revealed that, for different sets of primers, the annealing temperature varied from 55 to 60°C. For ICRSV-518/ICRSV-1420, CP-FW/CP-R-Eco and CP-FW/ICRSVICRSV-1420 required 57-60°C for annealing, while ICRSV-518/ICRSV-951 and ICRSV-1096/ICRSV-1420 required 55°C to 57°C. The conditions for amplification are 94°C for 2min,

94°C for 30 sec. 35 cycles, 55-60°C for 30 sec. 35 cycles, 72°C for 1 min 35 cycles, and final extension at 72°C for 10 min.

When the set of primers "ICRSV-1096/ICRSV-1420" was used, all the four isolates were amplified nicely with very clear band of ≈350 bp, which was later used for cloning. However, for amplification of longer fragments of the CP gene, the other set of primers (CP-FW/CP-R-Eco) was used (Fig.11).

The PCR products from these two sets of primers have been cloned in pGEMT-easy vector.

4.3.2. Cloning

The viral coat protein of all the four isolates was separately cloned by adopting the amplified RT- PCR product of ≈ 350 bp and ≈ 1000 bp which contained both A tail at two ends with T hanging of pGEM-T easy vector (Fig. 12). The presence of the ICRSV - CP insert in recombinant clones was confirmed through restriction analysis, miniprep DNA and colony PCR.

4.3.2.1. Analysis of Recombinant Clones

For selection of recombinant clones rapid screening method was used. The recombinant DNA was restricted with *EcoR* I enzymes and products resolved by electrophoresis in 1% agarose gel. Colonies having the desired inserts ≈ 350bp and ≈ 1kb size for each isolates were selected for further studies.

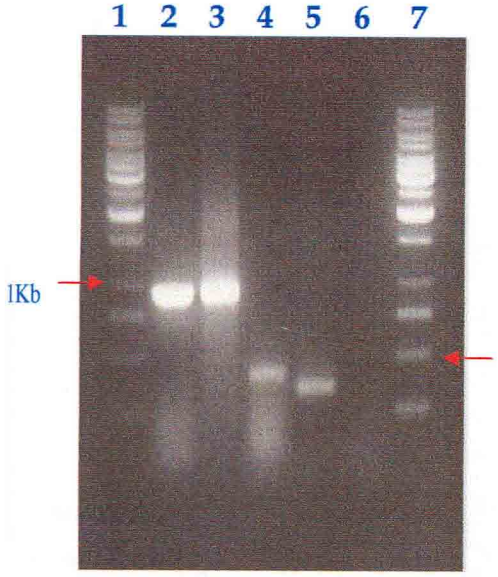


Fig. 9: PCR amplification of ICRSV-CP with 4 sets of primers.

Lane 1& 7: 1Kb DNA ladder
Lane 2: Product of ICRSV-518/ -1420 primers
Lane 3: Product of CP-FW/CP-R-Eco primers
Lane 4: Product of ICRSV-518/ -951 primers
Lane 5: Product of ICRSV-1096/ -1420 primers
Lane 6: Healthy control

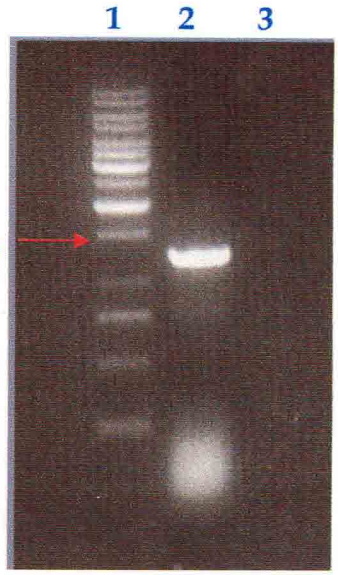


Fig. 10: PCR amplification of ICRSV- CP

Lane 1: 1Kb DNA ladder
Lane 2: Product of CP-FW/ ICRSV- 1420 primers
Lane 3: Healthy control

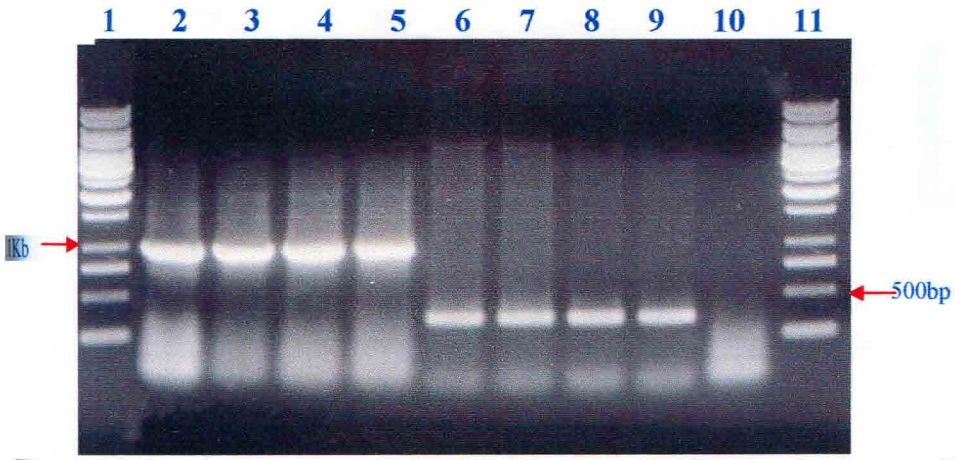
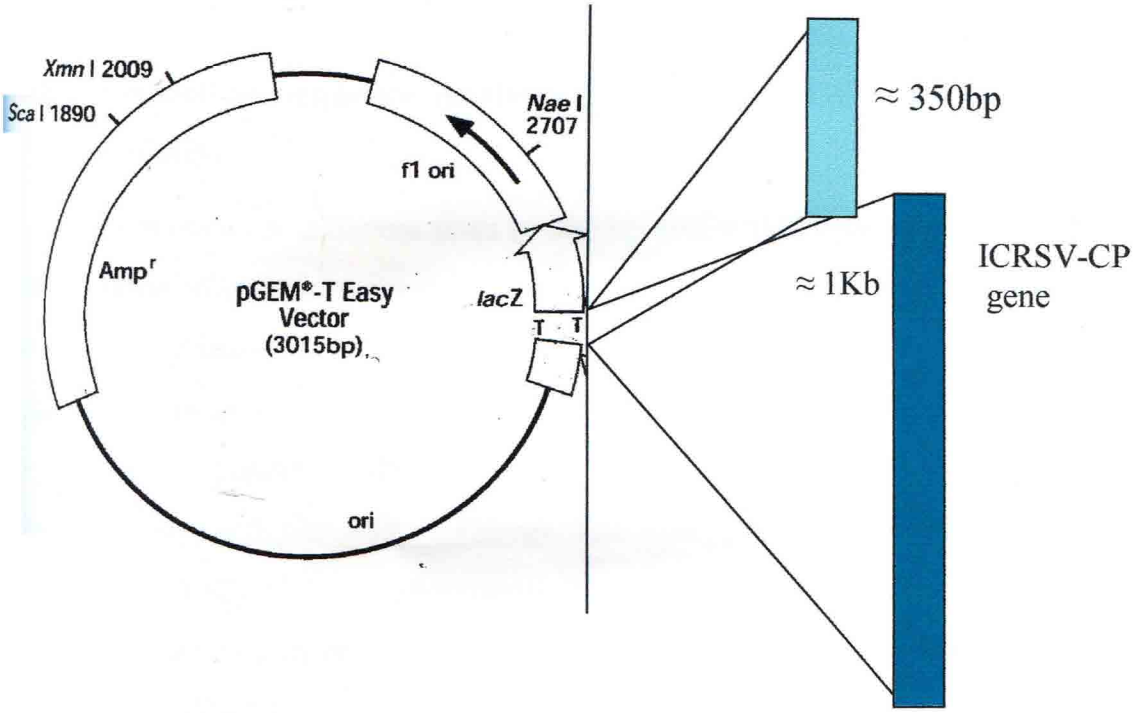


Fig. 11: PCR amplification of ICRSV-CP gene with 2 sets of primers, CP-FW/CP-R-Eco (lane2-5) and ICRSV-1096/ICRSV-1420 (lane 6-9).

Lane 1 and 11: 1Kb DNA ladder,
Lane 2, 6: ICRSV - Ab isolate
Lane 3, 7: ICRSV - Dl isolate
Lane 4, 8: ICRSV - Ah isolate
Lane 5, 9: ICRSV - Pu isolate
Lane 10: Healthy control



12: Schematic of cloning strategy to amplify the two overlap fragments of Coat Protein of ICRSV on pGEM-T easy vector

4.3.2.2. Miniprep DNA and colony PCR

Plasmid DNA was recovered either by 'miniprep' method and recombinant clones were confirmed by restricted with *EcoR* I or amplified by PCR using single white colony from master plate for colony DNA PCR. Both the methods used confirmed the presence of ≈350 for ICRSV-1096/ICRSV-1420 primers and ≈ 1kb size for CP-FW/CP-R-Eco primers of each isolates (Fig. 13 and 14).

4.3.3. Nucleotide Sequence Analysis of Coat Protein of four ICRSV isolates.

Sequences of different sizes (≈ 350 bp and ≈ 1kb) coat protein gene of the same isolate were overlap cut and aligned for one long sequence of ≈ 1300 bp by using Bioedit (version 5.0.6.). All the four isolates were done the same way and then the deduced amino acids were translated (Fig.15 A - D). Later they were analyzed in BLAST (http://www.ncbi.nlm.nil.gov./blast). The analysis was done in Clustal W (version 1.82) multiple alignments. The data showing percent identity in each other and in comparison with the reported ICRSV-D isolate are present in Table 15.

A total of 1283 nucleotide sequences in viral sense were anlysed in BLAST analysis and were further confirmed by Clustal analysis (Fig 16). A maximum of 99.5% and 97.8% identity alignment was observed in ICRSV-Ab isolate and ICRSV-Dl necrotic isolate with ICRSV-D reported earlier respectively and 98% identity alignment to each other. While ICRSV-Ah and ICRSV-Pu isolates showed only 80.2 and 80.0 % respectively but 99.6% identity alignment to each other. The result was confirmed with amino acid sequences analysis. The same results have been viewed (Fig

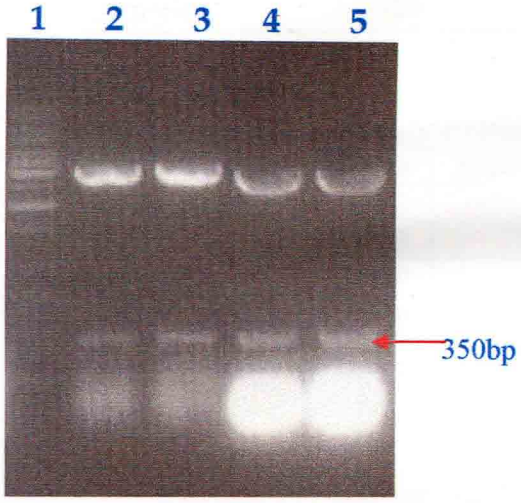


Fig 13: *Restriction of 350 bp clones in pGEM-T easy vector by EcoR* I

Lane 1: 1Kb DNA marker
Lane 2: ICRSV - Ab isolate
Lane 3: ICRSV - Dl isolate
Lane 4: ICRSV - Ah isolate
Lane 5: ICRSV Pu isolate

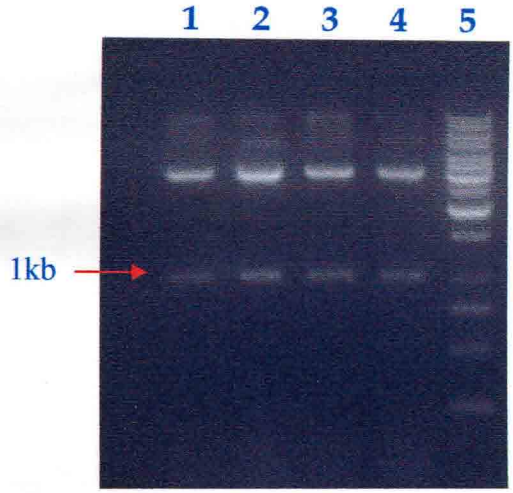


Fig 14: *Restriction of 1kb clones in pGEM-T easy vector by EcoR* I

Lane 1: ICRSV - Ab isolate
Lane 2: ICRSV - Dl isolate
Lane 3: ICRSV - Ah isolate
Lane 4: ICRSV - Pu isolate
Lane 5: 1Kb DNA marker

17). However, the amino acids homologous are more than that of nucleotides sequences (Table 15 & 16).

Table 15: *Percentage of nucleotide sequence identity of nucleocapsid gene between Indian Citrus Ringspot Virus isolates*

	ICRSV-D	ICRSV-Ab	ICRSV-Dl	ICRSV-Ah	ICRSV-Pu
ICRSV-D	**100.0**	**99.5**	**97.8**	80.2	80.0
ICRSV-Ab	-	**100.0**	**98.0**	80.2	80.0
ICRSV-Dl	-	-	**100.0**	80.8	80.6
ICRSV-Ah	-	-	-	**100.0**	**99.6**
ICRSV-Pu	-	-	-	-	**100.0**

Table 16: *Percentage of amino acid sequence identity of nucleocapsid gene between Indian Citrus Ringspot Virus isolates*

	ICRSV-D	ICRSV-Ab	ICRSV-Dl	ICRSV-Ah	ICRSV-Pu
ICRSV-D	**100.0**	-	-	-	-
ICRSV-Ab	**99.5**	**100.0**	-	-	-
ICRSV-Dl	**98.0**	**98.5**	**100.0**	-	-
ICRSV-Ah	85.0	85.0	85.2	**100.0**	-
ICRSV-Pu	84.5	85.5	84.7	**99.5**	**100.0**

```
1      ATG AGC TTT GAC TAC ACA GAT CCA ACC TTC CGT AAT TAC CCC TTC    45
1       M   S   F   D   Y   T   D   P   T   F   R   N   Y   P   F    15

46     CCG CAC TAT TGT GAT TTC GAC CGC CAC CAA CAC TGC GAT CAC GAC    90
16      P   H   Y   C   D   F   D   R   H   Q   H   C   D   H   D    30

91     CTA CGA ACC AAT CCA CCT CCA ACC GAG CCA CCA TCC CGA AAA TCC    135
31      L   R   T   N   P   P   P   T   E   P   P   S   R   K   S    45

136    AAA CTC ATG TCT ACC AGT GAG AAC AAA GGC AAA CAG CCG CTT CAC    180
46      K   L   M   S   T   S   E   N   K   G   K   Q   P   L   H    60

181    CCG CCT CCC ACC GAA GGC TTT CCA AAA CCT CCA CCA CCA CCG TCG    225
61      P   P   P   T   E   G   F   P   K   P   P   P   P   P   S    75

226    AGC ACT CCG ACT ACT CCT ACA CCG CCC GAC CAG ACG AAG GCT CCT    270
76      S   T   P   T   T   P   T   P   P   D   Q   T   K   A   P    90

271    GAA CCC ATT GAG AAA AGA ATC ATC CAC GCC TTC CAC GCT GAA CCT    315
91      E   P   I   E   K   R   I   I   H   A   F   H   A   E   P    105

316    AAA ACC CAC ACC AAT GGA GAA GCC CCC CCG GCA TTC AAT CCC AAT    360
106     K   T   H   T   N   G   E   A   P   P   A   F   N   P   N    120

361    AAC ATG AAC GCT GTG CCA CTC AAC CTA CTC AAC ATC AAC CTG AAG    405
121     N   M   N   A   V   P   L   N   L   L   N   I   N   L   K    135

406    TAT TCA CCA GTC ACC AAC TCC ATT GCG AAC CCG AAA CAG ACA GAA    450
136     Y   S   P   V   T   N   S   I   A   N   P   K   Q   T   E    150
```

```
451  GCT ATT GGC AAA GCC TGG GTC CGT ATC CTG CAA ATT GAC CCG GCG  495
151   A   I   G   K   A   W   V   R   I   L   Q   I   D   P   A  165

496  AAC GTA TTC CTC TAC GCC ATC GAT CTC GCC CGA GCC TGC GCT GAC  540
166   N   V   F   L   Y   A   I   D   L   A   R   A   C   A   D  180

541  GCT GGA AGC TCA CCC GAA GCC GAC ATT ATC GGC GCG AAC GAA GAC  585
181   A   G   S   S   P   E   A   D   I   I   G   A   N   E   D  195

586  CTC AAT CCA GTG GTC GAG CGA AAC GCC CTA GCT GGT GTT GTC CGA  630
196   L   N   P   V   V   E   R   N   A   L   A   G   V   V   R  210

631  GAC TTC TGC CCA CTG CGC GCC TTT TGC GCT TAC TAC TCC AGG GTG  675
211   D   F   C   P   L   R   A   F   C   A   Y   Y   S   R   V  225

676  GTA TGG AAC CTC ATG ATC AAA GCG GAT CAA CCA CCC GCC AAC TGG  720
226   V   W   N   L   M   I   K   A   D   Q   P   P   A   N   W  240

721  ATG AAA TCA GGG ATA GAC GAG GGA GCC AAA TTC GCA GCG TTC GAC  765
241   M   K   S   G   I   D   E   G   A   K   F   A   A   F   D  255

766  TTC TTC CAT GGC GTC CTT TCA CCC GCT TCC CTG TAT GTC CCT CTG  810
256   F   F   H   G   V   L   S   P   A   S   L   Y   V   P   L  270

811  GAA CGT CAC CCT ACA GCC GCG GAA CGC ATA GCC AAC CAA GCT ATG  855
271   E   R   H   P   T   A   A   E   R   I   A   N   Q   A   M  285

856  TTC GCT GTC AAA ATT GCG AAC GCG CCC GGC AAT GGT TCA GAG CTC  900
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901  ACG ATG GAC CAC GTT GCC TTC ACC AAA GGC CGG ATT ACA GCA GAC  945
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316   S   K   P   R   P   T   P   F   N   T   *   T   A   S   N  330

991  CCA TAC TCC TGG CCG CCC TGA GTG CTC TAC GGC CAC TAC CCC GAG 1035
331   P   Y   S   W   P   P   *   V   L   Y   G   H   Y   P   E  345

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346   I   S   K   L   P   *   Y   R   V   R   V   T   I   S   I  360

1081 CTG TAC GCT GTG CCA GTA GTC GGT ACT TAG GCT CAA GCC GTT CCG 1125
361   L   Y   A   V   P   V   V   G   T   *   A   Q   A   V   P  375

1126 CCG TCA AAA GGG GGG CTG CCA GGC TCA ATT ACT GCT ACA AGT GTG 1170
376   P   S   K   G   G   L   P   G   S   I   T   A   T   S   V  390

1171 GGC ACC CTC TCT ACT TAA ATA AAC CTC ACA CCT GCC GCC CAG GTC 1215
391   G   T   L   S   T   *   I   N   L   T   P   A   A   Q   V  405

1216 GTC TTT GCT CCG CGT CAA TTT CTG AAC GCT TGG CTC TAC TCC GCG 1260
406   V   F   A   P   R   Q   F   L   N   A   W   L   Y   S   A  420

1261 CGG GAC CGA TTA GGT CAT TGA  1281
421   R   D   R   L   G   H   *
```

Fig. 15A: Nucleotide (shown as DNA) and deduced amino acid sequences of the coat protein gene of ICRSV - Ab isolate

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1      ATG AGC TTT GAC TAC ACA GAT CCA ACC TTC CGT AAT TAC CCC TTC    45
1       M   S   F   D   Y   T   D   P   T   F   R   N   Y   P   F    15

46     CCG CAC TAC TGT GAT TTC GAC CGC CAC CAA CCC TGC GAC CAC GAC    90
16      P   H   Y   C   D   F   D   R   H   Q   P   C   D   H   D    30

91     CTA CGA ACC AAT CCA CCT CCA ACC GAG CCA CCA TCC CGA AAA TCC    135
31      L   R   T   N   P   P   P   T   E   P   P   S   R   K   S    45

136    AAA TTC ATG TCT ACC AGT GAG AAC AAA GGC AAA CAG CCT CTT CAC    180
46      K   F   M   S   T   S   E   N   K   G   K   Q   P   L   H    60

181    CCG CCT CCC ACC GAA GAC TTT TCA AAA CCT CCA CCA CCA CCG TCG    225
61      P   P   P   T   E   D   F   S   K   P   P   P   P   P   S    75

226    AGC ACC CCG GCT ACT CCT ACA CCG CCC GAC CAG ACG AAG GCT CTT    270
76      S   T   P   A   T   P   T   P   P   D   Q   T   K   A   L    90

271    GAA CCC ATT GAG AAA AGA ATC ATC CAC GCC TTC CAC GCT GAA CCC    315
91      E   P   I   E   K   R   I   I   H   A   F   H   A   E   P   105

316    AAA ACC CAC ACC AAT GGA GAA GCG CCC CCA GCA TTC AAT CCC AAT    360
106     K   T   H   T   N   G   E   A   P   P   A   F   N   P   N   120

361    AAC ATG AAC GCT GTG CCA CTC AAC CTA CTC AAC ATC AAC CTG AAG    405
121     N   M   N   A   V   P   L   N   L   L   N   I   N   L   K   135

406    TAT TCA CCA GTC ACC AAC TCC ATT GCG AAC CCG AAA CAG ACA GAA    450
136     Y   S   P   V   T   N   S   I   A   N   P   K   Q   T   E   150

451    GCT ATT GGC AAA GCC TGG GTC CGT ATC CTG CAA ATT GAT CCT GCG    495
151     A   I   G   K   A   W   V   R   I   L   Q   I   D   P   A   165

496    AAC GTA TTC CTC TAC GCC ATC GAC CTC GCC CGA GCT TGC GCT GAC    540
166     N   V   F   L   Y   A   I   D   L   A   R   A   C   A   D   180

541    GCT GGA AGC TCA CCC GAA GCC GAC ATT ATC GGC GCG AAC GAA GAC    585
181     A   G   S   S   P   E   A   D   I   I   G   A   N   E   D   195

586    CTC AAT CCA GTG GTC GAG CGA AAC GCC CTA GCT GGT GTT GTC CGA    630
196     L   N   P   V   V   E   R   N   A   L   A   G   V   V   R   210

631    GAC TTC TGC CCA TTG CGC GCC TTT TGC GCT TAC TAC TCC AGG GTA    675
211     D   F   C   P   L   R   A   F   C   A   Y   Y   S   R   V   225

676    GTA TGG AAC CTC ATG ATC AAA GCG GAT CAA CCA CCG GCC AAC TGG    720
226     V   W   N   L   M   I   K   A   D   Q   P   P   A   N   W   240

721    ATG AAA TCA GGG ATA GAC GAG GGA GCC AAA TTC GCA GCG TTC GAC    765
241     M   K   S   G   I   D   E   G   A   K   F   A   A   F   D   255

766    TTC TTC CAT GGC GTC CTT TCG CCC GCT TCC CTG TAT GTT CCT CTG    810
256     F   F   H   G   V   L   S   P   A   S   L   Y   V   P   L   270

811    GAA CGT CAC CCT ACT GCC GCG GAA CGC ATA GCC AAC CAA GCC ATG    855
271     E   R   H   P   T   A   A   E   R   I   A   N   Q   A   M   285

856    TTC GCT GTC AAA ATT GCG AAC GCG CCC GGC AAT GGT TCA GAG CTC    900
286     F   A   V   K   I   A   N   A   P   G   N   G   S   E   L   300

901    ACG ATG GAC CAC GTT GCC TTC ACC AAA GGC CGG ATT ACA GCA GAC    945
301     T   M   D   H   V   A   F   T   K   G   R   I   T   A   D   315
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946   TCC AAG CCC CAC CCG ACC CCT TTC AAC ACT TAA ACA GCT TCG AAC   990
316    S   K   P   H   P   T   P   F   N   T   *   T   A   S   N   330

991   CCA TAC TCC TGG CCG CCC TGA GTG CTC TAC GGC CAC TAC CCC GAG   1035
331    P   Y   S   W   P   P   *   V   L   Y   G   H   Y   P   E   345

1036  ATA TCC AAA TTA CCA TAA TAT CGT GTG CGT GTG ACT ATT TCC ATT   1080
346    I   S   K   L   P   *   Y   R   V   R   V   T   I   S   I   360

1081  CTG TAC GCT GTG CCA GTA GTC GGT ACT TAG GCT CAA GCC GTT CCG   1125
361    L   Y   A   V   P   V   V   G   T   *   A   Q   A   V   P   375

1126  CCG TCA AAA GGC GGG CTG CCA GGC TCA ATT ACT GCT ACA AGT GTG   1170
376    P   S   K   G   G   L   P   G   S   I   T   A   T   S   V   390

1171  GGC ACC CTC TCT ACT TAA ATA AAC CTC ACA CCT GCC GCC CAG GTC   1215
391    G   T   L   S   T   *   I   N   L   T   P   A   A   Q   V   405

1216  GTC TTT GCT CCG CGT CAA TTT CTG AAC GCT TGG CTC TAC TCC GCG   1260
406    V   F   A   P   R   Q   F   L   N   A   W   L   Y   S   A   420

1261  CGG GAC CGA TTA GGT CAT TGA   1281
421    R   D   R   L   G   H   *
```

Fig. 15B: Nucleotide (shown as DNA) and deduced amino acid sequences of the coat protein gene of ICRSV - DI isolate.

```
1     ATG AGC TTT GAC TAC ACA CAC CCT CTC TAC CGC AGC TAT CCA TTT   45
1      M   S   F   D   Y   T   H   P   L   Y   R   S   Y   P   F   15

46    CCA CAC TAC TGC GAG TTC GAC CGG CAC CAA CTC TGC GAC CAT CAT   90
16     P   H   Y   C   E   F   D   R   H   Q   L   C   D   H   H   30

91    CCA GTA CTC AAA CCT CCA ACT CAC AAA CCC AGT GCC CCG AAC TCT   135
31     P   V   L   K   P   P   T   H   K   P   S   A   P   N   S   45

136   CTC ATG TCT ACC GAC GAC AAC AAG GGC AAA CAA CCA CTT CAC CCG   180
46     L   M   S   T   D   D   N   K   G   K   Q   P   L   H   P   60

181   ACA CCT TCG GGC CCT AAC GAC AAG ACC CCA AAA CCT ACC CCC GTA   225
61     T   P   S   G   P   N   D   K   T   P   K   P   T   P   V   75

226   CTC ACT CCC TCA GCT ACG CCC ACA GCT GCA GGT AAG GAA AAC CAG   270
76     L   T   P   S   A   T   P   T   A   A   G   K   E   N   Q   90

271   GAG CCC ATC GAA AAG CGT ATC ATA CAC GCT TTC CAC GCT GAA GCA   315
91     E   P   I   E   K   R   I   I   H   A   F   H   A   E   A   105

316   AAA ACC CAC AAC AAT GGG GTC TCT CCA CCT GCC TTT AAC CCG AAC   360
106    K   T   H   N   N   G   V   S   P   P   A   F   N   P   N   120

361   AAC ATG AAC GCT GTG CCG CTG AAC CTG CTT AAC CTC AAC CTA AGA   405
121    N   M   N   A   V   P   L   N   L   L   N   L   N   L   R   135

406   TAC TCA CCG GTC ACC AAC TCC ATA GCC AAC CCT AAA CAG ACC GAG   450
136    Y   S   P   V   T   N   S   I   A   N   P   K   Q   T   E   150

451   GCT ATC GGA AAA GCT TGG GTC CGC ATT TTG AAC ATC GAT CCT GCC   495
151    A   I   G   K   A   W   V   R   I   L   N   I   D   P   A   165

496   AAC GTG TTC TTA TAC GCC ATT GAC CTC GCC AGA GCT TGC GCC GAC   540
166    N   V   F   L   Y   A   I   D   L   A   R   A   C   A   D   180

541   GCG GGC TCT TCT CCT GAA GCT GAT ATT ATT GGA GCG AAC GAA GAT   585
181    A   G   S   S   P   E   A   D   I   I   G   A   N   E   D   195

586   CTC AAC CCC GTT GTT GAA CGA AAC GCA TTG GCC CTA GTG GTT AGG   630
196    L   N   P   V   V   E   R   N   A   L   A   L   V   V   R   210

631   GAT TTC TGC CCG CTA CGC GCT TTT TGC GCT TAC TAC TCT CGA GTG   675
211    D   F   C   P   L   R   A   F   C   A   Y   Y   S   R   V   225

676   GTA TGG AAC CTC ATG ATC AAG GCG GAC CAG CCT CCG GCC AAC TGG   720
226    V   W   N   L   M   I   K   A   D   Q   P   P   A   N   W   240

721   ATG AAA TCC GGG GTA GAC GAG AAC GCG AAA TTC GCG GCA TTC GAT   765
241    M   K   S   G   V   D   E   N   A   K   F   A   A   F   D   255

766   TTC TTC CAT GGT ATC CTC TCG CCA GCT TCC CTG TAT GTG CCC CTA   810
256    F   F   H   G   I   L   S   P   A   S   L   Y   V   P   L   270

811   GAG AGA CAC CCT ACT TCC GCG GAG AGG ATC GCA AAT CAG GCC ATG   855
271    E   R   H   P   T   S   A   E   R   I   A   N   Q   A   M   285

856   TTC GCT GTG AAA ATT GCC AAC GCT CCA GGA AAT GGC ACG GAC CTC   900
286    F   A   V   K   I   A   N   A   P   G   N   G   T   D   L   300

901   ACG ATG GAC CAC GTT GCC TTC ACC AAA GGA AAG ATT ACC CAG CCC   945
301    T   M   D   H   V   A   F   T   K   G   K   I   T   Q   P   315
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```
946   TCC GGC CTC CGC CCG ACC CCT TTC AAC ACT TAA ACA GCT TCG AAC   990
316    S   G   L   R   P   T   P   F   N   T   *   T   A   S   N   330

991   CCA TAC TCC TGG CCG CCC TGA GTG CTC TAC GGC CAC TAC CCC GAG   1035
331    P   Y   S   W   P   P   *   V   L   Y   G   H   Y   P   E   345

1036  ATA TCC AAA TTA CCA TAA TAT CGT GTG CGT GTG ACT ATT TCC ATT   1080
346    I   S   K   L   P   *   Y   R   V   R   V   T   I   S   I   360

1081  CTG TAC GCT GTG CCA GTA GTC GGT ACT TAG GCT CAA GCC GTT CCG   1125
361    L   Y   A   V   P   V   V   G   T   *   A   Q   A   V   P   375

1126  CCG TCA AAA GGC GGG CTG CCA GGC TCA ATT ACT GCT ACA AGT GTG   1170
376    P   S   K   G   G   L   P   G   S   I   T   A   T   S   V   390

1171  GGC ACC CTC TCC ACT TAA ATA AAC CTC ACA CCT GCC GCC CAG GTC   1215
391    G   T   L   S   T   *   I   N   L   T   P   A   A   Q   V   405

1216  GTC TTT GCT CCG CGT CAA TTT CTG AAC GCT TGG CTC TAC TCC GCG   1260
406    V   F   A   P   R   Q   F   L   N   A   W   L   Y   S   A   420

1261  CGG GAC CGA TTA GGT CAT TGA   1281
421    R   D   R   L   G   H   *
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Fig. 15C: Nucleotide (shown as DNA) and deduced amino acid sequences of the coat protein gene of ICRSV - Ah isolate.

```
1     ATG AGC TTT GAC TAC ACA CAC CCT CTC TAC CGC AGC TAT CCA TTT   45
1      M   S   F   D   Y   T   H   P   L   Y   R   S   Y   P   F   15

46    CCA CAC TAC TGC GAG TTC GAC CGG CAC CAA CTC TGC GAC CAT CAT   90
16     P   H   Y   C   E   F   D   R   H   Q   L   C   D   H   H   30

91    CCA GTA CTC AAA CCT CCA ACT CAC AAA CCC AGT GCC CCG AAC TCT   135
31     P   V   L   K   P   P   T   H   K   P   S   A   P   N   S   45

136   CTC ATG TCT ACC GAC GAC AAC AAG GGC AAA CAA CCA CTT CAC CCG   180
46     L   M   S   T   D   D   N   K   G   K   Q   P   L   H   P   60

181   ACA CCT TCG GGC CCT AAC GAC AAG ACC CCA AAA CCT ACC CCC GTA   225
61     T   P   S   G   P   N   D   K   T   P   K   P   T   P   V   75

226   CTC ACT CCC TCA GCT ACG CCC ACA GCT GCA GGT AAG GAA AAC CAG   270
76     L   T   P   S   A   T   P   T   A   A   G   K   E   N   Q   90

271   GAG CCC ATC GAA AAG CGT ATC ATA CAC GCT TTC CAC GCT GAA GCA   315
91     E   P   I   E   K   R   I   I   H   A   F   H   A   E   A   105

316   AAA ACC CAC AAC AAT GGG GTC TCT CCA CCT GCC TTT AAC CCG AAC   360
106    K   T   H   N   N   G   V   S   P   P   A   F   N   P   N   120

361   AAC ATG AAC GCT GTG CCG CTG TAC CTG CTT AAC CTC AAC CTA AGA   405
121    N   M   N   A   V   P   L   Y   L   L   N   L   N   L   R   135

406   TAC TCA CCG GTC ACC AAC TCC ATA GCC AAC CCT AAA CAG ACC GAG   450
136    Y   S   P   V   T   N   S   I   A   N   P   K   Q   T   E   150

451   GCT ATC GGA AAA GCT TGG GTC CGC ATT TTG AAC ATC GAT CCT GCC   495
151    A   I   G   K   A   W   V   R   I   L   N   I   D   P   A   165

496   AAC GTG TTC TTA TAC GCC ATT GAC CTC GCC AGA GCT TGC GCC GAC   540
166    N   V   F   L   Y   A   I   D   L   A   R   A   C   A   D   180

541   GCG GGC TCT TCT CCT GAA GCT GAT ATT ATT GGA GCG AAC GAA GAT   585
181    A   G   S   S   P   E   A   D   I   I   G   A   N   E   D   195

586   CTC AAC CCC GTT GTT GAA CGA AAC GCA TTG GCC CTA GTG GTT AGG   630
196    L   N   P   V   V   E   R   N   A   L   A   L   V   V   R   210

631   GAT TTC TGC CCG CTA CGC GCT TTT TGC GCT TAC TAC TCT CGA GTG   675
211    D   F   C   P   L   R   A   F   C   A   Y   Y   S   R   V   225

676   GTA TGG AAC CTC ATG ATC AAG GCG GAC CAG CCT CCG GCC AAC TGG   720
226    V   W   N   L   M   I   K   A   D   Q   P   P   A   N   W   240

721   ATG AAA TCC GGG GTA GAC GAG AAC GCG AAA TTC GCG GCA TTC GAT   765
241    M   K   S   G   V   D   E   N   A   K   F   A   A   F   D   255

766   TTC TTC CAT GGT ATC CTC TCG CCA GCT TCC CTG TAT GTG CCC CTA   810
256    F   F   H   G   I   L   S   P   A   S   L   Y   V   P   L   270

811   GAG AGA CAC CCc ACT TCC GCG GAG AGG ATC GCA AAT CAG GCC ATG   855
271    E   R   H   P   T   S   A   E   R   I   A   N   Q   A   M   285

856   TTC GCT GTG AAA ATT GCC AAC GCT CCA GGA AAT GGC ACG GAC CTC   900
286    F   A   V   K   I   A   N   A   P   G   N   G   T   D   L   300

901   ACG ATG GAC CAC GTT GCC TTC ACC AAA GGA AAG ATT ACC CAG CCC   945
301    T   M   D   H   V   A   F   T   K   G   K   I   T   Q   P   315
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```
946   TCC GGC CTC CGC CCG ACC CCT TTC AAC ACT TAA ACA GCT TCG AAC   990
316    S   G   L   R   P   T   P   F   N   T   *   T   A   S   N   330

991   CCA TAC TCT TGG CCG CCC TGA GTG CTC TAC GGC CAC TAC CCC GAG   1035
331    P   Y   S   W   P   P   *   V   L   Y   G   H   Y   P   E   345

1036  ATA TCC AAA TTA CCA TAA TAT CGT GTG CGT GTG ACT ATT TCC ATT   1080
346    I   S   K   L   P   *   Y   R   V   R   V   T   I   S   I   360

1081  CTG TAC GCT GTG CCA GTA GTC AGT ACT TAG GCT CAA GCC GTT CCG   1125
361    L   Y   A   V   P   V   V   S   T   *   A   Q   A   V   P   375

1126  CCG TCA AAA GGC GGG CTG CCA GGC TCA ATT ACT GCT ACA AGT GTG   1170
376    P   S   K   G   G   L   P   G   S   I   T   A   T   S   V   390

1171  GGC ACC CTC TCT ACT TAA ATA AAC CTC ACA CCT GCC GCC CAG GTC   1215
391    G   T   L   S   T   *   I   N   L   T   P   A   A   Q   V   405

1216  GTC TTT GCT CCG CGT CAA TTT CTG AAC GCT TGG CTC TAC TCC GCG   1260
406    V   F   A   P   R   Q   F   L   N   A   W   L   Y   S   A   420

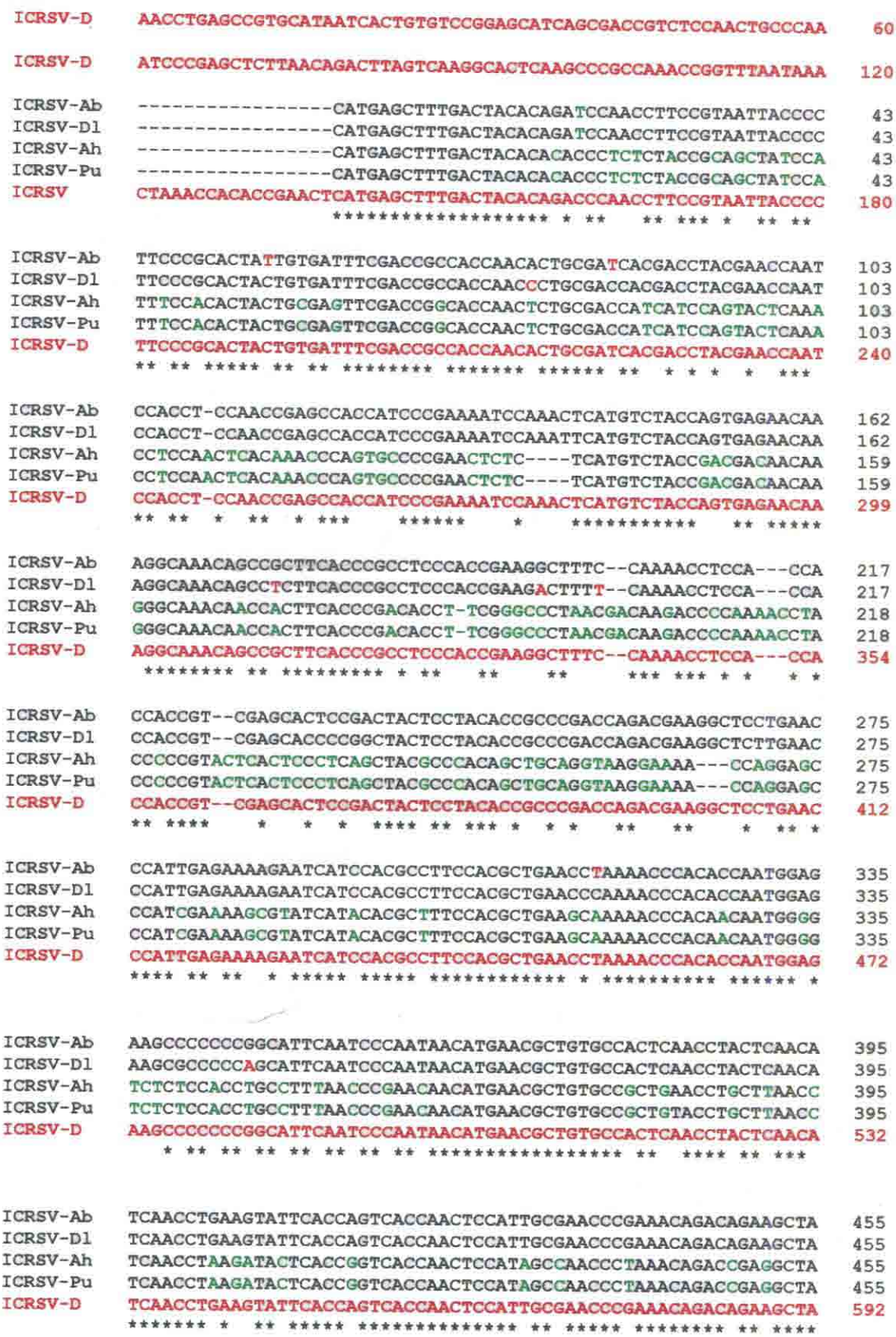
1261  CGG GAC CGA TTA GGT CAT TGA   1281
421    R   D   R   L   G   H   *
```

Fig. 15D: Nucleotide (shown as DNA) and deduced amino acid sequences of the coat protein gene of ICRSV - Pu isolate.

The phylogenetic tree constructed based on nucleotide sequences (Fig. 18), revealed very close relationship between ICRSV-Ab isolate with the reported ICRSV-D, following by ICRSV - Dl necrotic isolate, and both of them showing close relationship with each other, while the ICRSV-Ah and ICRSV - Pu isolates showed very close relationship to each other, but distant relationship to ICRSV - Dl, ICRSV-Ab and ICRSV-D isolates.

The phylogenetic tree constructed based on amino acid comparison of different isolates showed the similar results as observed in the phylogenetic tree based on nucleotide sequences (Fig. 19).

All the sequences with 1283 nucleotides of four isolates were submitted to genebank and the accession numbers have been released (Table 17)


ICRSV-D AACCTGAGCCGTGCATAATCACTGTGTCCGGAGCATCAGCGACCGTCTCCAACTGCCCAA 60
ICRSV-D ATCCCGAGCTCTTAACAGACTTAGTCAAGGCACTCAAGCCCGCCAAACCGGTTTAATAAA 120
ICRSV-Ab ----------CATGAGCTTTGACTACACAGATCCAACCTTCCGTAATTACCCC 43
ICRSV-Dl ----------CATGAGCTTTGACTACACAGATCCAACCTTCCGTAATTACCCC 43
ICRSV-Ah ----------CATGAGCTTTGACTACACACACCCTCTCTACCGCAGCTATCCA 43
ICRSV-Pu ----------CATGAGCTTTGACTACACACACCCTCTCTACCGCAGCTATCCA 43
ICRSV CTAAACCACACCGAACTCATGAGCTTTGACTACACAGACCCAACCTTCCGTAATTACCCC 180
ICRSV-Ab TTCCCGCACTATTGTGATTTCGACCGCCACCAACACTGCGATCACGACCTACGAACCAAT 103
ICRSV-Dl TTCCCGCACTACTGTGATTTCGACCGCCACCAACCCTGCGACCACGACCTACGAACCAAT 103
ICRSV-Ah TTTCCACACTACTGCGAGTTCGACCGGCACCAACTCTGCGACCATCATCCAGTACTCAAA 103
ICRSV-Pu TTTCCACACTACTGCGAGTTCGACCGGCACCAACTCTGCGACCATCATCCAGTACTCAAA 103
ICRSV-D TTCCCGCACTACTGTGATTTCGACCGCCACCAACACTGCGATCACGACCTACGAACCAAT 240
ICRSV-Ab CCACCT-CCAACCGAGCCACCATCCCGAAAATCCAAACTCATGTCTACCAGTGAGAACAA 162
ICRSV-Dl CCACCT-CCAACCGAGCCACCATCCCGAAAATCCAAATTCATGTCTACCAGTGAGAACAA 162
ICRSV-Ah CCTCCAACTCACAAACCCAGTGCCCCGAACTCTC----TCATGTCTACCGACGACAACAA 159
ICRSV-Pu CCTCCAACTCACAAACCCAGTGCCCCGAACTCTC----TCATGTCTACCGACGACAACAA 159
ICRSV-D CCACCT-CCAACCGAGCCACCATCCCGAAAATCCAAACTCATGTCTACCAGTGAGAACAA 299
ICRSV-Ab AGGCAAACAGCCGCTTCACCCGCCTCCCACCGAAGGCTTTC--CAAAACCTCCA---CCA 217
ICRSV-Dl AGGCAAACAGCCTCTTCACCCGCCTCCCACCGAAGACTTTT--CAAAACCTCCA---CCA 217
ICRSV-Ah GGGCAAACAACCACTTCACCCGACACCT-TCGGGCCCTAACGACAAGACCCCAAAACCTA 218
ICRSV-Pu GGGCAAACAACCACTTCACCCGACACCT-TCGGGCCCTAACGACAAGACCCCAAAACCTA 218
ICRSV-D AGGCAAACAGCCGCTTCACCCGCCTCCCACCGAAGGCTTTC--CAAAACCTCCA---CCA 354
ICRSV-Ab CCACCGT--CGAGCACTCCGACTACTCCTACACCGCCCGACCAGACGAAGGCTCCTGAAC 275
ICRSV-Dl CCACCGT--CGAGCACCCCGGCTACTCCTACACCGCCCGACCAGACGAAGGCTCTTGAAC 275
ICRSV-Ah CCCCCGTACTCACTCCCTCAGCTACGCCCACAGCTGCAGGTAAGGAAAA---CCAGGAGC 275
ICRSV-Pu CCCCCGTACTCACTCCCTCAGCTACGCCCACAGCTGCAGGTAAGGAAAA---CCAGGAGC 275
ICRSV-D CCACCGT--CGAGCACTCCGACTACTCCTACACCGCCCGACCAGACGAAGGCTCCTGAAC 412
ICRSV-Ab CCATTGAGAAAAGAATCATCCACGCCTTCCACGCTGAACCTAAAACCCACACCAATGGAG 335
ICRSV-Dl CCATTGAGAAAAGAATCATCCACGCCTTCCACGCTGAACCCAAAACCCACACCAATGGAG 335
ICRSV-Ah CCATCGAAAAGCGTATCATACACGCTTTCCACGCTGAAGCAAAAACCCACAACAATGGGG 335
ICRSV-Pu CCATCGAAAAGCGTATCATACACGCTTTCCACGCTGAAGCAAAAACCCACAACAATGGGG 335
ICRSV-D CCATTGAGAAAAGAATCATCCACGCCTTCCACGCTGAACCTAAAACCCACACCAATGGAG 472
ICRSV-Ab AAGCCCCCCCCGGCATTCAATCCCAATAACATGAACGCTGTGCCACTCAACCTACTCAACA 395
ICRSV-Dl AAGCGCCCCCAGCATTCAATCCCAATAACATGAACGCTGTGCCACTCAACCTACTCAACA 395
ICRSV-Ah TCTCTCCACCTGCCTTTAACCCGAACAACATGAACGCTGTGCCGCTGAACCTGCTTAACC 395
ICRSV-Pu TCTCTCCACCTGCCTTTAACCCGAACAACATGAACGCTGTGCCGCTGTACCTGCTTAACC 395
ICRSV-D AAGCCCCCCCCGGCATTCAATCCCAATAACATGAACGCTGTGCCACTCAACCTACTCAACA 532
ICRSV-Ab TCAACCTGAAGTATTCACCAGTCACCAACTCCATTGCGAACCCGAAACAGACAGAAGCTA 455
ICRSV-Dl TCAACCTGAAGTATTCACCAGTCACCAACTCCATTGCGAACCCGAAACAGACAGAAGCTA 455
ICRSV-Ah TCAACCTAAGATACTCACCGGTCACCAACTCCATAGCCAACCCTAAACAGACCGAGGCTA 455
ICRSV-Pu TCAACCTAAGATACTCACCGGTCACCAACTCCATAGCCAACCCTAAACAGACCGAGGCTA 455
ICRSV-D TCAACCTGAAGTATTCACCAGTCACCAACTCCATTGCGAACCCGAAACAGACAGAAGCTA 592

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ICRSV-Ab    TTGGCAAAGCCTGGGTCCGTATCCTGCAAATTGACCCGGCGAACGTATTCCTCTACGCCA   515
ICRSV-Dl    TTGGCAAAGCCTGGGTCCGTATCCTGCAAATTGATCCTGCGAACGTATTCCTCTACGCCA   515
ICRSV-Ah    TCGGAAAAGCTTGGGTCCGCATTTTGAACATCGATCCTGCCAACGTGTTCTTATACGCCA   515
ICRSV-Pu    TCGGAAAAGCTTGGGTCCGCATTTTGAACATCGATCCTGCCAACGTGTTCTTATACGCCA   515
ICRSV-D     TTGGCAAAGCCTGGGTCCGTATCCTGCAAATTGACCCGGCGAACGTATTCCTCTACGCCA   652
            * ** ***** ******** **  ** * ** ** ** ** ***** *** * *******

ICRSV-Ab    TCGATCTCGCCCGAGCCTGCGCTGACGCTGGAAGCTCACCCGAAGCCGACATTATCGGCG   575
ICRSV-Dl    TCGACCTCGCCCGAGCTTGCGCTGACGCTGGAAGCTCACCCGAAGCCGACATTATCGGCG   575
ICRSV-Ah    TTGACCTCGCCAGAGCTTGCGCCGACGCGGGCTCTTCTCCTGAAGCTGATATTATTGGAG   575
ICRSV-Pu    TTGACCTCGCCAGAGCTTGCGCCGACGCGGGCTCTTCTCCTGAAGCTGATATTATTGGAG   575
ICRSV-D     TCGACCTCGCCCGAGCCTGCGCTGACGCTGGAAGCTCACCCGAAGCCGACATTATCGGCG   712
            * ** ****** **** ***** ***** **    ** ** ***** ** ***** ** *

ICRSV-Ab    CGAACGAAGACCTCAATCCAGTGGTCGAGCGAAACGCCCTAGCTGGTGTTGTCCGAGACT   635
ICRSV-Dl    CGAACGAAGACCTCAATCCAGTGGTCGAGCGAAACGCCCTAGCTGGTGTTGTCCGAGACT   635
ICRSV-Ah    CGAACGAAGATCTCAACCCCGTTGTTGAACGAAACGCATTGGCCCTAGTGGTTAGGGATT   635
ICRSV-Pu    CGAACGAAGATCTCAACCCCGTTGTTGAACGAAACGCATTGGCCCTAGTGGTTAGGGATT   635
ICRSV-D     CGAACGAAGACCTCAATCCAGTGGTCGAGCGAAACGCCCTAGCTGGTGTTGTCCGAGACT   772
            ********** ***** ** ** ** ** ********  * **    ** **  * ** *

ICRSV-Ab    TCTGCCCACTGCGCGCCTTTTGCGCTTACTACTCCAGGGTGGTATGGAACCTCATGATCA   695
ICRSV-Dl    TCTGCCCATTGCGCGCCTTTTGCGCTTACTACTCCAGGGTAGTATGGAACCTCATGATCA   695
ICRSV-Ah    TCTGCCCGCTACGCGCTTTTTGCGCTTACTACTCTCGAGTGGTATGGAACCTCATGATCA   695
ICRSV-Pu    TCTGCCCGCTACGCGCTTTTTGCGCTTACTACTCTCGAGTGGTATGGAACCTCATGATCA   695
ICRSV-D     TCTGCCCACTGCGCGCCTTTTGCGCTTACTACTCCAGGGTGGTATGGAACCTCATGATCA   832
            *******  * ***** *****************  * ** ********************

ICRSV-Ab    AAGCGGATCAACCACCCGCCAACTGGATGAAATCAGGGATAGACGAGGGAGCCAAATTCG   755
ICRSV-Dl    AAGCGGATCAACCACCGGCCAACTGGATGAAATCAGGGATAGACGAGGGAGCCAAATTCG   755
ICRSV-Ah    AGGCGGACCAGCCTCCGGCCAACTGGATGAAATCCGGGGTAGACGAGAACGCGAAATTCG   755
ICRSV-Pu    AGGCGGACCAGCCTCCGGCCAACTGGATGAAATCCGGGGTAGACGAGAACGCGAAATTCG   755
ICRSV-D     AAGCGGATCAACCACCCGCCAACTGGATGAAATCAGGGATAGACGAGGGAGCCAAATTCG   892
            * ***** ** ** ** ***************** *** ********   ** *******

ICRSV-Ab    CAGCGTTCGACTTCTTCCATGGCGTCCTTTCACCCGCTTCCCTGTATGTCCCTCTGGAAC   815
ICRSV-Dl    CAGCGTTCGACTTCTTCCATGGCGTCCTTTCGCCCGCTTCCCTGTATGTTCCTCTGGAAC   815
ICRSV-Ah    CGGCATTCGATTTCTTCCATGGTATCCTCTCGCCAGCTTCCCTGTATGTGCCCCTAGAGA   815
ICRSV-Pu    CGGCATTCGATTTCTTCCATGGTATCCTCTCGCCAGCTTCCCTGTATGTGCCCCTAGAGA   815
ICRSV-D     CAGCGTTCGACTTCTTCCATGGCGTCCTTTCACCCGCTTCCCTGTATGTCCCTCTGGAAC   952
            * ** ***** ***********  **** ** ** ************** ** ** **

ICRSV-Ab    GTCACCCTACAGCCGCGGAACGCATAGCCAACCAAGCTATGTTCGCTGTCAAAATTGCGA   875
ICRSV-Dl    GTCACCCTACTGCCGCGGAACGCATAGCCAACCAAGCCATGTTCGCTGTCAAAATTGCGA   875
ICRSV-Ah    GACACCCTACTTCCGCGGAGAGGATCGCAAATCAGGCCATGTTCGCTGTGAAAATTGCCA   875
ICRSV-Pu    GACACCCCACTTCCGCGGAGAGGATCGCAAATCAGGCCATGTTCGCTGTGAAAATTGCCA   875
ICRSV-D     GTCACCCTACAGCCGCGGAACGCATAGCCAACCAAGCTATGTTCGCTGTCAAAATTGCGA  1012
            * ***** **  *******  * ** ** ** ** ** ********** ******** *

ICRSV-Ab    ACGCGCCCGGCAATGGTTCAGAGCTCACGATGGACCACGTTGCCTTCACCAAAGGCCGGA   935
ICRSV-Dl    ACGCGCCCGGCAATGGTTCAGAGCTCACGATGGACCACGTTGCCTTCACCAAAGGCCGGA   935
ICRSV-Ah    ACGCTCCAGGAAATGGCACGGACCTCACGATGGACCACGTTGCCTTCACCAAAGGAAAGA   935
ICRSV-Pu    ACGCTCCAGGAAATGGCACGGACCTCACGATGGACCACGTTGCCTTCACCAAAGGAAAGA   935
ICRSV-D     ACGCGCCCGGCAATGGTTCAGAGCTCACGATGGACCACGTTGCCTTCACCAAAGGCCGGA  1072
            **** ** ** *****  * ** ********************************   **

ICRSV-Ab    TTACAGCAGACTCCAAGCCCCGCCCGACCCCTTTCAACACTTAAACAGCTTCGAACCCAT   995
ICRSV-Dl    TTACAGCAGACTCCAAGCCCCACCCGACCCCTTTCAACACTTAAACAGCTTCGAACCCAT   995
ICRSV-Ah    TTACCCAGCCCTCCGGCCTCCGCCCGACCCCTTTCAACACTTAAACAGCTTCGAACCCAT   995
ICRSV-Pu    TTACCCAGCCCTCCGGCCTCCGCCCGACCCCTTTCAACACTTAAACAGCTTCGAACCCAT   995
ICRSV-D     TTACAGCAGACTCCAAGCCCCGCCCGACCCCTTTCAACACTTAAACAGCTTCGAACCCAT  1132
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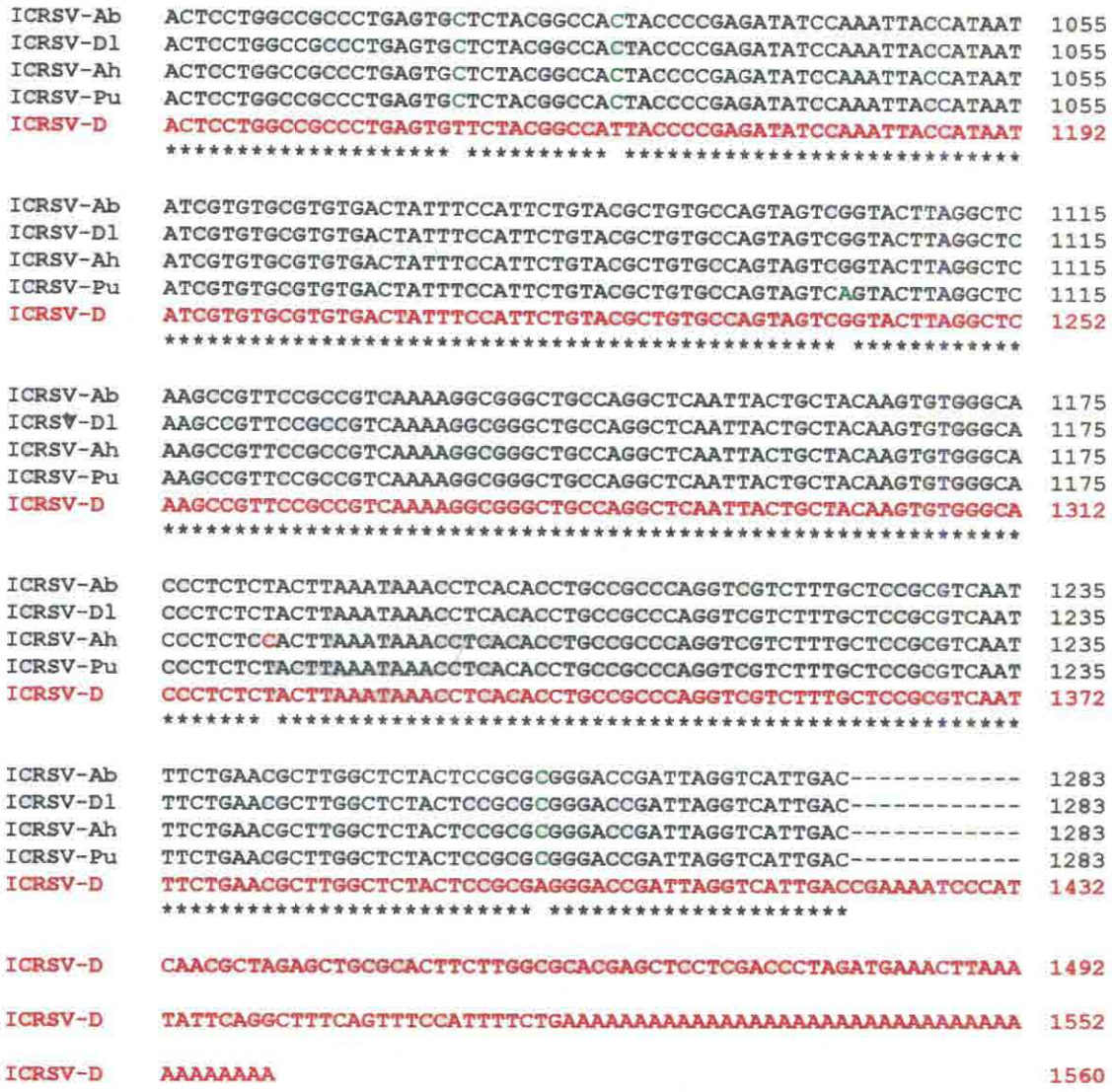

Fig. 16: Comparison of coat protein gene nucleotide sequences of four isolates of Indian Citrus Ringspot virus with the reported one (ICRSV-D).

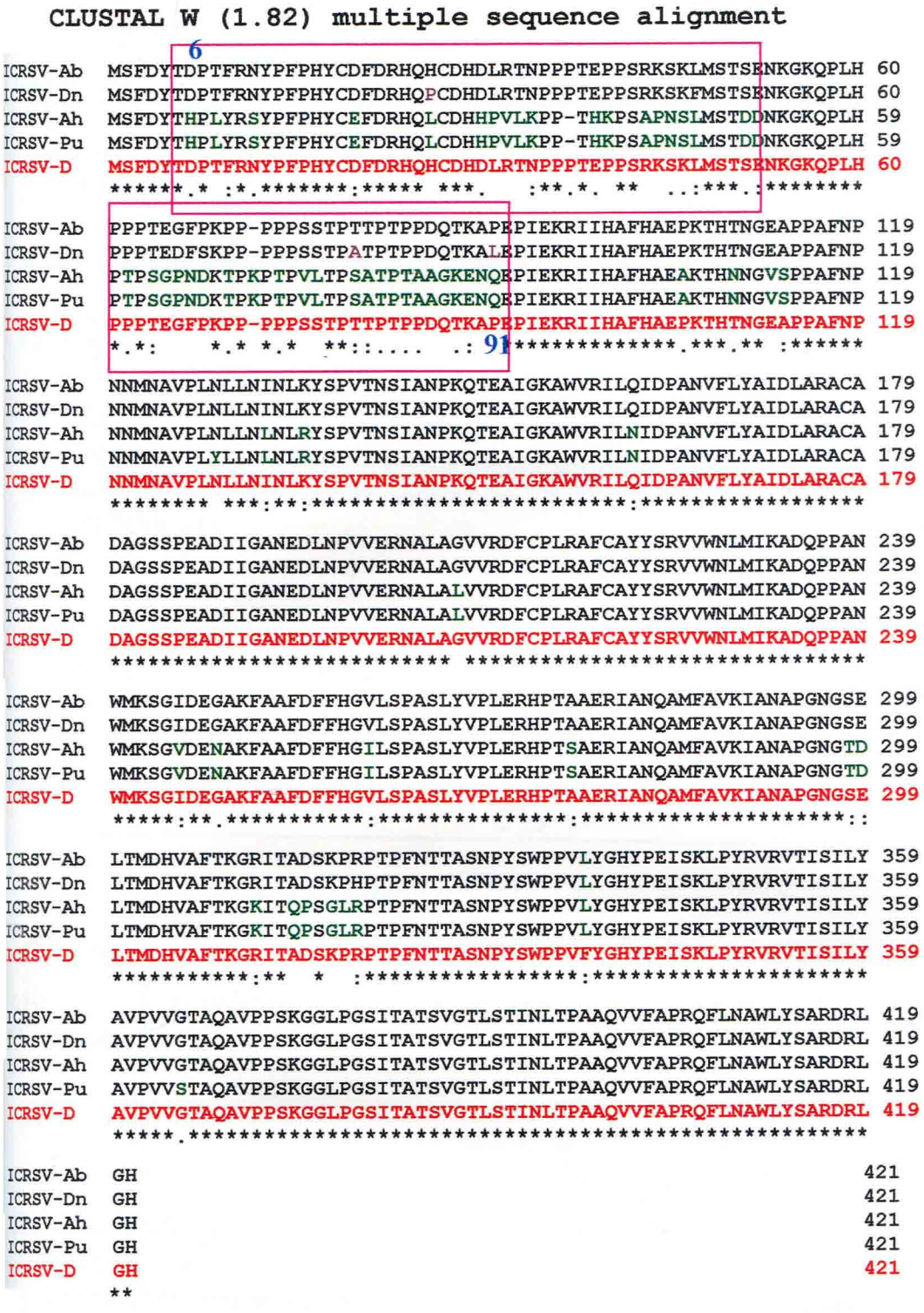

Fig. 17: Comparison of amino acid sequences of four isolates of Indian Citrus Ringspot virus with the reported one (ICRSV-D).

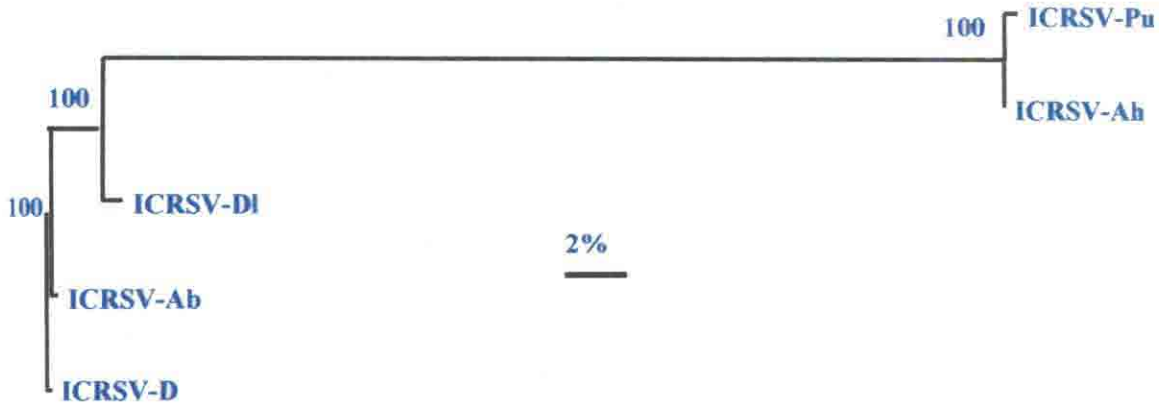

Fig. 18: Cluster dendrogram illustrating phylogenic clustering relationships using Treecon software based on the multiple alignments of the coat proein gene nucleotide sequences of 4 isolates with reported one (ICRSV-D).

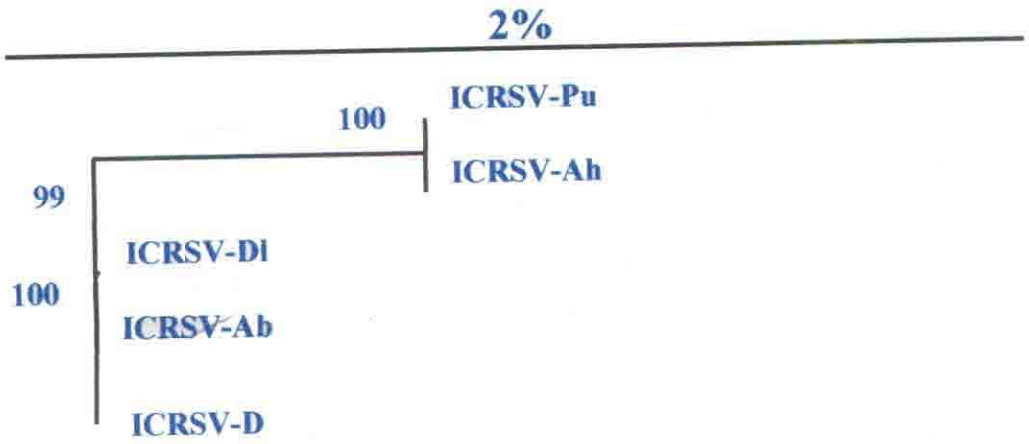

Fig. 19: Cluster dendrogram illustrating phylogenic clustering relationships using Treecon software based on the multiple alignments of the amino acids sequences of 4 isolates with the reported one (ICRSV-D).

Table 17: Accession numbers of sequences of four ICRSV isolates.

S. No.	Isolate	Accession No
1.	ICRSV - Dl	AY 255006
2.	ICRSV – Ah	AY 255007
3.	ICRSV - Pu	AY 255008
4.	ICRSV - Ab	AY 255009

4.3. 4. Restriction analysis

Restriction map of the four isolates were done based on the sequences by using Bioedit software. The results are shown in Fig. 20 and Table 18.

Table 18: Restriction sites present in all the four isolates and some sites of common restriction enzymes.

S. No.	Enzymes	Sites			
		ICRSV-Ab	ICRSV-Dl	ICRSV-Ah	ICRSV-Pu
1	Bsi E I	68	68	68	68
2	**Pst I**	-	-	**257**	**257**
3	**Cla I**	**517**	-	**487**	**487**
4	BspH I	688	688	688	688
5	Bcl I	691	691	691	691
6	Sty I	773	773	773	773
7	Nco I	773	773	773	773
8	Sac II	832	832	832	832
9	**Sac I**	**901**	**901**	-	-
10	BstB I	987	987	987	987
11	BsiC I	987	987	987	987
12	EcoR V	1039	1039	1039	1039

Result from Table 18 revealed that there are nine restriction sites present in all the four isolates, while there are three other sites of common restriction enzymes such as *Pst* I presents in ICRSV-Ah and ICRSV-Pu at 257 site, but it does not present in ICRSV-Dl and ICRSV-Ab. *Cla* I presents in ICRSV-Ah and ICRSV-Pu at 487 site and in ICRSV-Ab at 517 site, but it does not present in ICRSV-Dl. *Sac* I presents in ICRSV-Ab and ICRSV-Dl

at 901 site, but it does not present in ICRSV-Ah and ICRSV-Pu. They were further confirmed by restricting the PCR product (978bp) with these three enzymes (Fig. 21).

The amino acid sequences of ICRSV were used for blast separately in internet, the results shown some matching points with some other viruses belong to genus Luteo-, Potex- and Carlaviruses. The amino acid sequences of these viruses were used for multiple sequence alignment in Bioedit, the results came with some matching points for different viruses (Table 19 and Fig. 22). The position of matching was from 134 to 324 and percentage of matching was 32 to 47%. The multiple sequences alignment was again used to draw a phylogenetic tree using Treecon software (Fig. 23). The result showed that the ICRSV did not share any close relationship with any group of plant viruses reported worldwide.

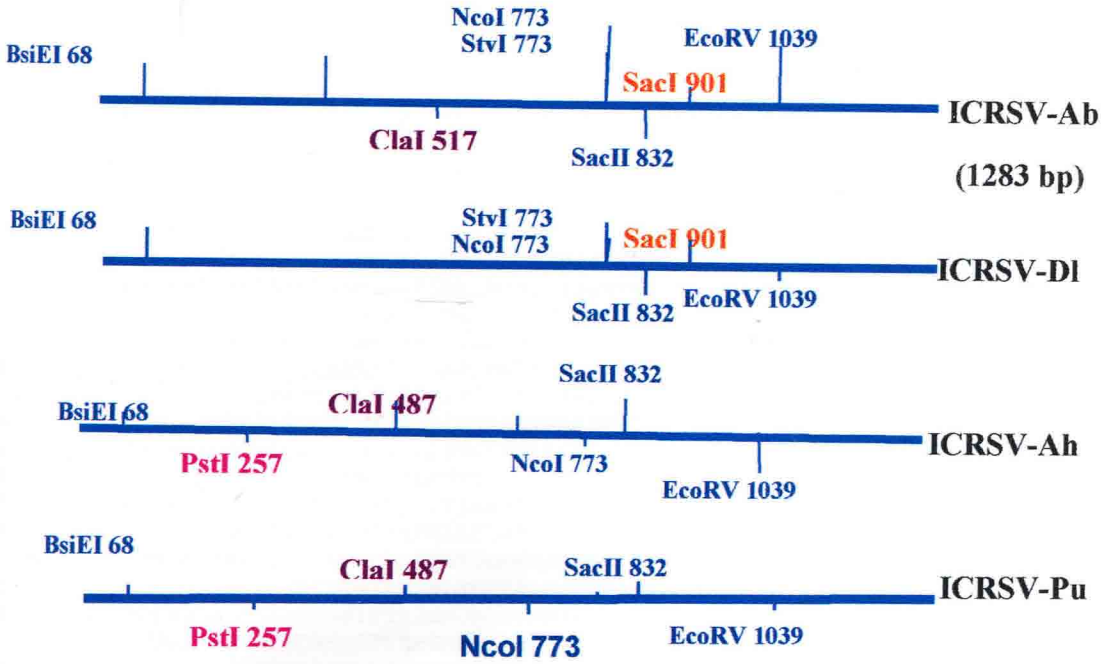

Fig. 20: Restriction map made in Bioedit of the four isolates of ICRSV

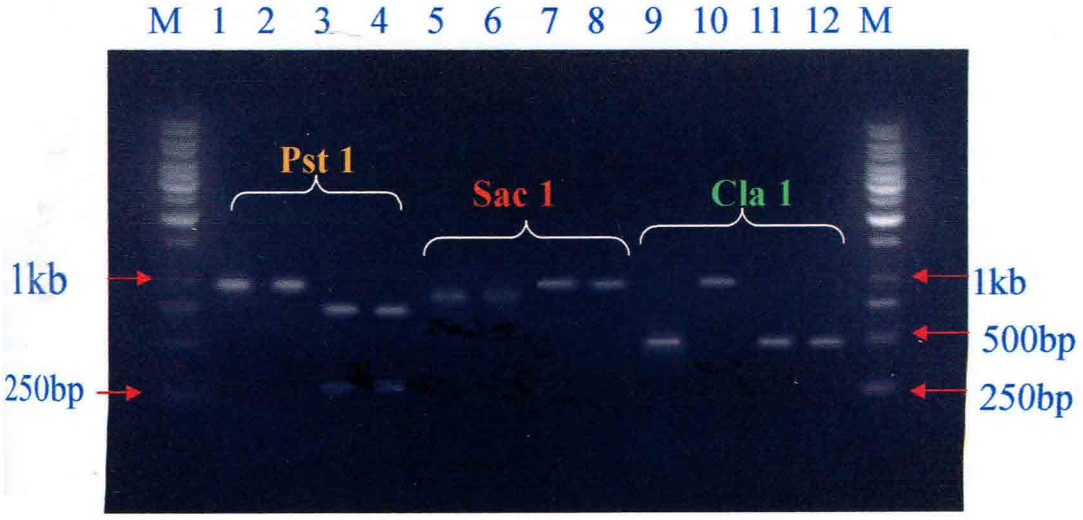

Fig. 21: Restriction analysis of 978bp PCR amplified CP gene by *Pst* I, *Sac* I and *Cla* I restriction enzymes.

M: 1 kb DNA Ladder,
Lane 1-4: ICRSV-Ab, ICRSV-Dl, ICRSV-Ah, ICRSV-Pu using *Pst* I
Lane 5-8: ICRSV-Ab, ICRSV-Dl, ICRSV-Ah, ICRSV-Pu using *Sac* I
Lane 9-12: ICRSV-Ab, ICRSV-Dl, ICRSV-Ah, ICRSV-Pu using *Cla* I

```
ICRSV-Ab    THTNGEAPPAFNPNNMNAVPLNLLNINLKYSPVTNSIANPKQTEAIGKAWVRILQIDPAN 166
ICRSV-D     THTNGEAPPAFNPNNMNAVPLNLLNINLKYSPVTNSIANPKQTEAIGKAWVRILQIDPAN 166
ICRSV-Dn    THTNGEAPPAFNPNNMNAVPLNLLNINLKYSPVTNSIANPKQTEAIGKAWVRILQIDPAN 166
ICRSV-Ah    THNNGVSPPAFNPNNMNAVPLNLLNLNLRYSPVTNSIANPKQTEAIGKAWVRILNIDPAN 166
ICRSV-Pu    THNNGVSPPAFNPNNMNAVPLYLLNLNLRYSPVTNSIANPKQTEAIGKAWVRILNIDPAN 166
SMYEV       ANQVGDPFRVLTPEELAAPIAAASNKVATREQIIQIVADLNALGFVG-----------D 86
CCMV        LDLKPASNLVASADALSAIAADWASLKVPTAQLMR------------------------- 71
PMV         LKVDPTSNLLPSPEQLKSVSALMVAAKVPAASVTT------------------------- 58
ASPV        LGNRAPRNATSNTGGMRRRLDSVGLKNIRYEPQAGVVASNQKIRAVGVALIM--GIPEHQ 237
GRSPAV      ---RIIENALSKTVDMREVLKHETVVISPNVMDEGAID--ELIRAFGES-----GIAEGV 99
CYMV        ---------APTDEQLDTLTLTIESNLVPSISELEAIAKDWKTLGLQEADFTANAIKIAW 61
CyMV        ---------TYSAADPTSAPKLADLAAIKYSPVTSSIATPEEIKAITQLWVNNLGLPADT 61
PoAMV       --QFLSAPKQFSASDVRSSPTLTDLDEIAYEVRTTSIASPAEIEAVSQLWIMNTEIPADK 88
WCMV        ----------MATTTATTPPSLTDIRALKYTSSTVSVASPAEIEAITKTWAETFKIPNDV 50
GLV         PKAQLADSLKGDASNIFTRPSMDALIVRNYAPESNNLATAEELAKIS-AKVQALGAPEES 134
SLV         PKAHLADSLKGDASNIFTRPSMDALLVRNYAPESNNLATAEELAKIS-AKVQALGAPEEC 134
BMMV        --------KEYDSSNMVTQPTLEQMAKFDFKNVCLDIATKAELEWISESWNMRLNLPKEK 80
CGRMV       LRARRKRVTFNPSDPTSSPSRSFINSIQETNPVTLNIASDDTVKAITSDWVEFLKIKQEE 110

ICRSV-Ab    VFLYAIDLARACADAGSSPEADIIGANEDLNPVVERNALAGVVRDF-CPLRAFCAYYSRV 225
ICRSV-D     VFLYAIDLARACADAGSSPEADIIGANEDLNPVVERNALAGVVRDF-CPLRAFCAYYSRV 225
ICRSV-Dn    VFLYAIDLARACADAGSSPEADIIGANEDLNPVVERNALAGVVRDF-CPLRAFCAYYSRV 225
ICRSV-Ah    VFLYAIDLARACADAGSSPEADIIGANEDLNPVVERNALALVVRDF-CPLRAFCAYYSRV 225
ICRSV-Pu    VFLYAIDLARACADAGSSPEADIIGANEDLNPVVERNALALVVRDF-CPLRAFCAYYSRV 225
SMYEV       PALGLFDLAFHCYDIGSSPSAQPVGASPFG---CSRMQVAAVVRNH-CTVRQLCMFYAPS 142
CCMV        ---HALDLVNFCFDSGSSKYTTVEGS--SPTPTIPRAALAGAVRKH-TTLRQFCRYYAKI 125
PMV         ---VAMELVNFCYDNGSSAYTTVVGP--SSVPEVSLAQLASIVKASGTSLRKFCRYFAPI 113
ASPV        LTEVGVYLARHCADVGASDKSTLLGTF-PGSDITLEEVGTMIKQTEGCTLRQYCAFYAKH 296
GRSPAV      QFDVAIDIARHCSDVGSSQRSTLIGKS-PFCDLNRSEIAGIIREVT--TLRRFCMYYAKI 156
CYMV        FCYHSGSSESVQVQGNSTSDKIPLYQL-AGVVRQHSTLRRFCRYFAKVIWNYALRKNQPP 120
CyMV        VGTAAIDLARAYADVGASKNATLLGFC-PTKPDVRRAALAAQIFVANVTPRQFCAYYAKV 120
PoAMV       VALIAIDMARAYADVGASRKAVLLDAP-ALAPTVARSRLAQLMAGAGISPRQFCSYYAKI 147
WCMV        LPLACWDLARAFADVGASSKSELTGDS-AALAGVSRKQLAQAIKIH-CTIRQFCMYFANI 108
GLV         LAEVFWDICMYCATAGSSPNVNPKGAISVAGKVVTRDMVVAVIKEY-TTLRQFCRCYAPV 193
SLV         LAEVFWDICMYCATAGSSPNVNPKGTISVGGKVVTRDMVVAVIKEY-TTLRQFCRCYAPV 193
BMMV        NFETALEIAEVCRHNGSSSDITFRGRS---RSGIEYSSLVAAIREI-CPLRQFCRAYANL 136
CGRMV       VFDCIFDVVLFCYHNSSSDKTKMVGKA---KNGIGLEDLASTVRSY-CSLRSFCSKYAPV 166
```

Fig. 22: Multiple alignment of amino acid sequences among ICRSV isolates and other viruses

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ICRSV-Ab    VWNLMIKADQPPANWMKSGIDEGAKFAAFDFFHGVLSPASLYVP--LERHPTAAERIANQ 283
ICRSV-D     VWNLMIKADQPPANWMKSGIDEGAKFAAFDFFHGVLSPASLYVP--LERHPTAAERIANQ 283
ICRSV-Dn    VWNLMIKADQPPANWMKSGIDEGAKFAAFDFFHGVLSPASLYVP--LERHPTAAERIANQ 283
ICRSV-Ah    VWNLMIKADQPPANWMKSGVDENAKFAAFDFFHGILSPASLYVP--LERHPTSAERIANQ 283
ICRSV-Pu    VWNLMIKADQPPANWMKSGVDENAKFAAFDFFHGILSPASLYVP--LERHPTSAERIANQ 283
SMYEV       VWNKAVKDNRPPGNWANLQFTPETKFAAFDFFDGVLNPASQQVP--LWRQPTPQEIYASA 200
CCMV        IWNARVKANIPPAGYANAHIKPEQAFAGFDFFDGVMNVAALEPSGGLVREPTPQEIIAAE 185
PMV         IWNIRTDK-TPPANWEAAGYKPNAKFAAFDFFDGVENPAAMQPPTGLIRSPTQEERIANA 172
ASPV        VWNLMLQTQSPPANWVGKEFKFETRYAAFDFFFGVESTASLEPADGLIRLPTQAERVANA 356
GRSPAV      VWNIHLETGIPPANWAKKGFNENEKFAAFDFFLGVTDESALEPKGGIKRAPTKAEMVANI 216
CYMV        ANWASQNYKEADRFAAFDFFEGVSSSAALSPPGGLIREPSPNERM-ANETNKNVHLYQTA 179
CyMV        VWNLMLATNDPPANWAKAGFQEDTRFAAFDFFDAVDSTAALEPAE-WQRRPTDRERAAHS 179
PoAMV       VWNLMLHKNEPPANWAKIGFKEDYKFAAFDFFDAVDSPAALEPSQ-WVRHPTDKERAAHG 206
WCMV        VWNIMLDTKTPPASWSKLGYKEESKFAGFDFFDGVNHPAALMPADGLIRGPSDAEILAHQ 168
GLV         VWNYMLLNEQPPANWDAKGFTENTKYAAFDTFDAVTNKAAIQPLEGLIRAPTDAERIAFA 253
SLV         VWNYMLLNEQPPANWDAKGFTENTKYAAFDTFDAVTNKAAIQPLEGLIRAPTDAERIAFA 253
BMMV        VWEKSLAEKNPPQHWQKRGFKERVKYAAFDFLDAVGSDAAIMPPTGISRLPTDEELNANL 196
CGRMV       VWNYGISNDLPPANWQRRKVVEGAKFAAFDFFEAVTSEAALQPVEGLVRNPTDKEMTAGA 226

ICRSV-Ab    AMFAVKIANAPGNGSELTMDHVAFTKGRITADSKPRPTPFNTTASNPYSWPPVLYGHYPE 343
ICRSV-D     AMFAVKIANAPGNGSELTMDHVAFTKGRITADSKPRPTPFNTTASNPYSWPPVFYGHYPE 343
ICRSV-Dn    AMFAVKIANAPGNGSELTMDHVAFTKGRITADSKPHPTPFNTTASNPYSWPPVLYGHYPE 343
ICRSV-Ah    AMFAVKIANAPGNGTDLTMDHVAFTKGKITQPSGLRPTPFNTTASNPYSWPPVLYGHYPE 343
ICRSV-Pu    AMFAVKIANAPGNGTDLTMDHVAFTKGKITQPSGLRPTPFNTTASNPYSWPPVLYGHYPE 343
SMYEV       THKDVATYRAASKGHERISNSTLLTKG---------------------------------- 227
CCMV        TARSLNLFEAQSKGNNLATNATQVTRG---------------------------------- 212
PMV         TNKQVHLFQAAAQDNNFASNSAFITKG---------------------------------- 199
ASPV        TSKEIQMYRIRSMEGTQAVNFGEVTGG---------------------------------- 383
GRSPAV      ASFEVQVLRQAMAEGKRSSNLGEISGG---------------------------------- 243
CYMV        SRGSNLATTSTVATKGAYSTNASNAGF---------------------------------- 206
CyMV        IGKYGALARQRIQNGNLITNIAEVTKG---------------------------------- 206
PoAMV       VVKWASLSRERLQEGTSITTVAELNKG---------------------------------- 233
WCMV        TAKQVALHRDAKPTWHKRCQLC--------------------------------------- 190
GLV         THKKLALAKN-SQNSRYANTSAEVTGG---------------------------------- 279
SLV         THKKLALAKN-SNNSRYANTSAEVTGG---------------------------------- 279
BMMV        AAKNIAVINSARKKGNNTVQNLEVTGG---------------------------------- 223
CGRMV       SLKEISLMRDEIRRGTSSTLMTEVTGG---------------------------------- 253
```

Fig. 22: Multiple alignment of amino acid sequences among ICRSV isolates and other viruses (Cont.)

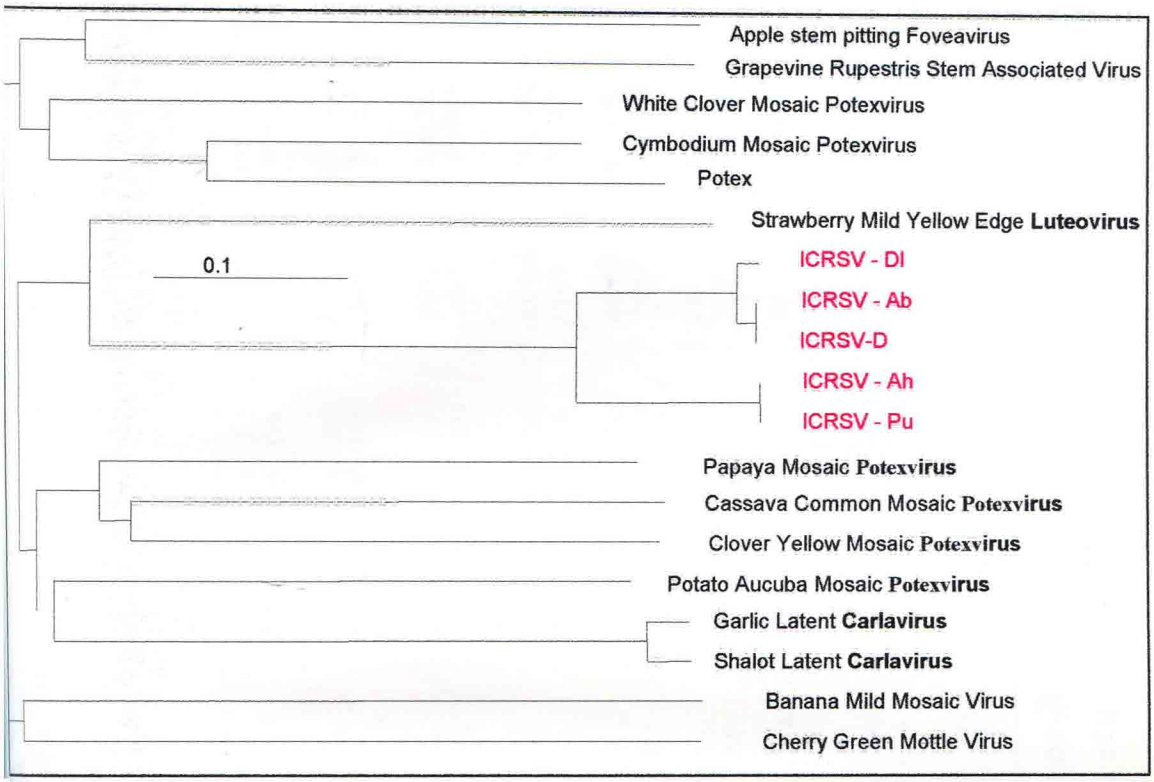


Fig. 23: Phylogenic tree showing the relationship of ICRSV with some other viruses

Table 19: Amino acid sequences identity of ICRSV isolates with other viruses showed some relationship

Virus	ICRSV - Ab		ICRSV - Dn		ICRSV - Ah		ICRSV - Pu	
	MP	I (%)	MP	I (%)	MP	I (%)	PM	I (%)
White clover mosaic virus	134 - 311 18 - 196	70/180 (38)	134 - 311 18 - 196	70/180 (38)	135 - 311 19 - 196	69/180 (38)	135 - 311 19 - 196	69/180 (38)
Cymbium mosaic virus	134 - 308 29 - 211	81/185 (43)	134 - 308 29 - 211	81/185 (43)	135 - 282 30 - 178	72/151 (47)	135 - 282 30 - 178	72/151 (47)
Grapevine Rupestris stem pitting associate virus	168 - 290 101 - 223	58/127 (45)	168 - 290 101 - 223	58/127 (45)	168 - 290 101 - 223	57/127 (44)	168-290 101-223	57/127 (44)
Potato aucuba mosaic virus	134 - 282 56 - 205	71/153 (46)	134 - 282 56 - 205	70/153 (46)	134 - 282 56 - 205	70/153 (46)	134 - 282 56 - 205	70/153 (46)
Potato virus X	140 - 313 49 - 222	69/176 (39)	140 - 313 49 - 222	69/176 (39)	140 - 313 49 - 222	68/176 (39)	140 - 313 49 - 222	68/176 (39)
Clover yellow mosaic virus	140 - 324 26 - 209	67/188 (35)	140 - 324 26 - 209	67/188 (35)	140 - 310 26 - 195	63/174 (36)	140 - 310 26 - 195	63/174 (36)
Cassava common mosaic virus	134 - 319 37 - 220	65/188 (34)	134 - 319 37 - 220	65/188 (34)	138 - 313 41 - 215	58/178 (32)	138 - 313 41 - 215	58/178 (32)
Cherry green ring mottle virus	128 - 285 72 - 228	62/161 (38)	137 - 285 81 - 225	60/152 (39)	137 - 285 81 - 228	59/152 (38)	137 - 285 81 - 228	59/152 (38)
Garlic latent virus	136 - 281 105 - 251	59/149 (39)	136 - 281 105 - 251	59/149 (39)	136 - 281 105 - 251	59/149 (390	136 - 281 105 - 251	59/149 (39)

MP: Matching point between ICRSV and other virus

I: Identity between two sequences (% identity)

4.3.5. Detection of ICRSV by RT-PCR

A primer set ICRSV-1096/ICRSV-1420 gave a product in the range of ICRSV - CP ≈ 350bp (Fig. 24).

The second and third sets of primers (ICRSV-579/ICRSV-951, ICRSV-518/ICRSV-1420) also used for detection of ICRSV isolates, the result showed that only two isolates ICRSV-Ab and ICRSV-Dl were able to amplify to a ≈ 450 bp and 900 bp product, while ICRSV-Ah and ICRSV-Pu isolate showed nonspecific amplification (Fig. 25).

4.3.4. Detection of ICRSV by Nucleic Acid hybridization

The PCR product of ICRSV - Ab isolate was used as a probe in this experiment. All the four isolates reacted with the probe making from the PCR product of ICRSV-1096/ICRSV-1420 primers (Fig. 26). The virus could be detected by this probe upto a dilution of 1:100. However, the PCR product of ICRSV-579/ICRSV-951 primers when used as probe, only ICRSV-Ab and ICRSV-Dl isolates strongly reacted with this probe. The ICRSV-Ah and ICRSV-Pu isolates showed faint dots suggesting mild reaction with this probe (Fig. 27).

4.4. PRODUCTION OF VIRUS - FREE NUCLEUS PLANTING MATERIALS

4.4.1. SHOOT TIP GRAFTNG

Sterilization of scion with 0.1% $HgCl_2$ for 3 min. at various concentrations showed maximum percentage of successful grafts (32.71%) as shown in Fig. 28. The treatment of shoot tip with 0.1% $HgCl_2$ for 3 min. for sterilization was therefore used for further the experiments.

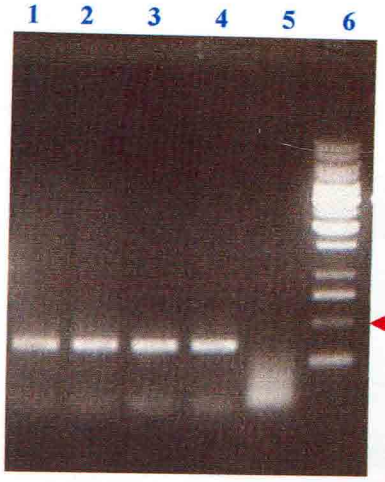


Fig. 24: PCR amplification of all four isolates using a set of primers **ICRSV-1096/ICRSV-1420** showing 350bp amplicon

Lane 1: ICRSV –Ab
Lane 2: ICRSV – Dl
Lane 3: ICRSV – Ah
Lane 4: ICRSV – Pu
Lane 5: Healthy
Lane 6: 1Kb DNA ladder

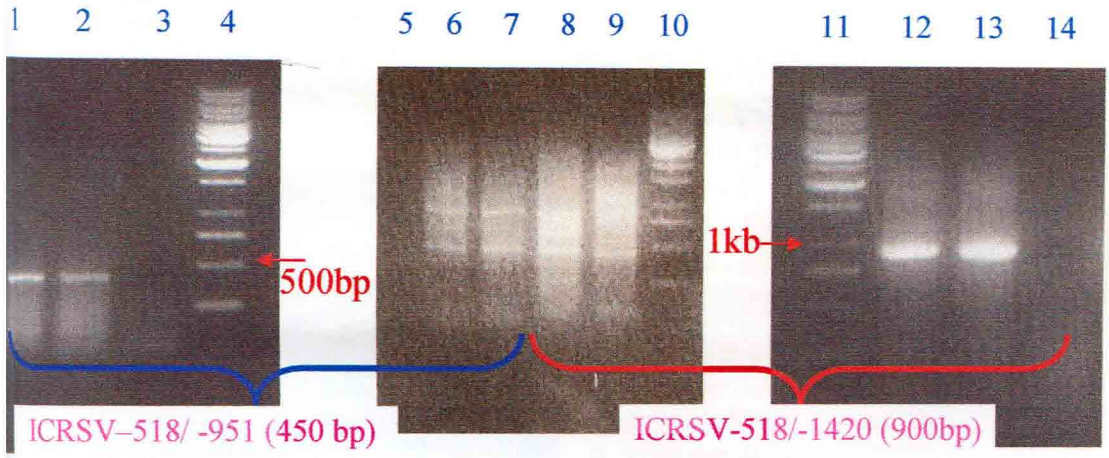


Fig. 25: PCR amplification of all four isolates using two sets of primers: ICRSV-518/ ICRSV-951(lane 1-7) and ICRSV-518/ICRSV-1420 (lane 8-14)

Lane 1: ICRSV- Dl
Lane 2: ICRSV – Ab
Lane 3, 5: Healthy control
Lane 4: 1kb DNA marker
Lane 6: ICRSV – Ah
Lane 7: ICRSV – Pu
Lane 8: ICRSV -Ah
Lane 9: ICRSV - Pu
Lane 10, 11: 1 kb DNA marker
Lane 12: ICRSV - Dl
Lane 13: ICRSV - Ab
Lane 14: Healthy control

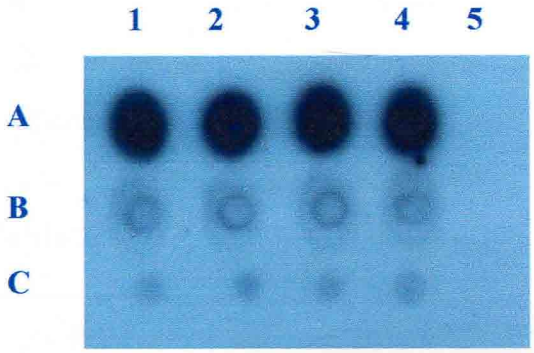


Fig. 26 : NASH test of 4 isolates by using γ-32P radiolabelled 350 bp PCR product as probe.

1: ICRSV - Pu isolate
2: ICRSV - Dl isolate
3: ICRSV - Ah isolate
4: ICRSV - Ab isolate
5: Healthy control
A: 1:1 dilution
B: 1:10 dilution
C: 1:100 dilution

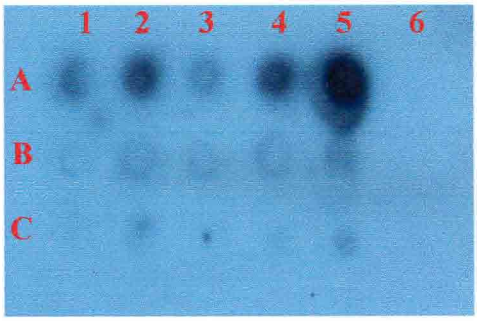


Fig. 27: NASH test of 4 isolates by using γ-32P radiolabelled 450 bp PCR product as probe.

1: ICRSV - Pu isolate
2: ICRSV - Dl isolate
3: ICRSV - Ah isolate
4: ICRSV - Ab isolate
5: Bean inoculated with ICRSV -Ab
6: Healthy control
A: 1:1 dilution
B: 1:10 dilution
C: 1:100 dilution

Among the different rootstocks tested for STG, Kinnow on Rough lemon was more successful (32.6%) than on Trifoliate orange, Mosambi, Malta sweet orange and on its own root (Table 20, Fig. 29). However, Mosambi on Mosambi rootstock gave highest success (28.2%) as compared to Rough lemon, Malta, Trifoliate and Kinnow mandarin. STG has been tried for the first time with Kinnow mandarin and Mosambi sweet orange. Since Rough lemon is used as rootstock in India, the same can also be used for Mosambi where successful grafts were 24% next to its own root.

Table 20: ***Selection of rootstock cultivar for successful grafting of Kinnow and Mosambi shoot-tips.***

Root stock	% Successful grafts with shoot tips of	
	Kinnow	Mosambi
Rough lemon	32.6	24.0
Trifoliate orange	28.3	20.7
Kinnow mandarin	20.1	19.6
Mosambi sweet orange	25.8	28.2
Malta sweet orange	24.6	22.7
Mean	26.28	23.04

Average of 24 grafts

Success of shoot tip grafts varied when different stages of Rough lemon rootstocks were used for grafting of Kinnow mandarin (Fig. 30). Maximum success of 33.33% was obtained when 15 days old seedlings were grafted. The percentage of successful grafts reduced with increasing age of seedlings.

The effect of light and darkness on the growth of rootstock seedlings was not significant except that the dark raised plants were pale green and soft, which helped for easier cutting and to insert meristem due to difference in color of rootstock and shoot tip.

Selection of sources for shoot tip grafting is very important as documented in Table 21 and Fig. 31. The results showed that shoot tips

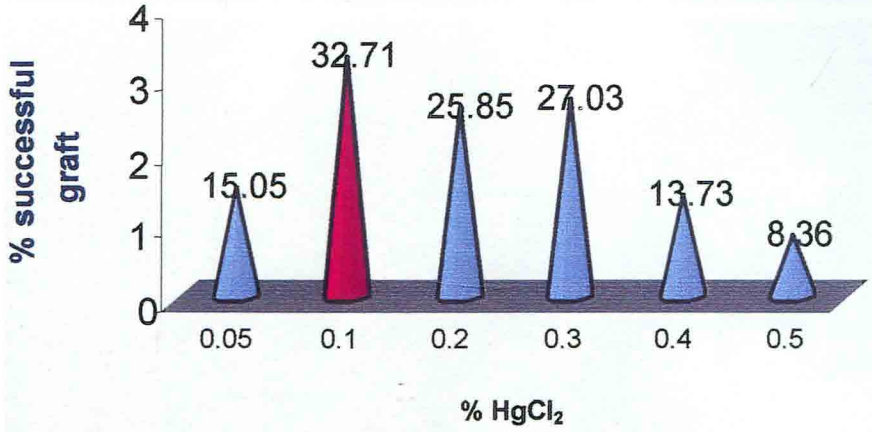


Fig 28: *Optimization of sterilization of shoot tip with $HgCl_2$ for 3 min treatment. (Average of 24 grafts)*

Fig. 29: Different rootstocks used for shoot tip grafting
(A): Left to right: Kinnow mandarin, Trifoliate orange
(B): Rough lemon

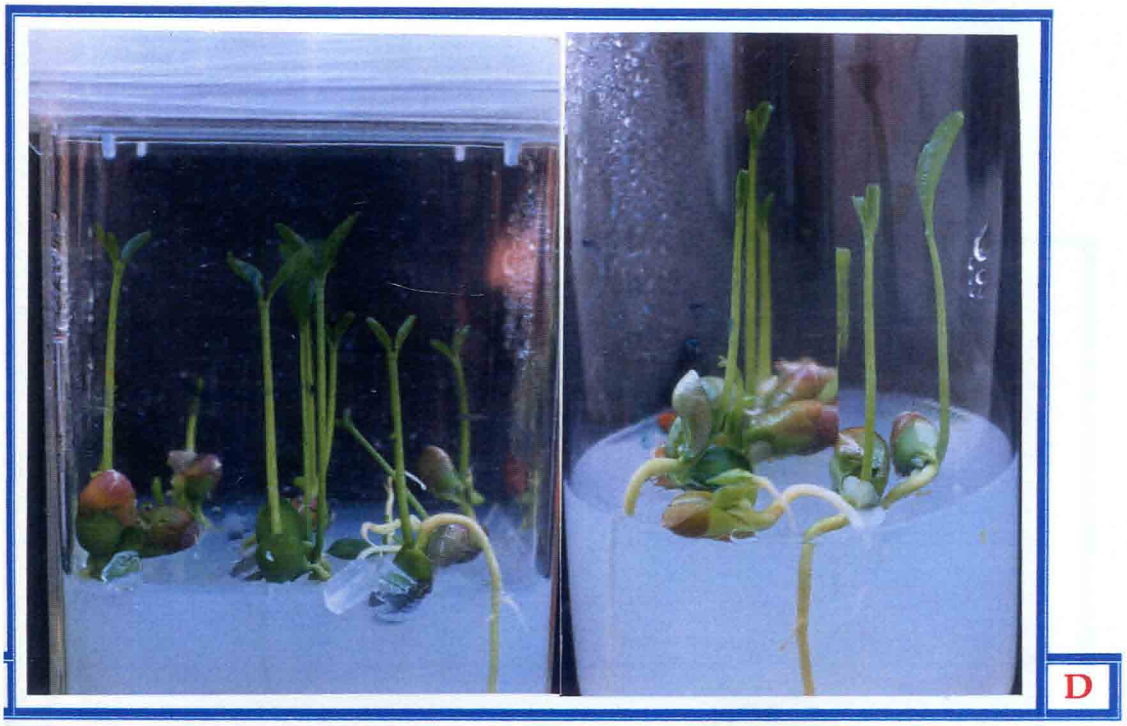


Fig. 29: Different rootstocks used for shoot tip grafting (Cont.)
(C): Malta sweet orange
(D): Mosambi sweet orange

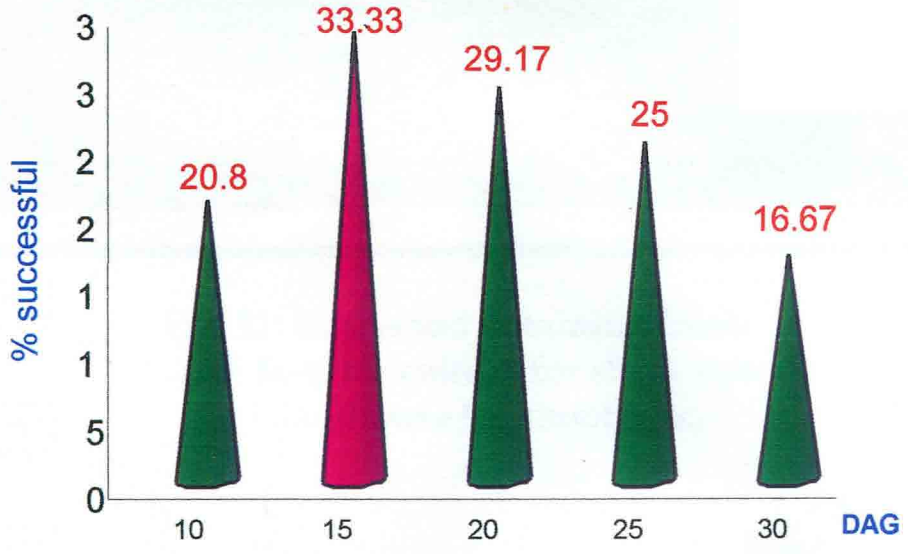


Fig 30: *Influence of age of Rough lemon seedlings on shoot-tip (Average of 24 grafts).*

Fig. 31: Bud wood obtaining from:
(A): *In-vitro* culture for shoot tips
(B): Glasshouse for shoot tips

from glasshouse raised plants followed by *in-vitro* culture were good source for STG. In a later experiment, it was found that pretreatment of the shoot tip with growth regulators and anti-oxidants (GR+AO) gave 55.56 and 64% successful grafts in *in-vitro* and glasshouse raised plants respectively.

Table 21: *Selection of source of shoot-tips for grafting (of 24 grafts)*

Source of shoot tips	% successful grafts	
	No treatment	Treatment With GR+AO
Field trees	18,18 (4/22)	-
Lateral buds cultured in vitro	29.16(7/24)	55.56(20/36)
Glasshouse grown plants	33.33 (8/24)	64.00(48/75)

The percentage of successful grafts depended on the size of the shoot tip (Table 22). Increase in the size of the shoot tip also increased percentage of successful grafts (41.67%).

Table 22: ***Effect of the size of excised shoot tip of Kinnow mandarin on successful grafts on Rough lemon rootstock.***

Shoot tip size	Size of tissue (mm)	No. of successful/ Attempted grafts	% successful grafts
Apical meristem alone	0.05 - 0.06	1/24	4.16
Apical meristem+ 2 primodia	0.10 - 0.15	5/24	20.83
Apical meristem+ 4 primodia	0.20 - 0.30	8/24	33.33
Apical meristem+ 6 primodia	0.40 - 0.70	10/24	41.67

The results of different methods of shoot tip insertion are given in Table 23 and Fig. 32. Triangular incision on rootstock gave best result with 55.48% of successful graft, followed by invert T incision (55.48%) and window cut method (40.00%).

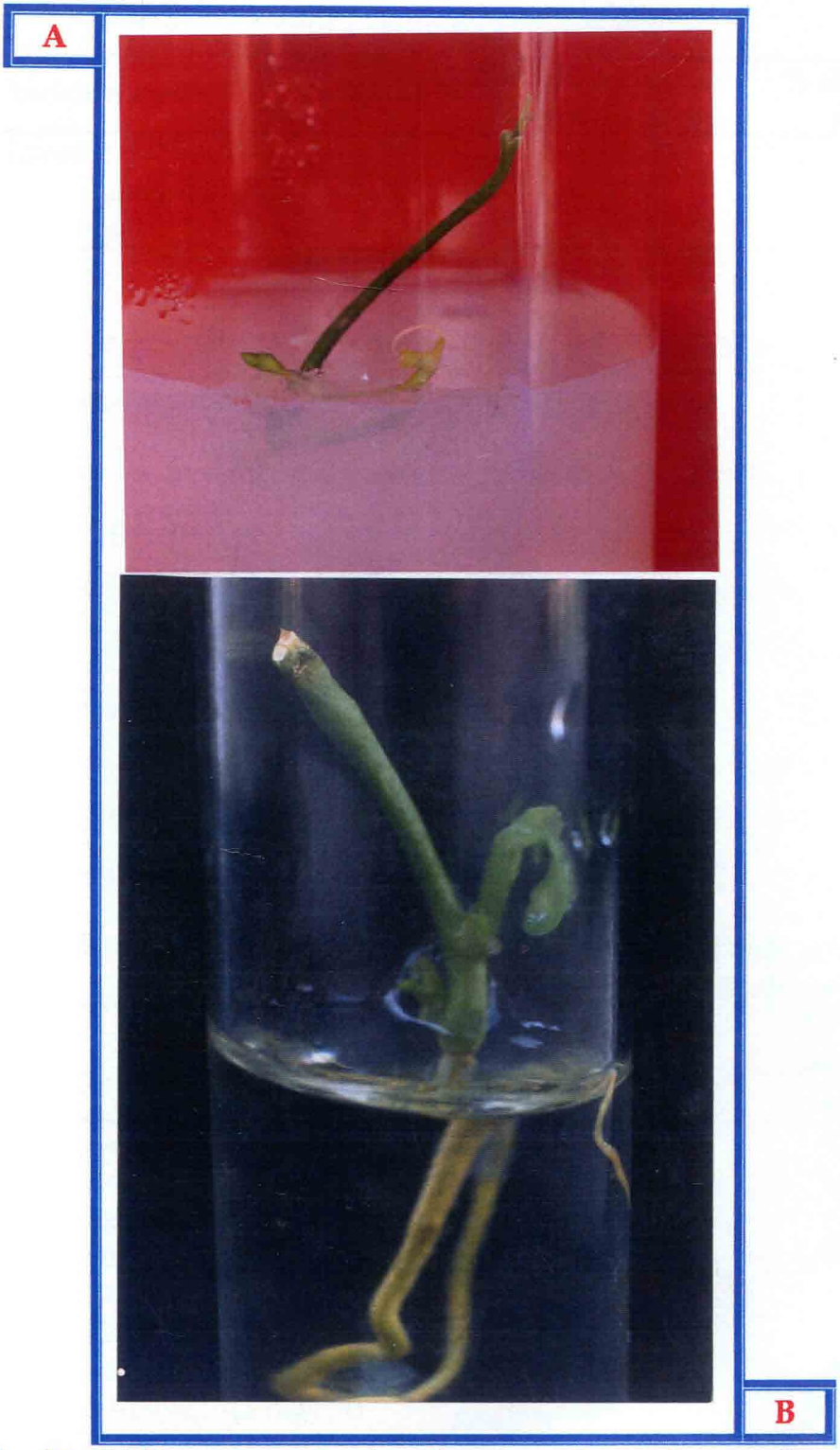


Fig. 32: Different methods of rootstock cutting and insertion of shoot tip
(A): Window cut; (B): Triangular cut

Table 23: *Comparative efficacy of method of placement of shoot-tips onto rootstock seedlings with different incisions.*

Incision method	No. of successful/ Attempted grafts	% successful grafts
Invert T	11/25	44.00
Triangular	86/155	55.48
Window cut	18/45	40.00

The grafted plants placed either in solid or liquid media did not show any significant difference in growth (Fig 33).

For increasing the percent successful graft, effect of different growth regulators and antioxidants for pretreatment of shoot tip explants was tested (Table 24). Among these, DIECA (100mg/lit) showed best result (63.64% successful), almost double to that of water control, followed by 2,4–D (10mg/lit) giving 60.11% and kinetin (1mg/lit) and 2,4-D (5mg/l) was also equally good, giving 58.33% successful graft. Increased dose of kinetin did not help to increase percentage of graft success, and BAP treatment was at par to water control.

Table 24: *Effect of growth regulators and anti-oxidants pre-treatment to shoot tips on successful graft percentage.*

Chemical used	No. of successful/ Attempted grafts	% successful grafts
➢ 2,4 - D (5 mg/lit)	14/24	58.33
➢ 2,4 - D (10 mg/lit)	11/18	60.11
➢ Zeatin (1 mg/lit)	13/24	54.16
➢ DIECA (100 mg/lit)	35/55	63.64
➢ Kinetin (1 mg/lit)	21/36	58.33
➢ Kinetin (5 mg/lit)	09/21	42.86
➢ Kinentin (10 mg/lit)	10/24	41.67
➢ BAP (1 mg/lit)	09/24	37.50
➢ BAP (5 mg/lit)	08/20	40.00
➢ Control (Water treatment)	09/24	37.50

33: Grafted plants grown in liquid (A) and solid (B) medium

Composition of the medium also plays an important role in successful STG as evident from the results of Table 25, where the MT medium gave more effective than that of MS medium (40 and 33.33% respectively), and when the MT medium supplemented with plant growth regulators showed higher percentage of successful grafts. MT medium supplemented with 1mg/lit of kinetin and 1mg/lit of IAA showed 61.17% successful grafts.

Table 25: ***Effect of growth regulators on percentage of successful grafts***

S. No	Type of media	No. of successful/ Attempted grafts	% successful grafts
1	Murashige & Skoog (MS)	4/12	33.33
2	Murashige & Tucker (MT)	6/15	40.00
3	MT+ 1mg Kinetin + 1mg IAA/lit	29/47	61.17
4	MT+ 1mg Kinetin + 1 mg NAA/lit	45/80	56.25
	MT+ 1mg Zeatin + 1 mg IAA/lit	24/42	57.14

The investigation to see differences between conventional hardening and double grafting technique showed that the percentage of success was higher in case of double grafting technique as compared with conventional hardening (Table 26), the double grafted plants grew faster than plants of conventional hardening (Fig. 34).

Indexing of STG plants against viruses: The selected plants after hardening and double grafting were tested by DAC - ELISA with antisera of ICRSV, citrus vein clearing, citrus tristeza virus, citrus yellow mosaic virus. The STG plants tested were raised with a shoot tip of 6 leaf primordial as there was a chance that virus may move with these shoot tip. The result showed that, all the plants tested were free from these viruses (Table 27). The following dilutions of the reagents were used in DAC-ELISA: Antigen dilution: 1:10; Antibody dilution: ICRSV, CTV and

Fig. 34: Hardening of STG plants

A: Direct hardening B: Double grafting
Comparison between conventional hardening (C) and Double grafting technique (D).

CVV = 1:500; and CYMV=1:250; Conjuagate dilution = 1:10,000; Substrate dilution: 0.6mg/ml.

Table 26: *Comparative efficacy of plants with conventional hardening and double grafted plants of Kinnow mandarin and Mosambi sweet orange.*

Scion variety	Months after grafting	Length of grafted plants in cm	
		Hardening	Double grafting
Kinnow	1	8.3	12.5
	2	15.9	18.2
	3	18.6	25.8
	4	32.5	40.5
	% of survival	68.5	80.7
Mosambi	1	09.1	13.6
	2	17.3	21.4
	3	25.7	32.5
	4	35.4	48.1
	% of survival	65.2	83.1

Table 27: *Detection of viruses in STG plants by DAC - ELISA*

Treatments (plant)	Absorbance at 405			
	ICRSV	CVV	CTV	CYMV
Kinnow STG - 1	0.061 (-)	0.105 (-)	0.143 (-)	0.148 (-)
- 2	0.076 (-)	0.207 (-)	0.072 (-)	0.118 (-)
- 3	0.041 (-)	0.218 (-)	0.150 (-)	0.149 (-)
- 4	0.108 (-)	0.085 (-)	0.169 (-)	0.042 (-)
- 5	0.106 (-)	0.147 (-)	0.157 (-)	0.083 (-)
- 6	0.072 (-)	0.150 (-)	0.087 (-)	0.115 (-)
- 7	0.070 (-)	0.077 (-)	0.123 (-)	0.114 (-)
- 8	0.090 (-)	0.159 (-)	0.170 (-)	0.109 (-)
- 9	0.103 (-)	0.087 (-)	0.164 (-)	0.111 (-)
- 10	0.038 (-)	0.169 (-)	0.153 (-)	0.096 (-)
Positive control	0.423 (+)	1.003 (+)	0.382 (+)	0.339 (+)
Healthy control	0.039	0.095	0.083	0.094
Mosambi STG - 1	0.121 (-)	0.120 (-)	Nt	0.102 (-)
- 2	0.098 (-)	0.095 (-)	Nt	0.158 (-)
- 3	0.067 (-)	0.146 (-)	Nt	0.135 (-)
- 4	0.095 (-)	0.123 (-)	Nt	0.149 (-)
- 5	0.188 (-)	0.164 (-)	Nt	0.104 (-)
Positive control	0.408 (+)	0.391 (+)	-	0.364 (+)
Healthy control	0.097	0.087	-	0.094

Nt: Not tested

Since the incidence of ICRSV was very high in Kinnow (up to 100% in some orchards). STG plants raised from known ICRSV infected source were also tested by dot blot hybridization and were found free (Fig. 35).

Multiplication of virus-free materials: The shoot tip grafted plants of Kinnow mandarin and Mosambi sweet orange after testing for viruses were further multiplied on Rough lemon rootstock in glasshouse (Fig. 36) and will be evaluated in the field in due course of time.

4.4.2. OVULE CULTURE

Ovules obtained from Kinnow mandarin and Mosambi sweet orange at different stages of flower growth as well as fertilized ovules from young fruits were used for ovule culture studies (Fig. 37).

4.4.2.1. Ovule culture from flowers of Kinnow mandarin and Mosambi sweet orange.

Ovules obtained from Kinnow mandarin and Mosambi sweet orange at different flowering stage, which were cultivated on MT medium (Fig. 38).

Table 28: *Percentage of ovule survived after one month of culture*

	Day after culture	No. of cultured	No. of survival (%)
Kinnow	26	126	101 (80.16)
Mosambi	30	107	96 (89.72)

Out of 126 ovules obtained from flowers of Kinnow, 101 survived which were transferred to MT medium plus BA and kinetin for callus

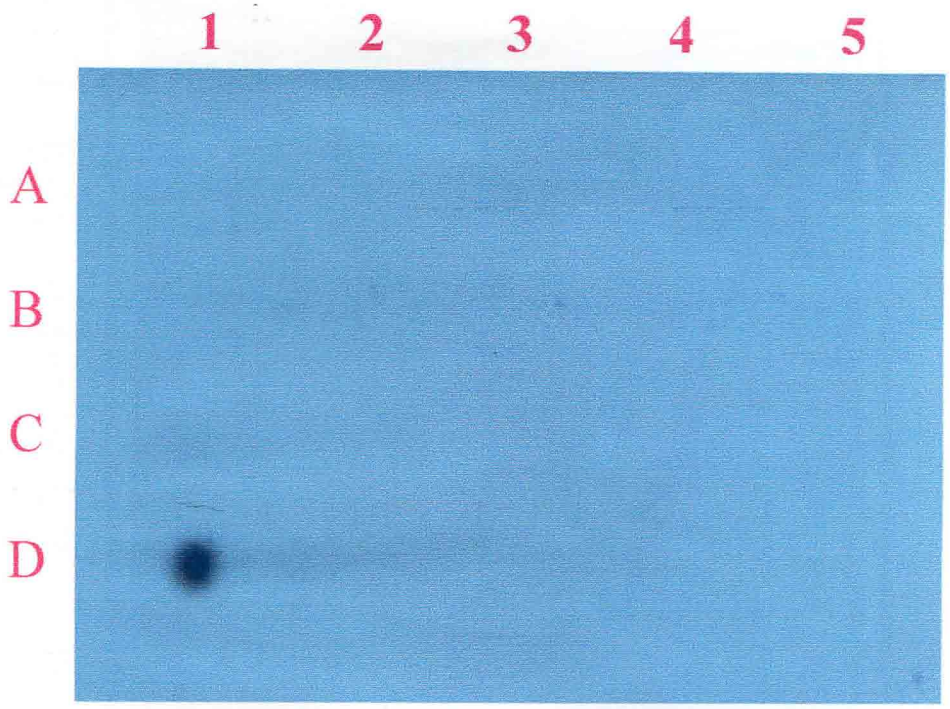


Fig. 35: Indexing of STG plants by Nucleic Acid Spot Hybridization using probe made from 350bp amplicon of ICRSV - Ab

D, 1: Citrus positive control
D, 2: Citrus negative control
A,1-5 and B, 1-5: Kinnow STG samples
C, 1-5: Mosambi STG samples
D,3-5: Replication of Kinnow STG samples

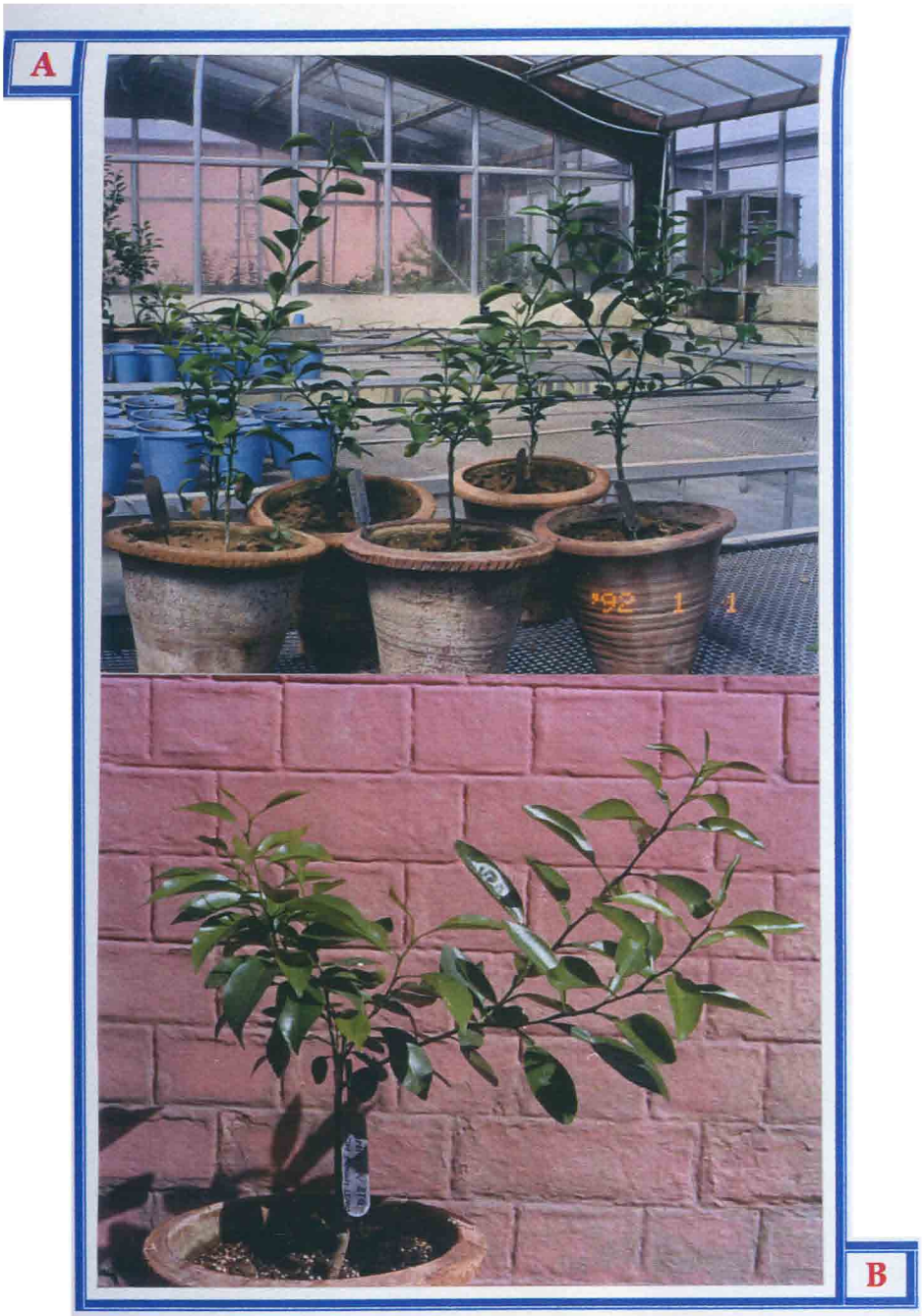
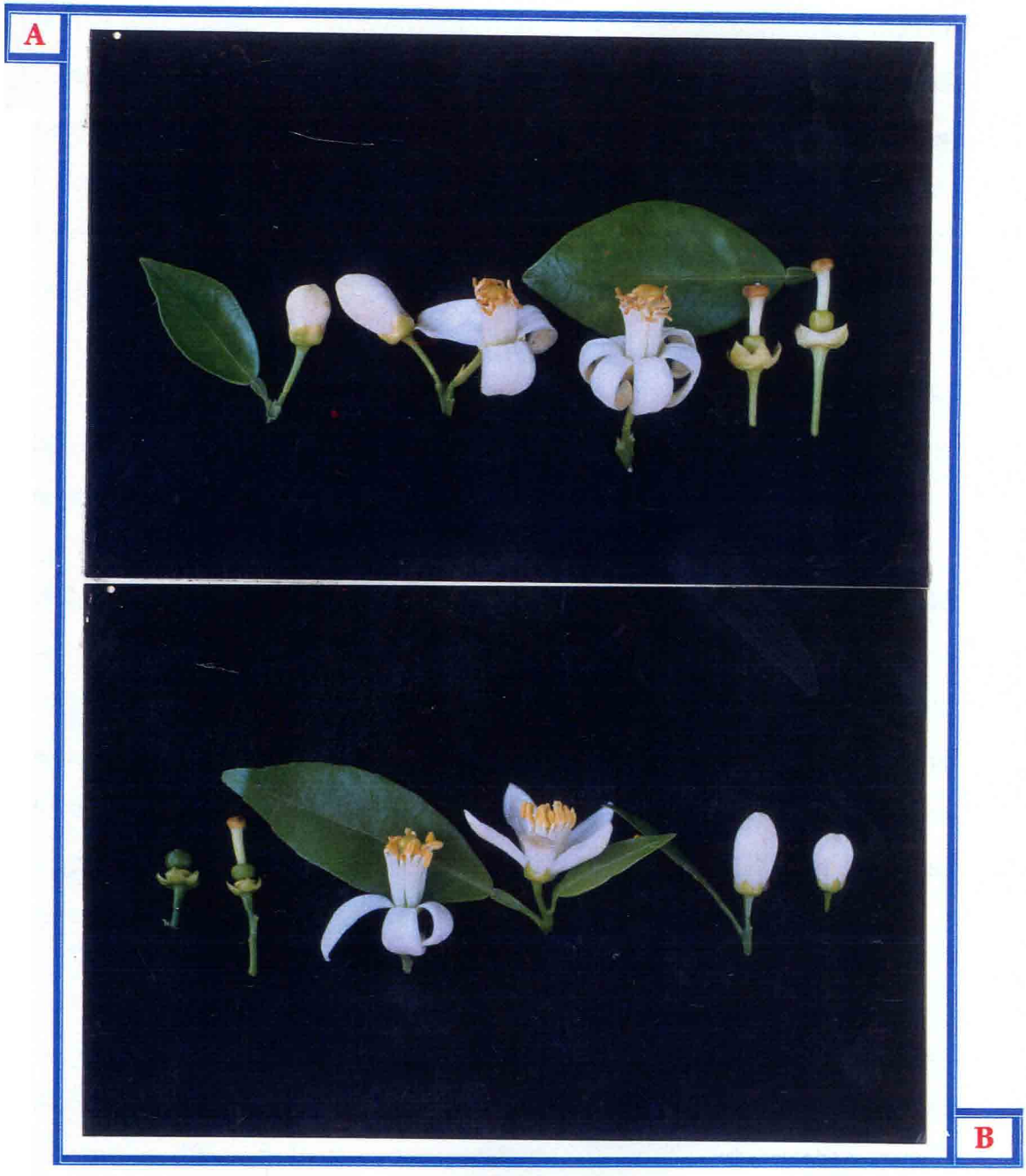


Fig. 36: Multiplication of virus-free STG plants
(A): Mass multiplication
(B): Stock plant



37: Different stages of flower of Mosambi sweet orange (A) and Kinnow mandarin (B) for obtaining ovule for culture

formation (Table 28). After three weeks only 5 ovules developed to green callus with different sizes and developed leaves of pale yellow colour which later changed to white colour, but no shoot emerged. When this plantlet was transferred to medium containing 1mg/lit NAA and 1 mg/lit kinetin, shoot starting to grow and gave true leaves with some yellow and green patches within two weeks time, and the root was also formed after 20 days when put on medium with 2mg/lit NAA and 2mg/lit Kinetin. However, the plant showing yellow and green patches on leaves (Fig. 39 and 40), subsequently died.

In case of Mosambi, percentage of ovule survival was higher (89%) than that of Kinnow (80%), but there was no callus formation after three months on MT medium.

4.4.2.2. Ovule culture of Kinnow and Mosambi from one month old fruits

The cultures of ovule of Kinnow and Mosambi from one month old fruits remained green up to one month without any sign of callus development (Fig. 41). They were transferred to MT medium supplemented with different concentration of 6-benzyladenine (BA) and Kinetin for callus induction.

Results (Table 29) showed that in the media containing different concentrations of growth regulators, callus formation took place, but percentage differed. In Kinnow mandarin, the highest percentage of calli formation was in MT medium supplemented with 5mg/l of BA and kinetin (36.67%), followed by BA (5mg/l) without kinetin (24.24%) and 1mg/l of BA and kinetin (23.33%). In Mosambi (Fig. 42), the result once again confirmed the effects of BA and kinetin at 5mg/l as well as in other

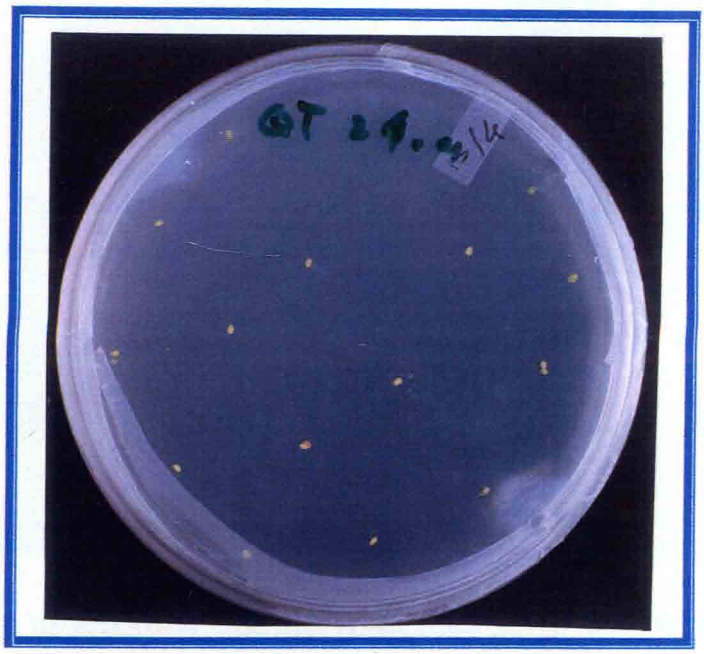


Fig. 38: Ovule culture on MT medium

39

Fig. 39: Embryo developed from ovule of Kinnow mandarin flower

40

Fig. 40: Two month old Kinnow plant obtained from flower stage showing yellow patches on the leaves.

Fig. 41: Culturing of Kinnow mandarin ovules from one month old fruits

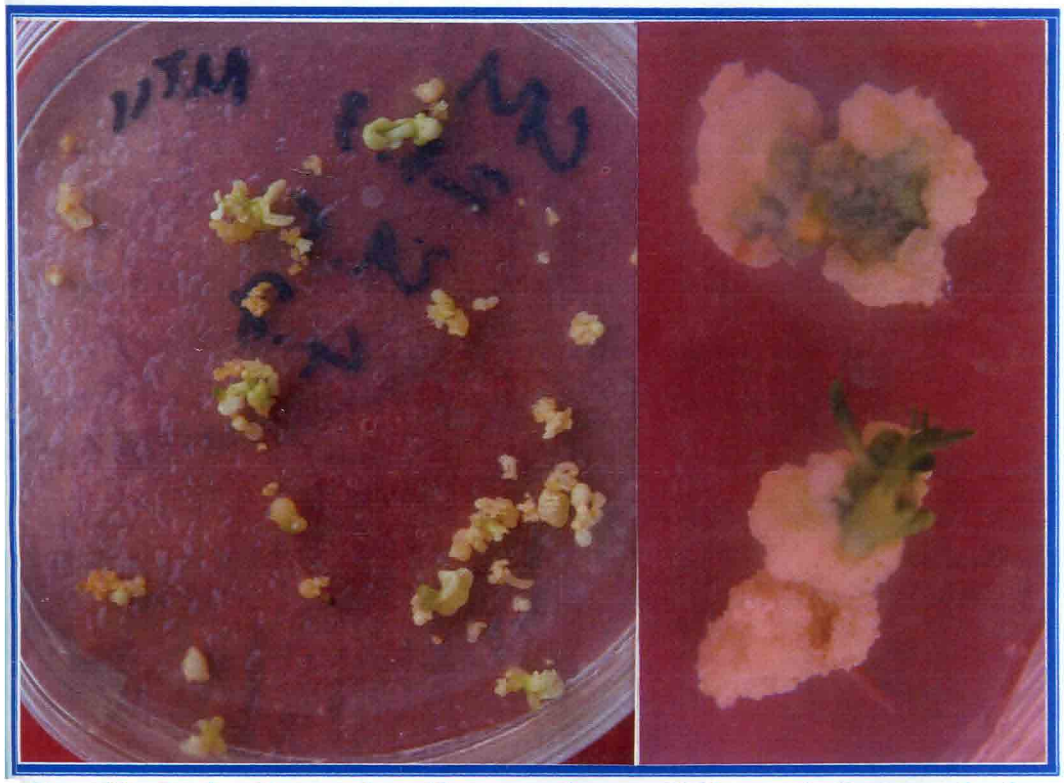


Fig. 42: Induction of callus, embryo differentiation and shoot proliferation in ovule culture of Mosambi sweet orange.

concentrations. The percentage of ovules showing callus formation was higher than that of Kinnow mandadrin (46.15, 34.78 and 42.14 % respectively)(Fig. 43 and 44).

Table 29: ***Effect of growth regulators on callus formation in ovule culture***

MT media Addenda (mg/lit)		Kinnow mandarin		Mosambi sweet orange	
BA	Kinetin	Ovule cultured	Responsive (%)	Ovule cultured	Responsive (%)
-	-	22	3 (13.36)	20	4 (20.00)
1	1	30	7 (23.33)	28	9 (32.14)
5	0	33	8 (24.24)	23	8 (34.78)
0	5	36	5 (13.89)	26	6 (23.08)
5	5	30	11 (36.67)	26	12 (46.15)

In both the cases, white to pale brown and green calli were formed and embryo differentiation occurred. All the calli looked normal.

For shoot and root formation, another experiment was carried out with different growth regulators and at different concentrations. The results are shown in Table 30 and Fig. 45.

Table 30: ***Effect of growth regulators on differentiation in calli from ovule culture***

MT media Addenda (mg/l)		Kinnow mandarin			Mosambi sweet orange		
		Callus cultured	Responsive (%)		Callus cultured	Responsive (%)	
			Roots	Shoots		Roots	Shoots
-	-	7	1 (14.29)	2 (28.71)	8	1 (12.50)	3(37.50)
NAA (1)	-	8	2 (25.00)	2 (25.00)	6	2 (33.33)	2(33.33)
NAA (2)	KT (2)	10	2 (20.00)	3 (30.00)	12	3 (25.00)	5(41.67)
IAA (1)	KT (2)	12	1 (08.33)	4 (33.33)	14	3 (21.43)	6(42.85)
BA (1)	GA (2)	16	5 (31.25)	4 (25.00)	11	3 (27.27)	4(36.63)
BA (2)	GA(2)	14	3 (21.43)	4 (28.57)	15	3 (20.00)	5(33.33)

When the calli were transferred to MT medium, the embryo differentiation and plantlets formation occurred in 15-20 days after inoculation as 28.71 and 37.5% in Kinnow and Mosambi respectively

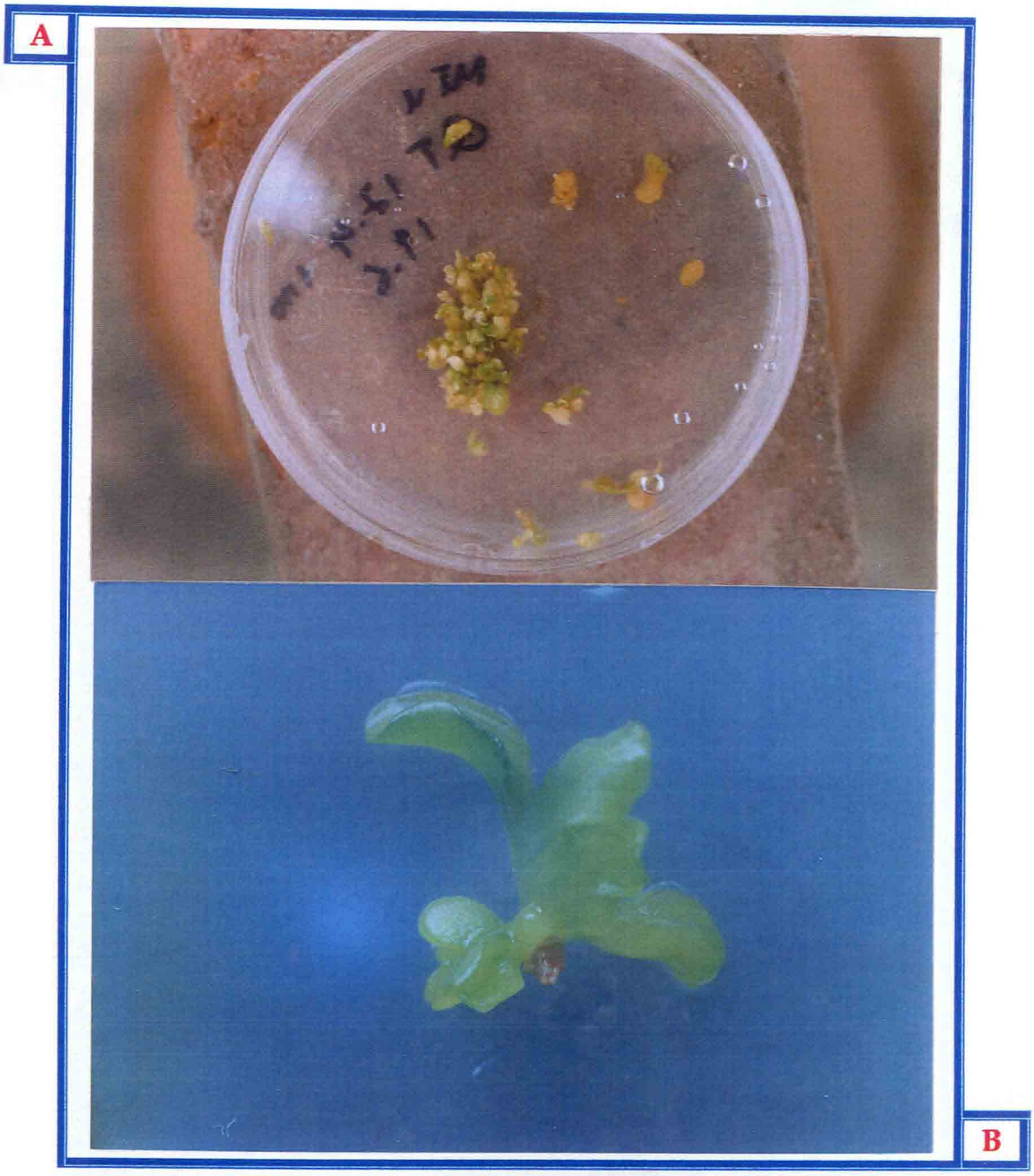


Fig. 43: Callus formation and embryo development (A) and plantlet growing (B) of Kinnow mandarin from ovule culture

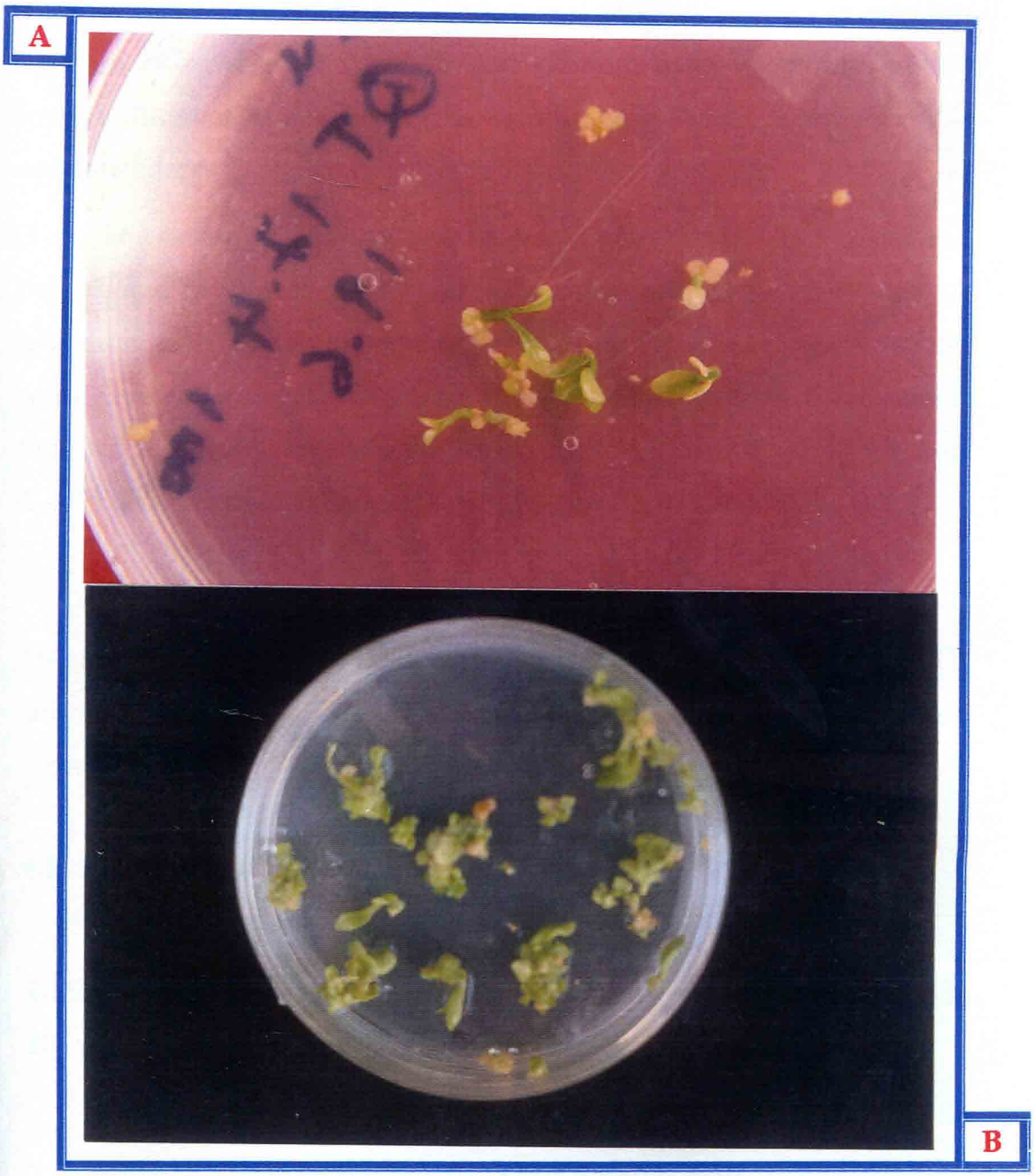


Fig. 44: Development of shoots and formation of plantlets in Kinnow mandarin (A) in comparison with Mosambi sweet orange (B)

which was higher than that of MT supplemented with NAA (1mg/l) and MT with BA (1mg/l) plus GA (2mg/l). However, the most effective medium was MT supplemented with 1mg/l IAA plus 2mg/l kinetin. In comparison between the two cultivars, Mosambi explants showed better embryo differentiation as it developed through globular, heart, torpedo, and cotyledonary stages, and produced secondary embryos.

The rate of root formation was low in both Kinnow and Mosambi, (14.29% and 12.5% respectively) on MT medium without any hormone. It took more time for development of root (20 - 30 days). The best responses were obtained when the calli were transferred MT medium supplement with NAA (1mg/l) for Mosambi (33.33%) and BA (1mg/l) plus 2mg/l of GA (31.25%) in case of Kinnow mandarin.

The plantlets wedge grafted on Mosambi seedlings developed faster and subsequently developed true leaves (Fig.46). Of the 14 grafts, 11 survived. The plants put to hardening directly took longer time for recovering (Fig. 47), and less number survived (3 out of 10 plants).

4.4.2.3. Indexing of regenerated plants for ICRSV

The plantlets derived from ovule through callus were tested by ELISA and nucleic acid hybridization for the presence of ICRSV (Fig. 48). The result showed that all the tested plants were free from ICRSV.

Fig. 45: Rooting of Kinnow mandarin embryo

46: Direct hardening (A) and embryo grafting on Kinnow mandarin (B)

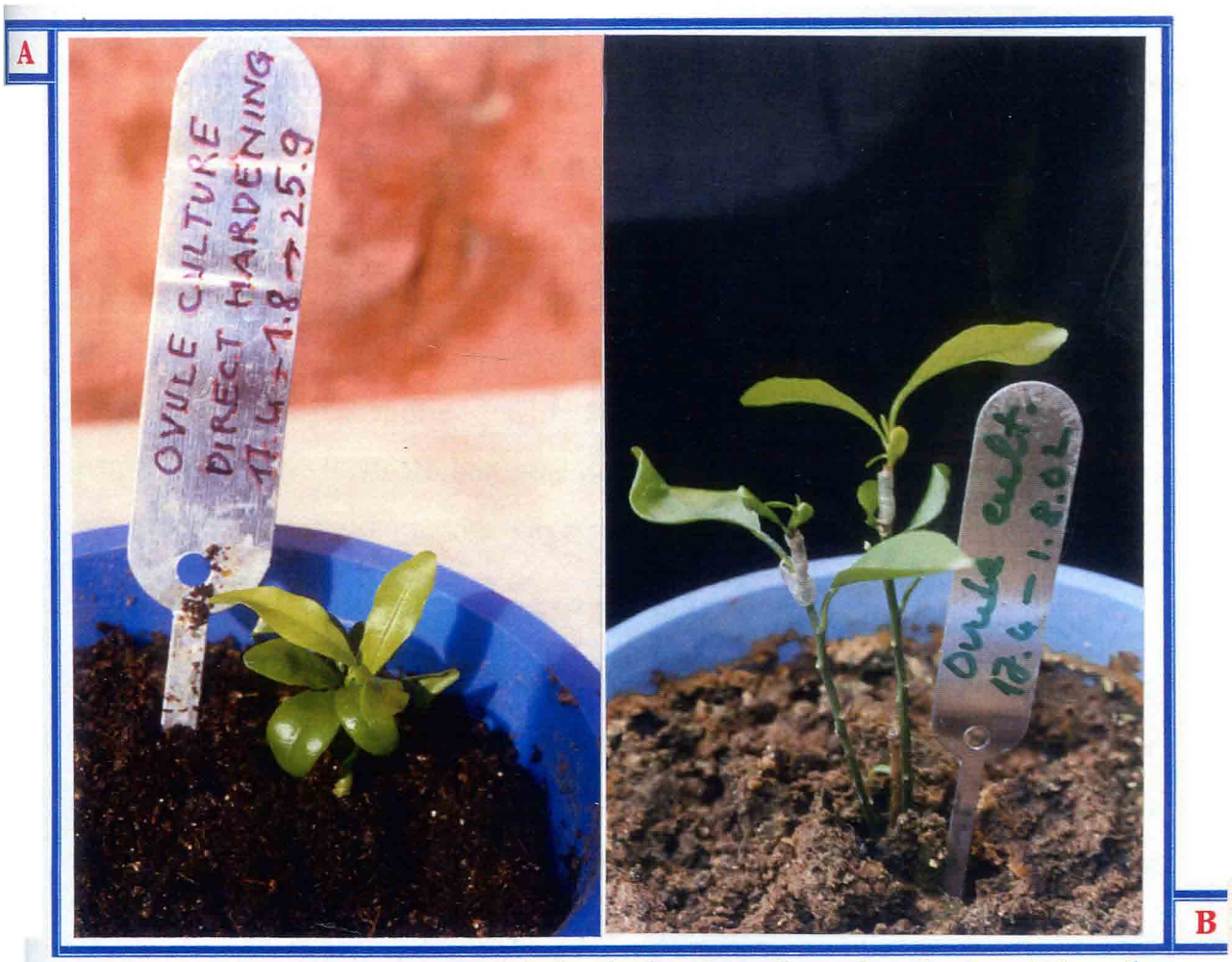


Fig. 47: Comparison between growth of planting direct hardening (A) and embryo grafted plants (B)

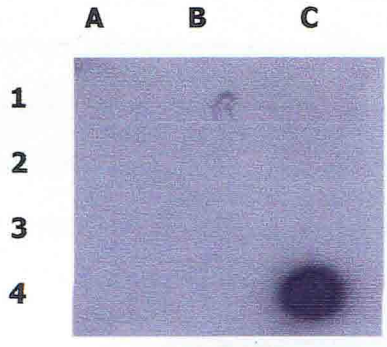


Fig. 48: Indexing of ovule cultured plants by Nucleic Acid Spot Hybridization using probe made from 350bp amplicon of ICRSV - Ab
+ 1-A,B,C; 2-A,B,C and 3-A,B, C: 11 tested samples
+ 4A+ 4B: Negative control and 4 C: Positive control

4.5. TRANSFORMATION OF TRUNCATED COAT PROTEIN GENE OF ICRSV-Ab ISOLATE INTO FRENCH BEAN AND CITRUS

4.5.1. Plasmid construction containing coat protein gene.

In the present investigation, coat protein gene from ICRSV-Ab isolate was used as a transgene. The fragment of CP gene (978bp) containing the starting codon ATG was amplified from the citrus plant using forward and reverse primers incorporated with *BamH* I and *EcoR* I restriction enzymes respectively. The PCR product was purified from gel and cloned in pGEM-T easy vector, and the sequences was obtained (Fig. 49).

4.5.2. Sense directional cloning in pBI121

The CP gene from pGEM-T easy vector was released at 5′ and 3′ ends by *Bam*H I and *Sac* I (from vector) restriction enzyme, respectively. The vector pBI121 was linearsed using *Bam*H I and *Sac* I (this removes β-glucoronidase gene (gus)) and sense directional cloning of CP gene was achieved by ligating *Bam*H I - *Sac* I digested CP gene insert with *Bam*H I - *Sac* I restricted pBI 121. Transformation of the resultant plasmid in *E. coli* strain DH 5 α yielded recombinant colonies. Among several colonies screened by colony PCR and restriction with the same enzymes, there was however no PCR product when amplified with the same set of primers (Fig. 50), while plasmids were isolated and restricted with *BamH* I and *Sac* I, a thick band on the gel, which was recorded at 900bp position (Fig. 51) and further confirmed by southern hybridization (Fig. 52) using probe made from PCR product amplified from ICRSV-Ab isolate with ICRSV-518/-951 primers.

Subsequently, sequence analysis revealed that there is another site of *Sac* I present at 900 bp position, and when another reverse primer (ICRSV-951) nearby this site was used, a PCR product of 814 bp was achieved (Fig. 53), and then a single colony was selected. The selected clone having CP gene in sense

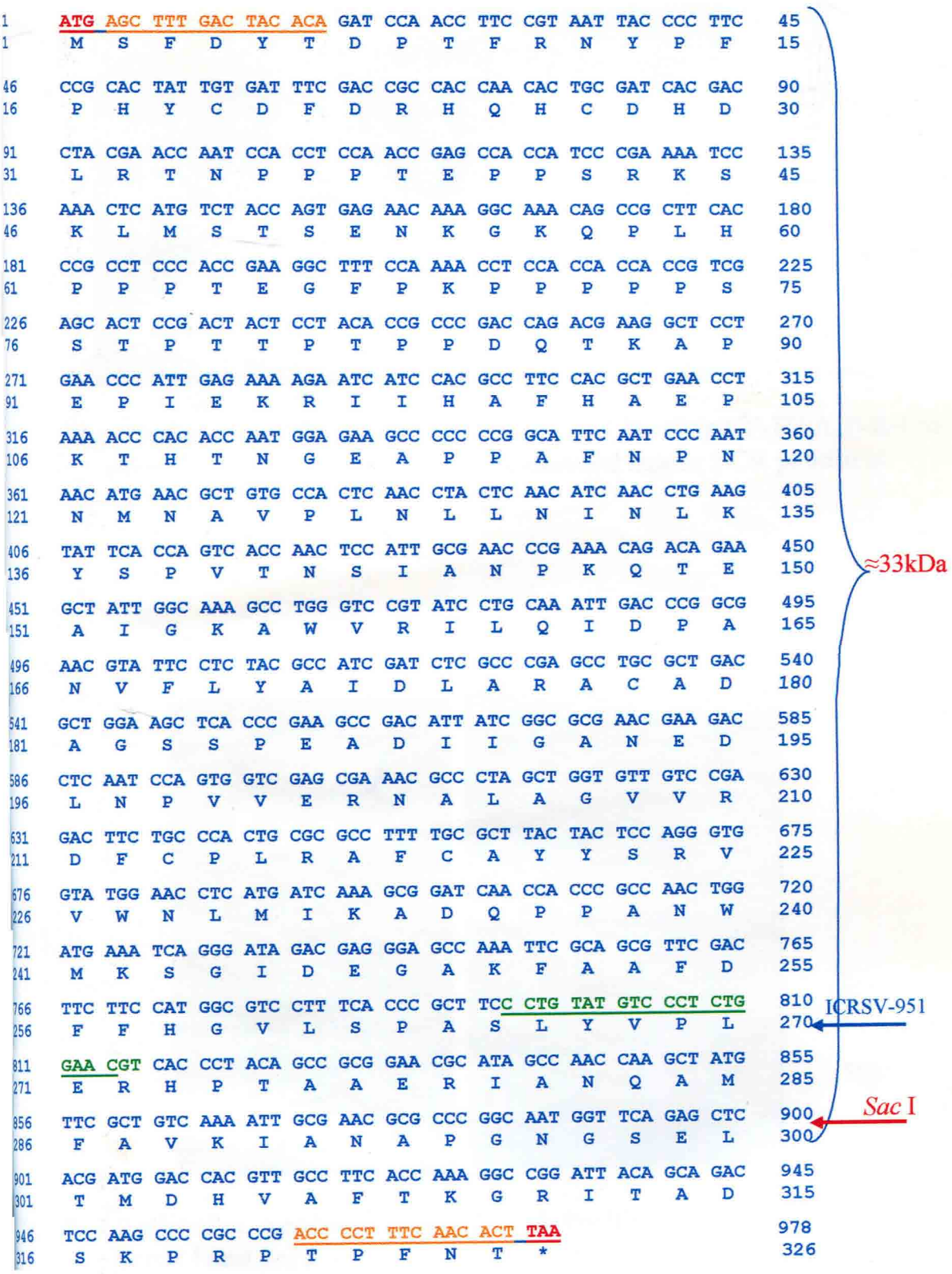


Fig. 49: Nucleotide (show as DNA) and deduced amino acid sequenes of CP gene of ICRSV – Ab isolate for construction.

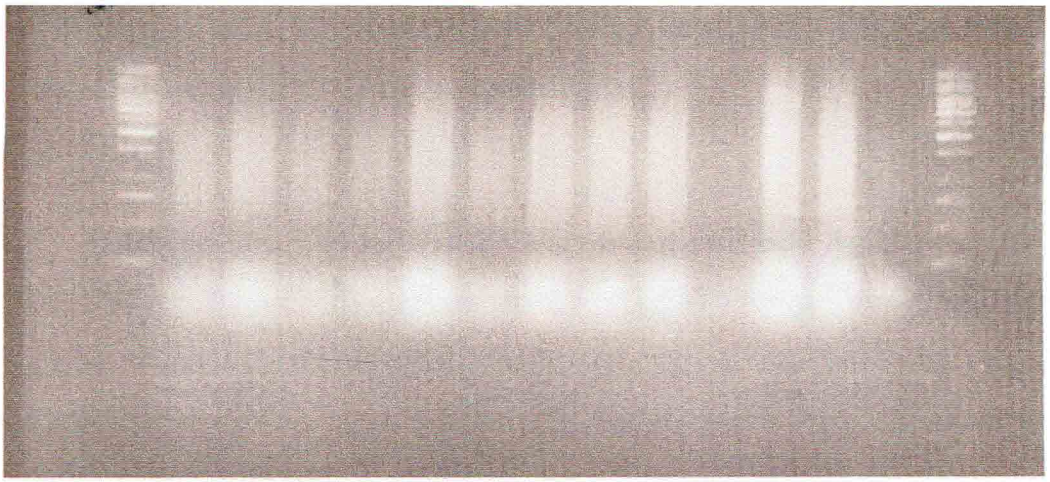
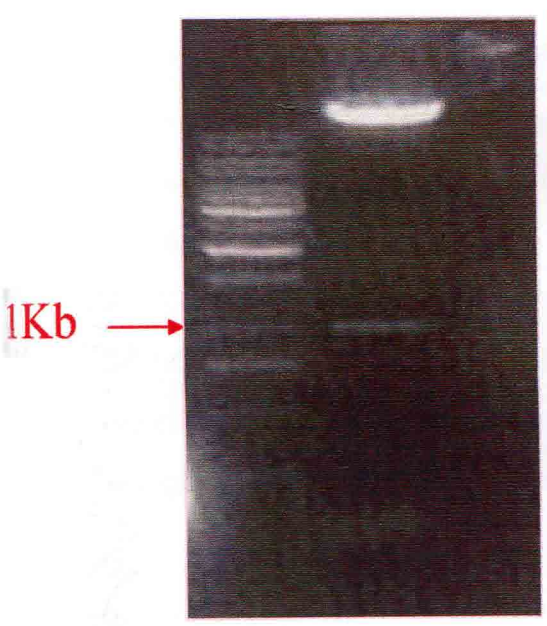


Fig. 50: Colony PCR from clones with pBI 121using CP-FW/CP-R-Eco primers, all most of the colonies showed smear PCR products



51: Restriction product using *BamH* I and *Sac* I

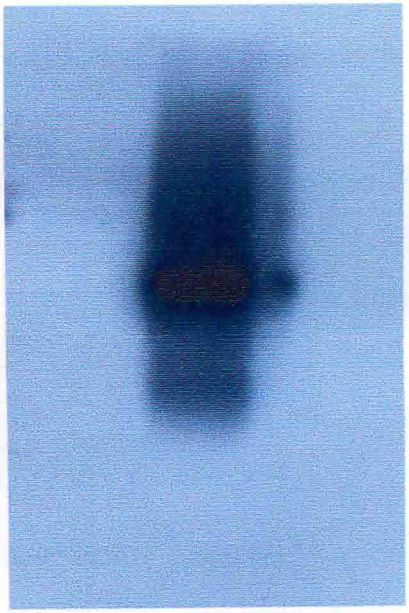


Fig. 52: Positive reaction of restriction product with probe made from ICRSV - Ab

orientation in pBI 121 was hitherto called as CpSTR (Coat protein Sense truncated) and used for plant transformation.

4.5.3. Transformation of mediated CP gene transfer into *Agrobacterium*

Truncated CP gene construct (CpSTR) was mobilised into *Agrobacterium tumefaciens* strain LBA4404 from *E. coli* with the help of pRK2013 plasmid in *E. coli.* The PCR amplification from *Agrobacterium* colony was confirmed. A single selected LBA4404 transconjugant colony revealed the successful mobilisation of CP gene construct to *A. tumefaciens.* (Fig. 54).

4.5.4. Transformation of French bean with coat protein gene

Internode explants of French bean (*Phaseolus vulgaris* var. *saxa*) were used for transfromation. French bean seeds sown in half strength MS medium with 0.8% agar, well-grown plants after 7-10 days of sowing were utilised for co-cultivation. Co-cultivation of explants were done by growing them in co-cultivation medium (CM) (Fig. 55). Selections of co-cultivated explants were done by growing them in selection medium (SM). Non-cocultivated explants grown in cocultivation medium (CM) and selection medium (SM) and explants cocultivated with LBA 4404 without any construct grown in SM severed as a control.

High transformation rate was observed in non-transformed explants growing in non-selection CM medium when compared to transformed explants on selection medium (SM). No transformants were observed in non-cocultivated plants growing on selection medium (SM) (Fig. 56), indicating the true transformant seem to be due to the CP gene construct along with the neomycin phosphotransferase II (NPT II) gene

Fig. 53: Colony PCR using CP-FW/ICRSV-951 primers

Lane 1: PCR product from pGEM-T vector cloned in *E.coli* DH 5α.

Lane 2: PCR product from pBI 121 vector cloned in *E.coli* DH5α.

Lane 3: 1 kb DNA marker

Fig. 54 : Colony PCR from *Agrobacterium* using CP-FW/ICRSV-951 Primers

Lane 1: 1 kb DNA marker

Lane 2: PCR product from transformed *Agrobacterium* (814bp)

conferring Kanamycin resistance in plant selection. The explants infected with LBA4404 without CP gene constructs grown in the SM selection medium produced shoot buds indicating that kanmycin did interfere with the regeneration of the explants (Fig.57).

The response of the co-cultivated plants in the selection medium was as follows: Initially after a week of transfer of the co-cultivated explants to the selection medium, enlargement of the explants was noticed. After 15 days, from the cut ends small callus and from them shooting was started (Fig. 58 A). A large number of small shoots buds were seen after 3-4 weeks followed by dark screening of explants (Fig. 58B). When the plantlets from the shoot buds attained 1-2 cm height with 2-3 tiny leaves after 5-6 weeks of cocultivation, they were transferred to the jam box. After 15 days interval, the plants were transferred to rooting medium (RM) to induce roots. Profused rooting was observed after 2-3 weeks of transfer (Fig. 59).

When the plants have 3-4 triple - leaves (one month after co-cultivation), 100 mg leaves of each individual plant were picked and the total DNA was extracted by using DNA plant mini kit (appendix II), and 10 µl was used for PCR. The result (Fig. 60A) showed that the PCR product had been amplified with specific primers CP-FW/ICRSV-951. This was further proved by southern hybridization (Fig. 60B). It was therefore confirmed that truncated protein gene of Coat protein of ICRSV-Ab isolate was integrated into French bean genome.

To further confirm that the truncated CP gene of ICRSV-Ab isolate has been incorporated in to the bean genome, NASH test was conducted from the same plants and results are given in Fig. 61.

Fig. 55: French bean segments developed roots and callus on co-cultivation medium

Fig. 56: Non co-cultivated bean segment died on selection medium (SM)

Fig.57: Poor development of segment when co-cultivated with *Agrobacterium* without transformed CP gene on SM

Fig. 58. Callus with shoot emerging (A) and
Plantlet on selection medium (B)

Fig. 59: Growing of bean explants (transformed) on SM
(A): First plant

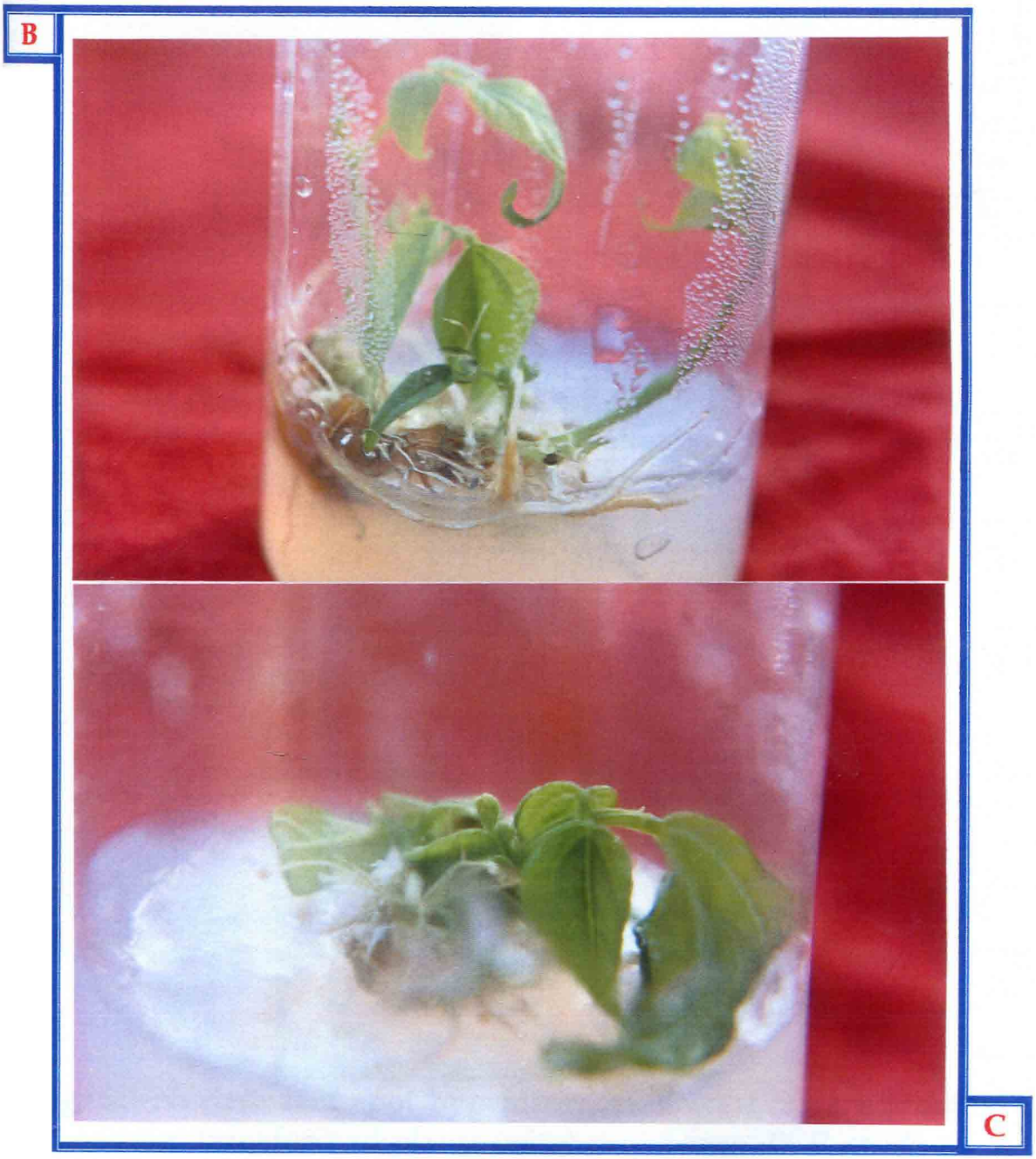


Fig. 59 (Cont.): Growing of bean explants (transformed) on SM
(B): Second plant
(C): Third plant

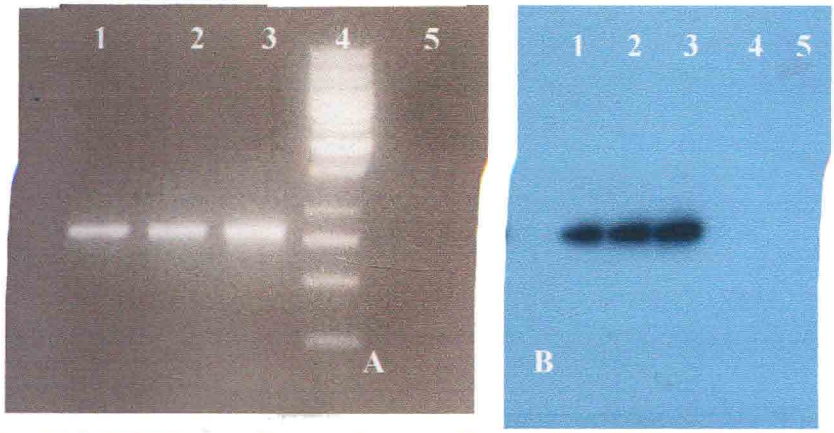


Fig. 60 : (A) PCR confirmation using CP-FW/ICRSV-951 primers (B): Southern hybridization using probe made from PCR product of ICRSV - Ab isolate

Lane 1: First bean explant; Lane 2: Second bean explant
Lane 3: Third bean explant; Lane 4: 1 Kb DNA ladder
Lane 5: Healthy bean

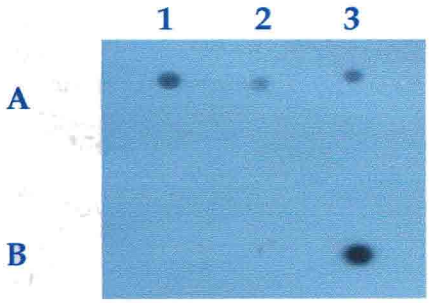


Fig. 61: Nucleic acid spot hybridization of total DNA extract from transformed plants using probe from PCR product of ICRSV - Ab isolate.

A1: First bean, A2: Second bean, A3: Third bean plant
B1, 2: Healthy bean control, B3: Infected bean plants

The expression of the coat protein gene in bean system was tested by DIBA using homonlogous antiserum. The results are shown in Fig. 62.

The result showed that all the bean plants tested expressed the presence of protein, which reacted with the homologous antiserum of ICRSV suggesting that the transformed CP gene has been integrated and acted in the bean genome.

4.5.5. Transformation of Kinnow mandarin with coat protein gene

During the time doing transformation on French bean, the hypocotyl segments of Kinnow mandarin were used for transformation and co-cultivation of explants were done by growing them in co-cultivation medium (CM)(Appendix III) for citrus, Selections of co-cultivated explants were done by growing them in selection medium (SM-for citrus) supplemented with hormone for callus formation (Appendix III). No transformant was observed in non-cocultivated plants growing on selection medium (SM), the segments died due to high concentration of kanamycin (200mg/lit), indicating the true transformant seem to be due to the CP gene construct along with the neomycin phosphotransferase II (NPT II) gene conferring Kanamycin resistance in plant selection. The explants infected with LBA4404 without CP gene constructs grown in the SM did not grow and subsequently died (Fig. 63). The response of the co-cultivated plants in the selection medium was as follows: The segments developed callus at the cut end after 2-3 weeks (Fig. 64). Later from the cut end the shoot emerged and developed into plantlet within one and haft month after co-cultivation (Fig. 65) and when they were transferred to tissue culture tube in SM of 200mg/lit kanamycin and 300mg/lit of Cefotaxime, the plants developed leaves (Fig. 66 & 67). However, at the moment the plants are not suitable for detection of CP gene transformation and it will be done within few weeks.

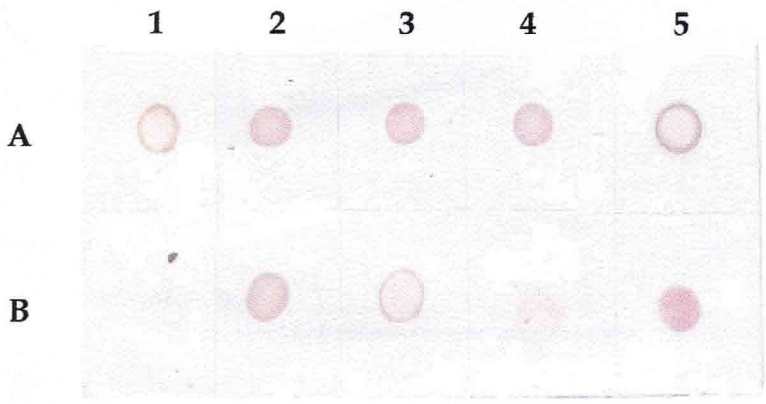


Fig.62: Expression of truncated coat protein of ICRSV-Ab in transformed *Phaseolus vulgaris* var. *saxa.* using DIBA with homologus antiserum

A, 1: Citrus positive control
B, 1: Buffer control
B, 5: Bean positive control
B, 4: Bean negative control
A, 2 - 5; B, 2,3: Transformed *P. v.* var *saxa* samples

Fig. 63: Kinnow segments without CP gene transformed died in the selection medium

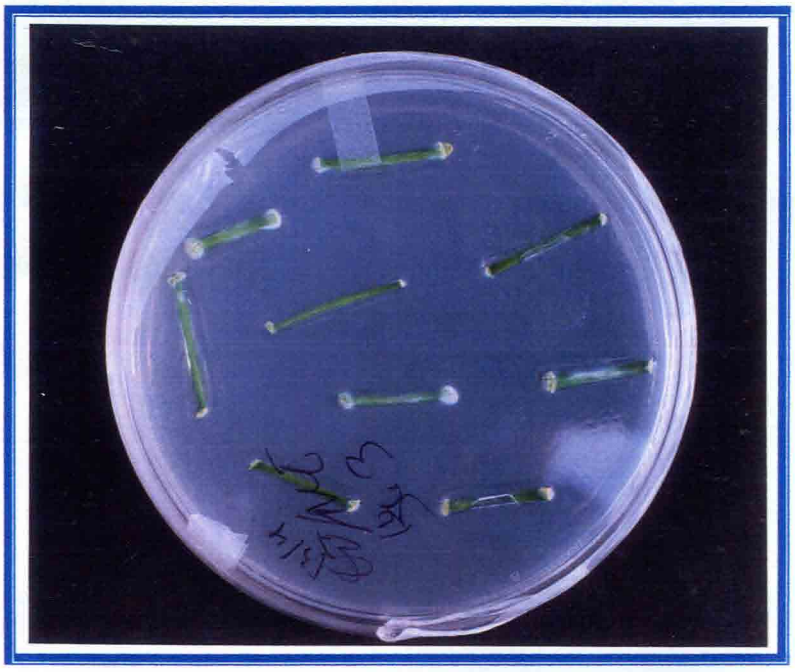


Fig. 64: Callus induction of transformed Kinnow mandarin segments on selection medium

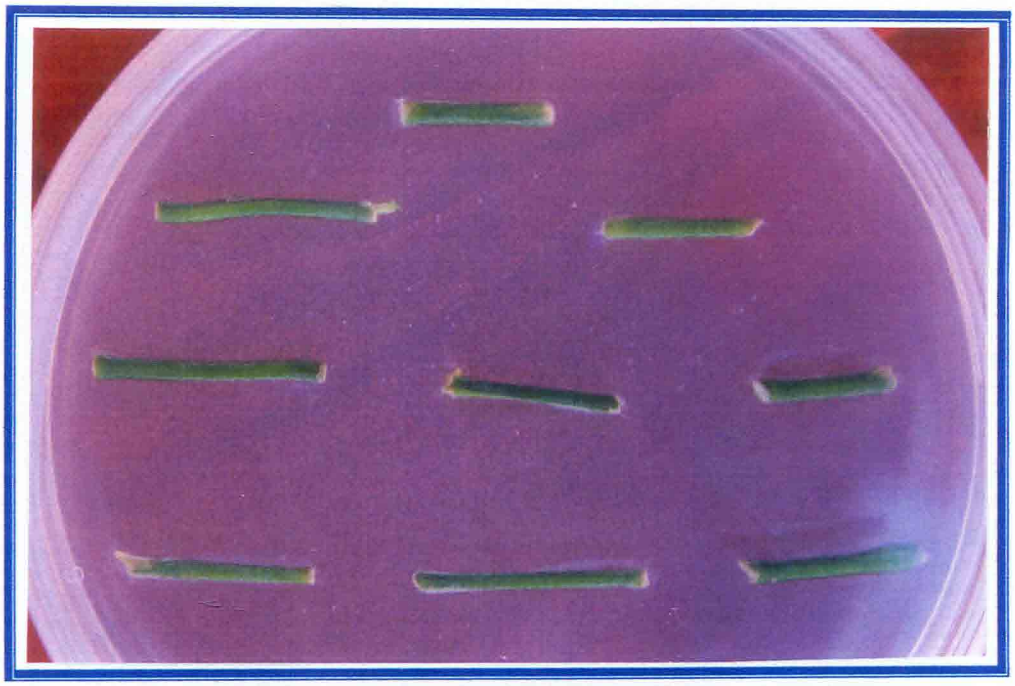


Fig. 65: Emerging of shoot from transformed Kinnow mandarin segment on selection medium

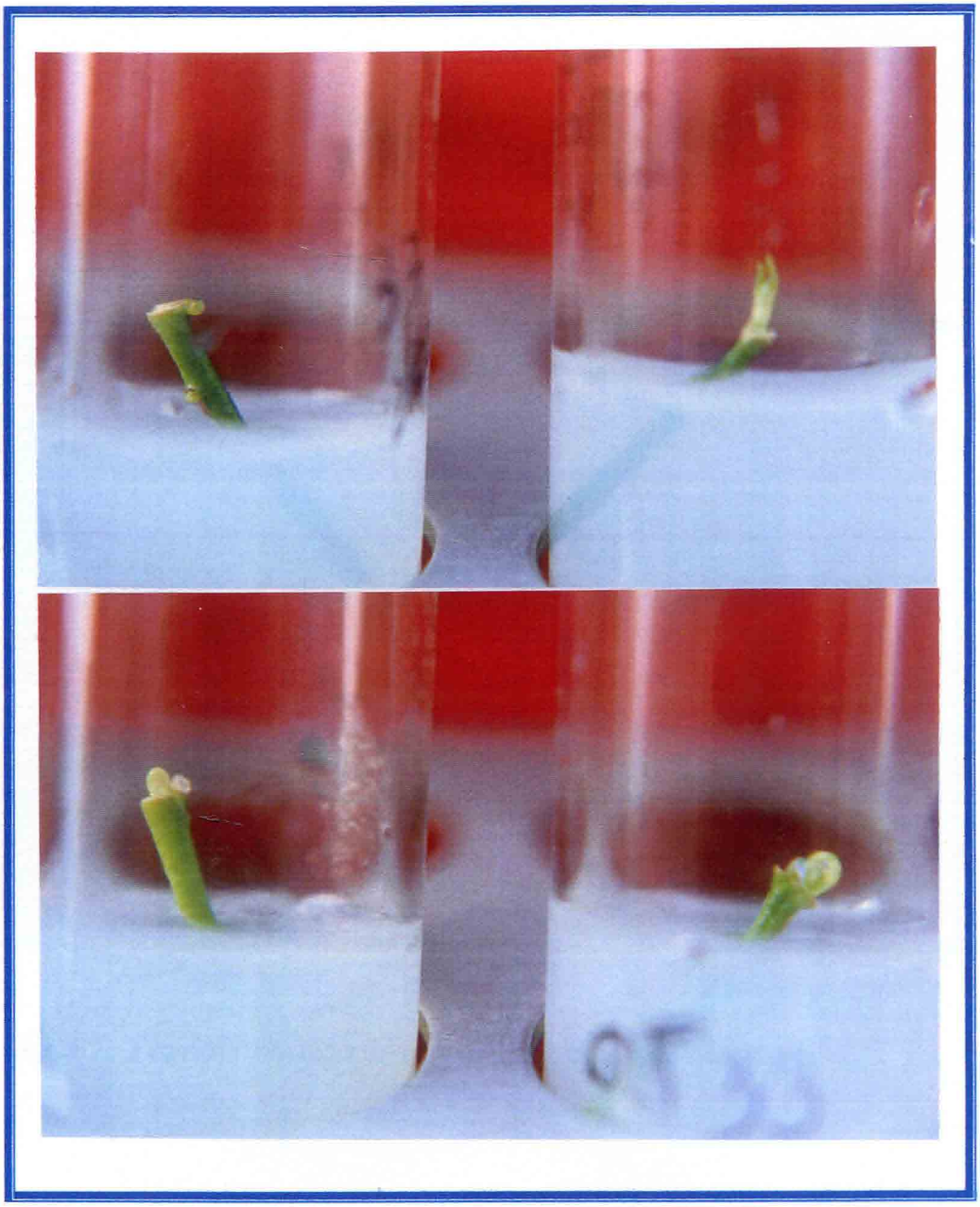


Fig. 66: Emerging of shoots from transformed Kinnow mandarin segments on tissue culture tubes containing SM

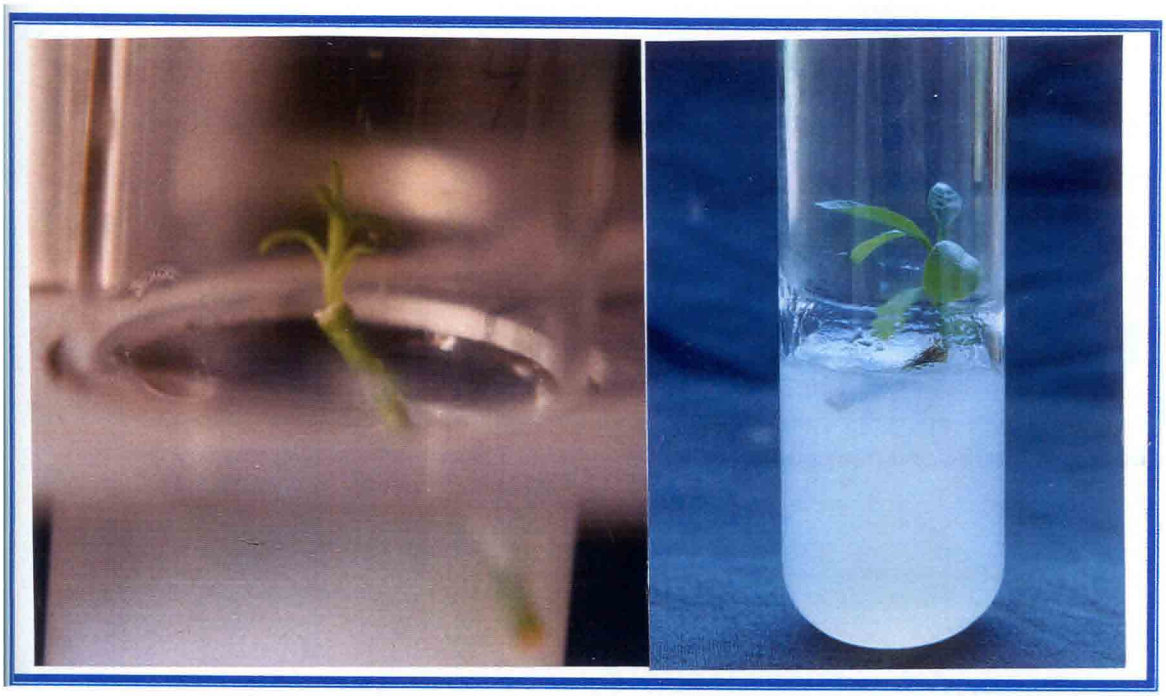


Fig. 67: Transformed plants developed leaves on SM.

DISCUSSION

5. DISCUSSION

Indian citrus ringspot virus (ICRSV) becomes one of the most destructive viruses affecting citrus plantations in India. The disease caused by this virus was first reported by Ahlawat (1989) on Mosambi trees in the states of Delhi, Maharashtra, Punjab and Andhra Pradesh. Later, surveys revealed that this disease was widely distributed in commercial citrus cultivars especially in mandarins and sweet oranges (Byadgi *et. al.,* 1993, Byadgi and Ahlawat, 1995). The affected cultivars were Malta, Mosambi, and Satgudi sweet oranges [*Citrus sinensis* (L) Pers.], Nagpur orange (*C. reticulata* Blanco), Kinnow mandarin, a hybrid (Willow x king mandarin) and Kagzi lime and Kagzi kalan [*C. aurantifolia* (Christen) Swingle]. The incidence of the disease ranged from 5 to 83.8% and the yield loss was estimated from 20.5 to 98.38% (Byadgi and Ahlawat, 1995). Recent report showed a decrease in fruit weight, size, granulation, TSS, TSS/acid ratio, vitamin C and reducing sugar in fruits of Kinnow mandarin from trees showing ringspot symptoms (Thind *et al.,* 1998).

Ringspot diseases reported from other parts of the world (Garsney and Timmer, 1970; Dehyar and Habashi, 1974; Garsney, 1975; Derrick, 1988), are distinct from the ringspot disease found in India (Byadgi and Ahlawat, 1995), and hence named as Indian citrus ringspot disease and the causal virus has been named as Indian citrus ringspot virus (ICRSV) (Ahlawat, 1997). The main symptoms of citrus ringspot in India are vein flecking of young leaves and chlorotic rings on mature leaves. The mode of natural spread of ICRSV is mainly through propagation of contaminated bud wood (Ahlawat, 1997). The causal virus is sap transmissible to French bean and *Chenopodium quinoa* (Pant *et al.,* 2000).

Indian isolates of ringspot virus showed two types of virion measuring 640 x 15 nm and 690 x 9 nm and sometimes with tubule-like structures (Byadgi and Ahlawat, 1995). The virus of Indian isolates did not react in ISEM with antisera of the ringspot viruses reported from other countries (Pant and Ahlawat, 1998). The distinct morphology and serological non-reactivity suggested that ICRSV is different from other CRSVs reports else where and hence this virus was tentatively identified as a member of Capillovirus group (Pant and Ahlawat, 1998). However, no work on molecular characterization of ICRSV isolates has been done so far from India for comparison to other CRSVs. Its incidence is more than 80% in Kinnow mandarin and Sweet oranges, which demands its management quickly to avoid its further spread in the country. These studies were therefore planned with these two major programmes.

Total four different isolates of ICRSV from Delhi (ICRSV - Dl), Abohar in Punjab (ICRSV - Ab), Ahmedabad in Gujarat (ICRSV - Ah) and Pune in Maharastra (ICRSV - Pu) were collected and cultures maintained in the glasshouse on *Citrus* spp. Immunosorbent electron microscopy tests were conducted with ICRSV antiserum available at The Advanced Centre for Plant Virology to identify the pure culture of these isolates. The ISEM test of all the four isolates, all the virus particles were decorated with ICRSV antiserum suggesting that they were infected with one virus. All the four isolates were wedge grafted transmission on different citrus species like Kinnow, King mandarin, Mosambi, Malta sweet orange and Rough lemon, but no difference in symptoms was observed. Therefore, these *Citrus* spp. can not be used to differentiate between isolates. The virus was transmissible to *Phaseolus vulgaris* var, *saxa, P.v.* var. *singtamy, Vignia sinensis, Glycine max, Vicia faba major* L., *Chenopodium quinoa* and *C. amaranticolor,* as reports earlier (Byadgi *et al.*, 1993, Pant and Ahlawat, 1998, Pant *et al.*, 2000 and Rustici *et al.*, 2000).

The physical properties of the virus (ICRSV - Ab) were studied for the first time from French bean to French bean. The results showed that the DEP of the virus was 10^{-5}, the LIV to 8 hours at room temperature and TIP between 65 to 70°C. No data are available on physical properties of CRSV for comparison.

Antiserum of the virus was developed from purified preparations of the virus obtained from infected plants of French bean (*Phaseolus vulgaris* var. *singtamy*). Two methods of virus purification were used (Byadgi *et al.* 1993 and Rustici *et al.* 2000), but the method of Rustici *et al* (2000) provided more concentration of purified virions. The A_{260}/A_{280} ratio was determined as 1.11 suggesting that the purified preparation was the nucleoprotein, and the virus has filamentous particles of 650 nm in length and 15 nm in width. These results are similar to the reported earlier by Byadgi and Ahlawat, 1995, except that a better protocol was developed for virus purification.

The IgGs were isolated from the whole serum and used for the detection of the virus in both DAS and DAC - ELISA. The optimization of the two protocols was done. For DAS-ELISA, the optimum concentration of IgG was 1µg/ml, antigen and conjugate dilution 1:10 and 1:2000 respectively. The results of ELISA indicated that a better antiserum than earlier antisera (Byadgi and Ahlawat, 1995 and Rustici *et al.* 2000) was produced. For DAC - ELISA, the concentration of IgG used was 1µg/ml and antigen dilution 1:10, the concentration of commercial antirabit-antibodies conjugate with antibody produced was 1:10,000. The results of DAC-ELISA were similar to the report of Baranwal *et al* (2000). These two ELISA systems effectivity detected the ICRSV from field samples of various *Citrus* spp. as shown in the results earlier.

The dot blot immuno assay (DIBA) was also tested for detection of ICRSV, it was observed that all the four isolates were detected by ICRSV -

Ab antiserum suggesting that there is no difference in serological reactivity of the four isolates of ICRSV. The best results in DIBA were obtained with 1:1 dilution of antigen and 1µg/ml concentration of antiserum.

RT-PCR is very sensitive technique, which is used for detection viruses with virus specific primers. During this study, we used three sets of primers for ICRSV detection. One set of primers named ICRSV-1096/ICRSV-1420, was used, amplified approximately 350 bp amplicons, and detected all the four isolates of ICRSV (ICRSV-Dl, ICRSV-Ab, ICRSV-Ah and ICRSV-Pu). Whereas two other sets of primers ICRSV-518/ICRSV-951 and ICRSV- 518/ICRSV-1420 specifically amplified product of ≈450 bp and 900 bp respectively only in cases of ICRSV-Dl and ICRSV-Ab, these two sets of primers, however, showed non-specific reaction with ICRSV-Ah and ICRSV-Pu strains. These non-specific amplifications appears to be due to the change of 4 nucleotides in forward primer ICRSV- 518 site and 3 nucleotides in reversed primer ICRSV-951 site in both the ICRSV-Ah and ICRSV-Pu. Therefore, these two sets of primers ICRSV-518/ICRSV-951 and ICRSV-518/ICRSV-1420 were used for differentiation of these two isolates ICRSV-Dn and ICRSV-Ab from two others ICRSV-Ah and ICRSV-Pu.

Nucleic acid spot hybridization (NASH) is another useful technique for specific detection of viruses. For NASH tests we made two probes have been made from 350 bp PCR amplicon of ICRSV- 1096/ICRSV-1420 primers, which detected all the four isolates and 450bp PCR amplicon of ICRSV-518/ICRSV-951 primers, which detected ICRSV-Ab and ICRSV-Dl. The nucleic acids of all the four isolates were extracted from bean and citrus and used for NASH test. The first probe (350bp amplicon) detected all the four isolates of ICRSV, whereas the 450 bp amplicon probe showed strong reaction with ICRSV-Dl and ICRSV-Ab, and weak reaction with

ICRSV-Ah and ICRSV-Pu. It may be due to the variation in the nucleotide sequences of both ICRSV-Ah and ICRSV-Pu. Therefore NASH test was not found suitable for isolates differentiation.

Under the present investigations, the coat protein of the four ICRSV was amplified in RT-PCR by specific primers designed based on the public sequence of ICRSV-D (Rustici *et al.*, 2000). The PCR conditions were standardized by using ICRSV-Dl isolate which combined both the cDNA synthesis and PCR in one tube, the reversed transcriptase was put along with PCR elements and run in PCR machine at once, first at 42°C for 45 min and following by PCR condition of 94°C for 2 min, and 94°C for 1 min (35 cycles) and annealing temperature varied from 55 to 60°C for 2 min. (35 cycles), and 72°C for 3 min (35 cycles), and the extension period was 45 min at 72°C. The results showed amplification of expected size by the five sets of primers, two sets of primers CP-FW/CP-R-Eco and CP-FW/ICRSV-1420 amplified at annealing temperature of 57-60°C, while other sets of primers ICRSV-518/ICRSV-951, ICRSV-518/ICRSV-1420 and ICRSV1096/ICRSV-1420 amplified at annealing temperature of 55 - 57°C. These differences in annealing temperatures may be due to different ratio of G+C content in the primers.

Two sets of primers, ICRSV-1096/ICRSV-1420 and CP-FW/CP-R-Eco have been used to amplify ≈350bp and ≈1kb respectively of all the four isolates ICRSV-Dl, ICRSV-Ab, ICRSV-Ah and ICRSV-Pu. The results were as expected for all the four isolates, but two fragments overlaped with few nucleotides. All the PCR amplicons have been eluted and cloned in pGEM-T easy vector and sequenced. Sequence comparison and analyses both at nucleotide and amino acid level showed variation in the N terminus region and more conservation in the core region of the coat protein. The amino acid sequences varied starting from position 6 to 91, while at other end were more reserved. The variation occurred mainly in

isolates ICRSV-Ah and ICRSV-Pu. The nucleotide and amino acid sequences identity and phylogenic tree view showed that two isolates ICRSV-Dl and ICRSV-Ab shared close relationship with the reported one: ICRSV-D (Rustici *et al.*, 2000), and they were also closely related to each other. The two isolates ICRSV-Ah and ICRSV-Pu were somehow distantly related with the earlier two and ICRSV-D, but they were very close to each other. Thus they are considered as different strains of the same virus ICRSV. Nucleotide sequences of all the four ICRSV isolates were submitted to gene bank and public in internet. The accession numbers are as followed AY 255006 (ICRSV - Dl), AY 255007 (ICRSV-Ah), AY 255008 (ICRSV - Pu) and AY 255009 (ICRSV - Ab).

When the amino acids of these four isolates were taken for comparison with other viruses in internet by BLAST, the results showed some matching with viruses, strawberry mild yellow edge luteovirus, papaya mosaic potexvirus, cassava common mosaic potexvirus, clover yellow mosaic potexvirus, potato aucuba mosaic potexvirus, garlic latent carlavirus and shallot latent carlavirus, and with some other viruses, the matching position was from 134 to 324 and the percentage of matching varied from 32 to 47%, there were more matching point in the middle of the sequences. These results are similar to report of Rustici *et al* (2000, 2002). These analyses showed that ICRSV does not belong to any group of virus reported worldwide. The International Committee on Virus Taxonomy (ICTV) asked to prepare a proposal for a new genus and a new species to accommodate ICRSV. The species name will be "Indian Citrus Ringspot Virus" and the genus name was proposed as KIMAVIRUS (Kinnow and Mandarin virus)(Dr. Y. S. Ahlawat - personal communication).

The restriction map of all the four isolates, which were generated, contained some restriction sites of all the four like *BsiE* 1 (68), *Nco* I and

Sty I (773), *EcoR*V (1039). There are three common restriction sites present in one or two isolates but not in others, such as *Pst* I, present in ICRSV-Ah and ICRSV-Pu but not in ICRSV-Dl and ICRSV-Ab. *Sac* I present in ICRSV-Dl and ICRSV-Ab but not in ICRSV-Ah and ICRSV-Pu. The *Cla* I was present in all the cases except ICRSV-Dl. All these common restriction sites were confirmed their presence or absence in different isolates by restricted them by these above enzymes. These results are very useful for further making construct of the CP gene in bacterial transformation or plant transformation vectors for expressing of the coat protein of the virus.

Since ICRSV has no vector for transmission, shoot tip grafting strategy for management of the disease would be the best option. During these investigations, therefore the protocols for shoot tip grafting (STG) of Kinnow mandarin and Mosambi sweet orange have been standardized and ICRSV - free nucleus materials of the two cultivars has been developed.

For successful STG, sterilization of scion with $HgCl_2$ (0.1%) for 3 min. showed best result. Seedling age of 15 days after germination used as rootstock showed highest percentage of successful grafts. This is similar to observations reported by Navaro *et al.*, 1975 and Singh, 2000. The reduction in STG success with older rootstock may be due to harder stem, which was difficult to cut and insert the scion on it. The less success in younger seedlings appears to be due to precocious callus formation, which may bury the scion within it.

Among the different rootstocks tested for STG of Kinnow mandarin, Rough lemon was more suitable than Trifoliate orange, Mosambi and Malta sweet orange. Howerever, Mosambi on Mosambi rootstock gave highest success. These results are different from the report by Vijaya Kumari *et al.* (2000), who got better result on Trifoliate orange with shoot tips of Nagpur mandarin. This is due to compatibility of scion

and rootstock varieties. However, STG technique has been developed for the first time with Kinnow mandarin and Rough lemon can be recommended as a suitable rootstock for Kinnow and Mosambi for STG.

The effect of light and darkness on the growth of rootstock seedlings was not significant except that the dark-raised plants were pale green and soft, which helped for easier cutting and insertion of meristem due to difference in color of rootstock and shoot tip.

The percentage of successful grafts depended on the size of the shoot tip. In our studies, maximum success was achieved with a shoot tip of apical meristem plus 6 primordia. These results are similar to the reports of Navaro *et al.*, 1975; Juarez *et al.*, 1990 and Vijaya Kumari and Singh, 2000.

Pretreatment of shoot tip with five growth regulators and anti-oxidants for 5 min before grafting have increased percentage of successful grafting except BAP (1mg/lit). Among these, DIECA (100mg/lit) showed best result, almost double to that of water control, followed by 2,4 - D (10 and 5 mg/lit) and Kinetin (1mg/lit). Increased dose of kinetin did not help to increase percentage of graft success, and BAP treatment was at par to water control. These results are different from the report of Mishra and Yadav, 2000, that DIECA at 100mg/lit was less effective than that of 2,4 - D, and zeatin. In case of kinetin treatment, the results were similar to report of Edriss and Burger, 1984 and Vijaya Kumari *et al.*, 2000. They also found that a concentration of 1mg/lit was more effective than that of 10mg/lit. However, since STG with Kinnow mandarin has not been done else where, no comparison can be made.

Composition of the medium also played an important role in successful STG. The MT medium supplemented with plant growth regulators showed higher percentage of successful grafts in our studies.

Selection of scion for shoot tip grafting is very important. During our studies, more STG plants survived when shoot tips were prepared from glasshouse as compared to field trees. This result is similar to the reports of Navarro *et al.*, (1975) and Vijaya Kumari and Singh (2000 c). STG was more successful when shoot tip was inserted in triangular cut of rootstock as compared to invert T or window cut, in the report of Navarro *et al*, (1975), they got more successful grafts using invert - T graft in comparison with open mouth method. Double grafting technique gave better result as compared to direct hardening by conventional method, this result is similar to the report of Vijiya Kumari *et al.*(2000). The reasons for this probably because the scions can uptake nutrients direct from rootstock and the distance from graft union to the soil surface is more and hence pathogens can not attack the scion.

The STG plants thus developed were tested in ELISA and ISEM for the presence of ICRSV, Citrus vein clearing, citrus tristeza virus and citrus yellow mosaic virus. Interestingly, all the STG plants produced were free from these viruses suggesting that the protocol developed under these studies can be safely used for developing virus(es) - free planting materials. Since the main objective of these studies was to eliminate ICRSV from contaminated plants, STG plants were further tested by dot blot hybridization and were also found free.

The Kinnow and Mosambi plants were multiplied on rough lemon rootstock taking bud from STG plants and maintained as virus - free nucleus materials at The Advanced Centre for Plant virology and also supplied to The Division of horticulture for multiplication.

Ovule culture is another approach used for improvement of citrus varieties (Starrantino and Caponnetto, 1990; Gmitter *et al.*, 1990), and the embryos developed by this method can be used for grafting (Ollitrault, 1991), or ovule culture plants can be used for transformation purpose.

During the present investigations, ovules from Kinnow mandarin and Mosambi sweet orange at flowering stage were used for culture. The percentage of ovule survival in the medium was high, but they developed abnormal embryos and plantlets, which died very soon. It may be due to unfertilized ovules obtained in the experiment, which is not suitable for growing normal plants. In case of Mosambi, percentage of ovule survival was higher than that of kinnow, but there was no callus formation even after three months on MT medium and all the ovules subsequently died.

The culture of ovule of Kinnow and Mosambi from one month's old fruits, although remained green but there was no sign of embryo development. When such ovules were transferred to MT medium supplement with different concentration of 6 - benzyladenine (BA) and Kinetin, there were calli induction, embryo formation and rooting.

The rate of callus formation on MT medium alone or supplemented with different concentration of 6 - benzyladenine (BA) and kinetin was different at different concentration of growth regulators. In Kinnow, the highest percentage of calli formation was in MT medium supplemented with 5mg/l of BA and kinetin, followed by BA (5mg/l) without kinetin and 1mg/l of BA and kinetin. While in Mosambi, best results were once again confirmed in MT plus BA and kinetin at 5mg/l. However, in this case the rate of calli formation was higher than that of kinnow madadrin. Similar results were reported by Starrantino and Caponnetto (1990) with Mosambi sweet orange, and Gmitter *et al.* (1990) with Duncan grapefruit.

When the calli were transferred to MT medium, the embryo differentiation was observed and plantlets developed at 15-20 days after inoculation. The most effective medium was MT supplemented with 1mg/l IAA plus 2mg/l kinetin. Between the two cultivars, Mosambi had better ability for embryo differentiation. It developed globular, heart, torpedo, and cotyledonary stages and finally produced secondary

embryos. It took more time for development of root normally 20-30 days after inoculation. The best responses were obtained when the calli were transferred on MT supplemented with NAA (1mg/l) for Mosambi. These results were similar to the results of Vijaya Kumari and Shyam Singh (2000b). However, in case of Kinnow mandarin the result was similar to the one report by Gmitter *et al* (1990) that the medium MT plus BA (1mg/l) plus 2mg/l of GA gave the best result.

The plantlets were put to hardening by either conventional method or the embryos were grafted to rootstock seedlings in the glasshouse as described by Ollitrault (1991). The plants were tested by DAC-ELISA and NASH for viruses and multiplied.

To develop virus - resistance plants using pathogen-derived sequences (PDSs) is the recent method of choice (Lomonosoff, 1995). The most commonly used PDS was from the viral coat protein gene (Powell - Abel *et al.*, 1986); however other PDSs, including truncated movement proteins and replicase genes, were also used for this purpose (Anderson *et al.*, 1992; Palukaitis and Zaitlin, 1997).

In the present investigation, the CP gene of ICRSV-Ab from pGEMT-easy vector was released at 5′ and 3′ ends by *Bam*H I and *Sac* I (from vector) restriction enzyme, respectively. The vector pBI121 was lenierised using *Bam*H I and *Sac* I (this removes β-glucoronidase gene (gus)) and sense directional cloning of CP gene was achieved by ligating *Bam*H I - *Sac* I digested CP gene insert with *Bam*H I - *Sac* I restricted pBI 121. Transformation of the resultant plasmid in *E. coli* strain DH 5 α yielded recombinant colonies. Colony PCR and restriction with the same enzyme were done, but there was no PCR product amplified with the same set of primers. When plasmids were isolated and restricted with *BamH* I and *Sac* I, a thick band on the gel, which was recorded at 900bp position, was confirmed by southern hybridization using probe made from

PCR product amplified from ICRSV-Ab with ICRSV-518/-951 primers. Subsequently, we analyzed the sequence, there is another site of *Sac* I present at 900 bp position suggesting that the reversed primer site was removed and when another reversed primer (ICRSV-951) nearby this site was used, a PCR product of 814 bp was achieved, and then a single colony was selected. The selected clone having CP gene in sense orientation in pBI 121 was hitherto called as CpSTR (Coat protein Sense truncated) and used for plant transformation.

Truncated CP gene construct (CpSTR) was mobilised into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) from *E. coli* with the help of pRK2013 plasmid in *E. coli.* (Comoi *et al., 1983)* by triparental mating method (Ditta *et al.*, 1980). The colony PCR amplification from *Agrobacterium* colony was confirmed. Internodes explants of French bean (*Phaseolus vulgaris* var. *saxa*) were used for transformation. Co-cultivation of explants was done by growing them in co-cultivation medium (CM)(Genga *et al.*, 1990). Selections of co-cultivated explants were done by growing them in selection medium (Genga, 1991 and Venketeswaran *et al.* 1990). The true transformant seem to be due to the CP gene construct along with the NPT II gene conferring Kanamycin resistance in plant selection.

When the plants have 3-4 triple-leaves (one month after co-cultivation), 100 mg leaves of each individual plant were picked and the total DNA were extracted by using DNA plant mini kit, and 10 μl were used for PCR. The result showed that the PCR product had been amplified with specific primers CP-FW/ICRSV-951. The result was also more clear in Southern hybridization suggesting that truncated protein gene of Coat protein of ICRSV-Ab has been integrated into French bean genome. Once again, the result from nucleic acid spot hybridization of these plants

confirmed that the truncated CP gene of ICRSV-Ab isolate has incorporated in to the bean genome.

For checking the expression of the coat protein gene in bean system, DIBA test was done using homologous antiserum (ICRSV-Ab antiserum). Interestingly, all the samples tested reacted with ICRSV-Ab antiserum suggesting that the transformed plants with CP gene expressed the gene by translating the virus specific protein. Similar result of expression of truncated protein gene of grapevine fanleaf virus was reported by Golles *et al.*(1997a & b, 2000).

In case of citrus, the Kinnow segment developed callus and then shoots from cut end on the selection medium containing high concentration of antibiotic 200mg/lit kanamycin and 300mg/lit cefotaxime, which are more likely to carry the transformed CP gene, whereas those had not got the transformation died very fast when they were transferred to SM medium.

The following new information/products have been generated during the present studies:

1. *Polyclonal antiserum against ICRSV – Ab isolate has been produced and successfully used for the detection of the virus in DAS, DAC - ELISA and DIBA systems.*
2. *One set of primer (ICRSV-1096/-1420) identified for detection of all the four ICRSV isolates, but two other sets of primer (ICRSV-518/-951 and ICRSV-518/-1420) identified for the ICRSV-Dl and ICRSV-Ab isolates.*
3. *Probe made from 350 bp amplicon of primer ICRSV – 1096/-1420 was successfully used for detection of all the four isolates in NASH system..*
4. *1282 bp of coat protein of four ICRSV isolates have been cloned in pGEM-T easy vector and sequenced. Nucleotide and deduced amino acid*

sequences have been analyzed showing variation at N terminus and conserved in the core region.

5. *Sequence analysis and phylogenic tree of both nucleotide and amino acid sequences showed the two isolates ICRSV -Ab and ICRSV- Dl shared close relation with ICRSV-D and formed one cluster, while the two isolates ICRSV -Ah and ICRSV - Pu were far related to first cluster and formed another cluster and hence they were considered as different strains of the same virus.*
6. *The restriction maps were generated and confirmed the sites of restriction by cutting with the common enzymes like Sac I, Pst I and Cla I.*
7. *Protocol for STG has been standardized in case of Mosambi and Kinnow and virus-free (CTV, ICRSV, CVV, CYMV) nucleus materials of Kinnow and Mosambi were produced, multiplied and given to The Division of Horticulture for developing Foundation Block.*
8. *Transformed French bean plants with truncated coat protein gene of ICRSV–Ab were developed and gene expression was proved in transformed bean plants.*

Future thrusts

- *To ascertain protection conferred by coat protein against ICRSV in citrus and subsequent field trials to evaluated transgenic plants.*
- *Bud wood certification programmes must be initiated using the technology of STG.*

SUMMARY

6. SUMMARY

During the present investigation, four isolates of Indian citrus ringspot virus (ICRSV), IVRSV-Dl, ICRSV-Ab, ICRSV-Ah and ICRSV-Pu, have been studied specially for their molecular characterization. The methods for detection of ICRSV evolved and virus-free planting material developed by shoot-tip grafting (STG) and using the transgene have been developed.

Four isolates of ICRSV were collected from different parts of the country and name as ICRSV- Dl (Necrotic isolate from Delhi on mandarin), ICRSV-Ab (Abohar isolate on Mosambi), ICRSV-Ah (Ahmedabad isolate on Rough lemon), and ICRSV-Pu (Pune isolate on Rough lemon). The pure cultures were identified by testing them on ISEM with ICRSV antiserum developed earlier by Ahlawat and his team. The pure culture of ICRSV-Ab was sap transmitted to French bean (*Phaseolus vulgaris* var. *singtamy*). The dilution end point, longevity *in-vitro* and thermal inactivation point were worked out as 10^{-5} dilution, 8 hrs at room temperature and 70°C respectively.

The virus was purified from infected French bean (*Phaseolus vulgaris* var. *singtamy*). The A_{260}/A_{280} ratio of purified preparation was 1.11 suggesting that the preparation was a nucleoprotein. Polyclonal antiserum of ICRSV was prepared and IgG were purified and diluted as 1mg/ml for the use of serodiagnosis of ICRSV. The titer of the antiserum was determined by ISEM and ELISA. The optimum conditions for DAS-ELISA and DAC-ELISA were determined.

The methods of ICRSV detection by RT-PCR with specific primers and Nucleic acid spot hybridization were developed. During the present studies, we also standardized the primers and probes for these two techniques. The primers ICRSV-1096/ICRSV-1420 was found to detect all the four isolates of ICRSV, whereas primers ICRSV-518/ICRSV-951 and ICRSV-518/ICRSV-1420 were specifically detected the isolates ICRSV-Dl and ICRSV-Ab. The probe made from 350bp amplicon of the PCR product from primers ICRSV-1096/ICRSV-1420 also detected all the four isolates in NASH tests.

The coat protein of all the four isolates have been amplified by CP-FW/CP-R-Eco and ICRSV-1096/ICRSV-1420 primers, cloned in pGEM-T easy vector and sequenced. The analyses of both nucleotide and amino acid sequence levels and comparison with ICRSV-D (reported from Italy) showed that the variation occurred at N terminus and conserved in the core region of the CP. The nucleotide and amino acid sequence identity matrix and phylogenetic tree showed that the ICRSV-Dl and ICRSV-Ab shared close relation to ICRSV-D, whereas ICRSV-Ah and ICRSV-Pu was distantly related to ICRSV-D, but the two were closely related to each other. The results indicated strain's variation in ICRSV.

The nucleotide sequences of all the four isolates were used for generation of restriction map using Bioedit software, *Pst* I is present in ICRSV-Ah and ICRSV-Pu, but not in ICRSV-Ab and ICRSV-Dl. *Sac* I is present in ICRSV-Ab and ICRSV-Dl, but not in ICRSV-Ah and ICRSV-Pu. *Cla* I is present in all the cases except in ICRSV-Dl. These restriction sites played importance role when we planned to make the construct of the CP gene.

When the amino acid sequences of these isolates were used for blasting in the internet, they were matching with some other virus groups like Luteo-, Carla- and Potexvirus. But the matching point varies from 134 - 324 position of the sequences and showed a range from 32 to 47% identity. The results convinced that the virus does not belong to any known group of viruses. Therefore, ICRSV was suggested to the genus name as KIMAVIRUS and species name as "Indian citrus ringspot virus" (Dr. Y.S. Ahlawat - Personal communication).

Protocols for the production of virus-free nucleus materials of Kinnow and Mosambi have been standardized. The STG plants developed during the studies were free from ICRSV, CTV, CVV, CYMV. These are multiplied and maintained in the glasshouse condition as nucleus material. Some virus-free plants have been given to the Horticulture Division for multiplication and development of Foundation Block.

Another strategy developed for management of the virus was the use of CP gene of ICRSV-Ab for construction. The gene constructed in pBI 121 binary vector was mobilized to *Agrobacterium* strain LBA 4404 and co-cultivated in French bean (*Phaseolus vulgaris* var. *saxa*). The tranformant was a truncated version of CP gene of 900 bp. The presence of gene was confirmed in bean system by PCR and southern hybridization of this PCR product, and also by dot blot hybridization with the total DNA extracted from bean transformed plants. The coat protein expressing in the bean system was also checked for positive reaction by ICRSV-Ab antiserum in DIBA system.

The following new information/products have been generated during the present studies:

1. *Polyclonal antiserum against ICRSV – Ab isolate has been produced and successfully used for the detection of the virus in DAS, DAC - ELISA and DIBA systems.*
2. *One set of primer (ICRSV-1096/-1420) identified for detection of all the four ICRSV isolates, but two other sets of primer (ICRSV-518/-951 and ICRSV-518/-1420) identified for the ICRSV-Dl and ICRSV-Ab isolates.*
3. *Probe made from 350 bp amplicon of primer ICRSV – 1096/-1420 was successfully used for detection of all the four isolates in NASH system.*
4. *1282 bp of coat protein of four ICRSV isolates have been cloned in pGEM-T easy vector and sequenced. Nucleotide and deduced amino acid sequences have been analyzed showing variation at N terminus and conserved in the core region.*
5. *Sequence analysis and phylogenic tree of both nucleotide and amino acid sequences showed the two isolates ICRSV -Ab and ICRSV- Dn shared close relation with ICRSV-D and formed one cluster, while the two isolates ICRSV -Ah and ICRSV - Pu were far related to first cluster and formed another cluster and hence they were considered as different strains of the same virus.*
6. *The restriction maps were generated and confirmed the sites of restriction by cutting with the common enzymes like Sac I, Pst I and Cla I.*
7. *Protocol for STG has been standardized in case of Mosambi and Kinnow and virus-free (CTV, ICRSV, CVV, CYMV) nucleus materials of Kinnow and Mosambi were produced, multiplied and given to The Division of Horticulture for developing Foundation Block.*

8. *Transformed French bean plants with truncated coat protein gene of ICRSV–Ab were developed and gene expression was proved in transformed bean plants.*

Future thrusts

- *To ascertain protection conferred by coat protein against ICRSV in citrus and subsequent field trials to evaluated transgenic plants.*
- *Bud wood certification programmes must be initiated using the techonology of STG.*

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7. REFERENCES

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APPENDIX I

1. Buffers for Mechanical Transmission and Purification

1.1. Inoculation buffer (Phosphate buffer for 1L, 0.01 M (pH 7.2)

(i) Potassium dihydrogen phosphate (KH_2PO_4) - 1.362g/l
(ii) Disodium hydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$) - 1.781g/l

Mixed 285 ml of sol.(i) with 715 ml sol. (ii) gives 1 liter of phosphate buffer pH 7.2 and 0.01M

1.2. Pufication buffer (Phosphate buffer for 1L, 0.05 M (pH 7.8)

(iii) Potassium dihydrogen phosphate (KH_2PO_4) - 6.810g/l
(iv) Disodium hydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$) - 8.905g/l

Mixed 86 ml of sol. (i) with 914 sol. (ii) gives 1 liter of phosphate buffer pH 7.8 and 0.05M

2. Enzyme-Link Immunosorbent Assay (ELISA)

(i). Phosphate buffer saline (PBS) pH 7.4 (1 X)

NaCl	- 8.0 g
KH_2PO_4	- 0.2 g
$Na_2HPO_4.2H_2O$	- 2.9 g
KCl	- 0.2 g
NaN_3	- 0.2 g
Distilled water	- 1000 ml

(ii). Coating buffer (pH 9.2)

Na_2CO_3	- 1.59 g
$NaHCO_3$	- 2.93 g
NaN_3	- 0.20 g
Distilled water	- 1000 ml

(iii). Washing buffer (PBS-T)

1 X PBS	- 100 ml
Tween 20	- 900 ml

(iv). Antigen extraction buffer

Coating buffer containing 2% solute polyvinyl pyrrolidone (MW 44,000)

(v). Enzyme conjugate buffer

PBS-T	- 1000 ml
Polyvinyl Pyrrodone (PVP)	- 20.0 g
Ovalbumin – 40	- 2.0 g

(vi). Blocking solution

Add 5 g of Bovine Serum Albumin (BSA) to 1000 ml coating buffer

(vii). Substrate buffer (pH 9.8)

Diethanol amine	- 97 ml
Distilled water	- 800 ml
NaN_3	- 0.20 g

The pH adjusted to 9.8 with 1 N HCl and make upto 1000 ml with distilled water.

APPENDIX II

Common Reagents, Buffer and Media Used For Cloning and Confirmation of Clone.

(i) Antibiotics

Ampicillin: Stock solution (50mg/ml) of the antibiotic was made in distilled water, filter sterilized (through 0.22 micron filter) and distributed into 200µl aliquots and stored at - 20°C. It was used at a concentration of 50µg/ml.

(ii) Electrophoresis reagents

50X TAE	Tris base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100.0 ml
	Distilled water to 1 liter.	

Loading dye	1% Bromophenol blue	200 µl
	Glycerol	200 µl
	10% SDS	60 µl
	0.5 M EDTA	50 µl
	10 X TAE	60 µl
	Distilled water	30 µl

(iii) Plasmid isolation buffers

Sol. I (resuspension buffer)	25 mM Tris HCl (pH 8.0) 50 mM Glucose 10 mM EDTA
Sol. II (lysis buffer)	0.2 N NaOH 1% SDS
Sol. III (neutralization buffer)	3M Sodium acetate pH4.8

(iv) Hybridization solutions

Denaturing solution	1.5 M NaCl 0.5 M NaOH
Neutralizing solution	1.5 M NaCl 1 M Tris. HCl (pH 7.4)
Denhardt's reagents (50X)	1gPolyvinylpyrilidone (Sigma) 1g Ficoli (Type 400, Sigma) 1g Bovine serum albumin (Fraction V. Sigma) 100 ml Double distilled water.

This stock solution was stored at - 20°C. It was used at working concentration of 5X Denhardt's reagent.

Standard saline citrate (SSC) buffer 20 X, pH 7.0

NaCl (3.0M)	175.3 g
Sodium Citrate (0.3 M)	88.2 g

Dissolved in 800 ml distilled water. Adjusted pH to 7.0 and made up the volume to 1 litre. Dispensed into aliquots and sterilized before storing at room temperature.

(v) Prehybridization solution

5 X Denhardt's reagent
6 X SSC
0.3% SDS
100 μg ml^{-1} yeast RNA

(vi) Other buffer

Membrane washing buffer	0.1% SDS 2 X SSC
Dephophorylation buffer	10 mM Mg Cl_2 100 mM Tris.HCl (pH 8.3)

10X Ligation buffer	0.5 M Tris. HCl (pH 7.6) 0.5 M Mg Cl_2 0.1 M Dithiothreitol 500μgml^{-1} BSA (Sigma Chemical Co., A 8022)

(vii) DNA molecular weight marker

1 Kilobase (1Kb) DNA ladder of MBI Fermentas was used as marker. The ladder formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb.

(viii) Preparation of commonly used stock solution

Solution	Method of preparation
M Adenosine Triphosphate (ATP)	60.0 mg of ATP was dissolved in 0.8 ml of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH and volume made upto 1ml with distilled water. The solution was dispensed into small aliquots and stored at – 70°C.
1 M CaCl	54.0 g $CaCl_2$. $2H_2O$ was dissolved in 200 ml of pure water. (Mill-Q or equivalent). The solution was sterilized by passing through a 0.22 micron filter and stored in 1 ml aliquots at 4°C.
0.5 M EDTA (pH 8.0)	186.1 g of ethylenediamine tetra acetic acid disodium salt, $2H_2O$ was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.

Ethidium bromide (10 mg ml^{-1})	1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature.
Phenol : Chloroform : Isoamyl alcohol	Buffer saturated phenol, chloroform and isoemyl alcohol were mixed in the ratio of 25:24:1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4°C dark glass bottle.
Chloroform : Isoamyl alcohol	Buffer saturated chloroform and isoamyl alcohol were mixed in the ratio of 24:1.
IPTG (Isopropyl-β-D-thiogalactopyranoside)	A solution of IPTG was made by dissolving 2.0 g of IPTG in 8 ml of distilled water. Volume was made upto 10 ml with distilled water and sterilized by filtration through a 0.22 micron disposable filter. The solution was dispensed into 1 ml of aliquots and stored at – 20°C.
1 M $MgCl_2$	203.3 g of $MgCl_2$. $6H_2O$ was dissolved in 800 ml distilled water. The volume was made upto 1L, dispensed into aliquots and sterilized by autoclaving.
3 M Solution acetate (pH 4.8)	408.1 g of $NaOAc.3H_2O$ was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acid. Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
5 M NaCl	233.8 g of NaCl was dissolved in 800 ml of distilled water, volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
10% solution dodecyl sulphate (SDS)	100 g of electophoresis grade SDS was dissolved in 900 ml distilled water, heated at 68C to assist dissolution and pH was adjusted to 7.2 by adding few drops of

	concentrated HCl. The volume was made upto 1 L with distilled water, dispensed in aliquots.
10 (N) NaOH	Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1 L with distilled water.
1 M Tris HCl	121.1 g of Tris. Base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated HCl (for pH 7.4, HCl 70 ml; for pH 8.0, HCl 42 ml). The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made up to 1 litre with distilled water, dispensed into aliquots and sterilized by autoclaving.
X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside	The stock solution was made by dissolving X gal in dimethyl formamide to make 20mg/ml solution and stored at –20°C.

(ix). Materials, source, catalogue number of chemicals and reagents.

Material	Source	Catalogue No.
Vector		
+ pGEMT –easy	Promega	
+ pBI 121	Clonetech	1996/1997
Cells		
+*Escherichia coli* DH 5α	Stratagene, USA	200233
+*Agrobacterium. tumefaciens* LBA 4404	(Hoekema *et al.*, 1983)	
Marker		
+ 1kb DNA ladder	MBI Fermentas	SM 0311
Enzymes		
+Restriction endonucleases	MBI Fermentas	GA 2151
+RNase A	Sigma chemical Co.	R5000
+ Proteinase K	Ambion	2548

+ T4 Ligase	Amersham, USA	E2050Y
Radioisotope (α-^{32}P) dCTP Sp. Activity 4000 Ci m/Mol	Board of Radiation and Isotope Technology (BRIT), Bombay	LCP 102
Nitrocellulose Membrane (NCM)	Amersham, USA	088120
5-bromo-4-chloro-3-indolyl-β-D-galacto pyranoside (X gal)	USB	12385
Isopropylthio-β-D-galactoside (IPTG)	USB	17886
Antibiotics		
+Ampicillin (Sodium)	Sigma chemical Co.	A0104-10g
+ Kanamycin monosulf.		K0126-5g
+ Rifampicin		R0146-1g
+ Cefotaxime sodium		C0111-5g
Kits		
+Random primer labeling kit	Bangalore genei Pvt. Ltd.	KT-04
+ RNeasy plant mini kit	QIAGEN, USA	74104
+ Gel extraction kit	QIAGEN, USA	28704
+ Taq PCR core kit (250)	QIAGEN, USA	201223
+ Omniscript RT kit (50)	QIAGEN, USA	205111
+ DNeasy plant mini kit	QIAGEN, USA	69104

APPENDIX III

Stock solutions for Murashige and Skoog's medium (MS)

Constitutions	Amount (mg l^{-1})
Stock solution I	
- NH_4NO_3	33000
- KNO_3	38000
- $CaCl_2.2H_2O$	8800
- $MgSO_4.7H_2O$	7400
- KH_2PO_4	3400
Stock solution II	
- KI	166
- H_3BO_3	1240
- $MnSO_4.7H_2O$	4460
- $ZnSO_4.7H_2O$	1720
- $Na_2MoO_4.2H_2O$	50
- $CuSO_4.5H_2O$	5
- $CoCl_2.6H_2O$	5
Stock solution III	
- $FeSO_4.7H_2O$	5560
- $Na_2.EDTA.2H_2O$	7460
Stock solution IV	
- Inositol	20000
- Nicotinic acid	100
- Pyridoxine HCl	100
- Thiamine HCl	100
- Glycine	400

To prepare 1 lit of medium take 50ml of stock I, 5 ml of stock II, 5 ml of stock III, and 5ml of stock IV

Dissolve $FeSO_4.7H_2O$ and $Na_2.EDTA.2H_2O$ separately in 450 ml distilled water by heating and constant stirring. Mix the two solutions, adjust the pH to 5.7 and add distilled water to make up the final volume to 1 liter.

To make MT medium, make MS medium first with the above solution and then add myo-inositol (100mg/l), nicotinic acid (vitamin B 3)

(5mg/l), pyridoxin HCl (Vit. B6)(10mg/l), thiamin HCl (Vit. B1)(10mg/l), glysine (2mg/l), Sucrose (5%).

Antibiotic solutions for construction of CP gene and transformation

Name	Stock solution (mg/ml)	Solvent
Ampicillin	50	Water
Kanamycin	100	Water
Cefotaxime	250	Water
Rifampicin	50	Methanol

All antibiotics which are water soluble were sterilized by filtration (0.22μm pore size) and then stored as frozen stock solutions at - 20°C. Antibiotics were added directly to liquid medium or to molten and precooled (45 -55°C) agar solidified media.

Modified MS medium for French bean transformation and regeneration

Seed germination medium

MS medium without any hormone

Co-Cultivation medium:

MS + 1μg/ml each of 2,4-D & NAA

Selection medium:

(100μg/ml Kan + 300μg/ml Cef.)

+ *Callus formation:*
MS + 1μg/ml BA+ 1μg/ml NAA

+ *Shooting medium:*
MS + 1μg/ml BA + 1μg/ml kinetin

+ *Rooting medium:*
MS + 1μg/ml IAA

Modified MT medium for citrus transformation and regeneration

Seed germination medium

MS medium without any hormone

Co-Cultivation medium:

MT + 1µg/ml each of IAA + KT

Selection medium:

MT plus (200µg/ml Kan + 300µg/ml Cef.) and supplement with hormone:

Culture	Kinnow (mg/l)	Mosambi
+ *Callus formation:*	BA (1)+KT (1)	BA (1)+KT (1)
+ *Shooting medium:*	IAA (1)+KT (2)	IAA (1)+KT (2)
+ *Rooting medium:*	BA (2)+ GA (2)	NAA (1)

BA: 6 - benzyladenine; KT: Kinetin; IAA: Indole -3- Acetic Acid; NAA: Naphthalene Acetic Acid; 2,4-D: 2,4-Dichlorophenoxy Acetic Acid.