Characterization of a Filamentous Virus Associated with Citrus Ringspot in India

A. S. Byadgi, Y. S. Ahlawat, N. K. Chakraborty, A. Varma, M. Srivastava, and R. G. Milne

ABSTRACT. A ringspot disease of citrus affecting most of the citrus cultivars is widely distributed in India. It was readily transmissible by graft inoculation and dodder (*Cuscuta reflexa*) but not by sap inoculations to herbaceous hosts, aphids, seed or soil. Two type of filamentous particles measuring 640 x 15 nm (C) and 690 x 9 nm (L) as well as tubules of 2250 x 40 nm (T) were associated with the disease. C and L particles had a nucleoprotein UV-absorption spectrum and contained about 6% ssRNA, whereas, T particles contained only protein. A 29-kDa protein was associated to C and T particles. Polyclonal antisera prepared against each individual component cross reacted to all components suggesting the presence of common epitopes. The present isolate was not serologically related to other CRSV or psorosis-A and hence considered to be a new citrus virus and designated CRSV-I. ELISA and ISEM proved useful in detecting the virus even in field infected trees. Disease spread occurs mainly through CRSV-I contaminated budwood.

Index words. citrus ringspot virus, characterization, filamentous virus, tubules.

A ringspot disease of citrus was first described by Wallace and Drake (23). Later it was found that ringspot shares with psorosis B, the severe form of psorosis bark and leaf symptoms (22) as well as other characteristics which led to the conclusion that psorosis and ringspot might be synonymous. In India, a common disease inducing a shock reaction on inoculated Mosambi sweet orange seedlings followed by flecking on young leaves and ringspots on mature leaves has been identified as a strain of psorosis A (1). However, field trees do not show bark lesion symptoms and the young leaf symptoms in inoculated Mosambi plants persist until the leaves mature. These symptoms suggest that the disease is similar to citrus ringspot except that bark lesions are not produced. The disease was observed on field trees of Kinnow mandarin, Malta, Satgudi, Mosambi sweet orange and acid limes. Ringspot on leaves of the lower canopy were the main diagnostic symptoms in the field. Incidence of the disease was observed up to 100% in one orchard of Malta sweet orange containing 2000 trees and a Kinnow mandarin orchard with 300 trees. Studies on the disease and its putative causal agent are reported in this paper.

MATERIALS AND METHODS

Transmission. The isolate of Indian ringspot (CRSV-I) used in these studies was from a 14-yr-old Mosambi sweet orange tree propagated on rough lemon rootstock in an orchard at New Delhi. Glasshouse grown Mosambi seedlings were inoculated by side grafting a budstick from a symptomatic branch. The inoculated plants showing severe shock reaction, leaf flecking in young flush and conspicuous rings on mature leaves were further propagated on Mosambi seedlings, to serve as source of inoculum. One-year-old seedlings of citrus cultivars Mosambi, Satgudi and Malta sweet orange; Darjeeling , Nagpur and Kinnow mandarin; acid limes, lemons, Star Ruby grapefruit, calamondin, Etrog citron, *Citrus indica*, rough lemon, sour orange and sweet lime were inoculated by side grafting from glasshouse maintained isolate of CRSV-I. The inoculated plants remaining symptomless were either indexed back to Mosambi indicator or samples were observed in the electron microscope for the presence of virions. *Chenopodium amaranticolor, C. quinoa, Nicotiana glutinosa, N. tabacum, Phaseolus vulgaris, Catharanthus*

roseus, *Cucumis sativus and Gomphrena globosa* were sap-inoculated with inoculum prepared in TACM buffer (8,11). *Aphis citricola*, *A. gossypii*, *A. craccivora* and *Myzus persicae*, were tested as potential vectors (3). Seed and soil transmission of CRSV-I was done by sowing 1000 seeds each from fruits of diseased and apparently healthy Kinnow trees in sterilized soil and the soil collected from CRSV-I affected orchards respectively. Dodder, *Cuscuta reflexa*, bridge between diseased and healthy plants of Mosambi sweet orange and Kinnow mandarin was maintained at least for 30 days for transmission of CRSV-I.

Purification. Purification and concentration of CRSV-I was attempted by various methods (2,9,10,18) as also a protocol developed and standardized in the present studies (Table 1). Purified preparations diluted 1:10 in 0.05 phosphate buffer (pH 7.6) were analysed for UV absorbance from 220 to 300 nm in a Pye Unichem SP-8-500 UV/VIS spectrophotometer.

Electron Microscopy. Leaf dip preparations stained with 2% aqueous uranyl acetate were used for the study of virus morphology and size of particles. EM was also used to monitor CRSV-I concentration during purification.

Nucleic acid and protein analyses. SDS - treated virus preparations were subjected to RNase and DNase treatments in 0.3M NaCl, electrophoresed in 1% agarose gel and stained with 0.1% ethidium bromide. Preparation without nuclease treatment served as control. Nucleic acid percentage was determined (13). The method of SDS-PAGE (15) on 12% polyacrylamide gel was employed to determine the molecular weight of CRSV-I coat and tubular proteins in purified preparations. The mobility of proteins compared with that of the following molecular weight markers: ovalbumin-50 kDa, carbonic anhydrase-39 kDa, soybean trypsin inhibitor-25 kDa, lysozyme-17 kDa and capsid protein of Henbane mosaic potyvirus-40 kDa. The gel was stained with Coomassie blue R-250.

Serology. Polyclonal antisera were prepared in New Zealand white rabbits against three components of CRSV-I: capillovirus- like (C), long flexuous (L)

TABLE 1
FLOW CHART OF THE STANDARD PROCEDURE FOR PURIFICATION OF CRSV-I

Material discarded		Material retained
	:	100 g symptomatic leaves of Kinnow
	:	1) Homogenize the tissue in 3 vol. of PB (0.05 M phosphate buffer,
	:	pH 7.6 + 0.05% Thioglycolic acid)
Debris	:	2) Strain through cheese cloth
	:	3) Add 10% cold n-Butanol while stirring for 15 min
	:	4) Centrifuge at 7,500 x *g*, 10 min at 4 C
Alcohol phase, pellet	:	5) Aqueous phase; filter through filter paper
	:	6) Centrifuge at 100,000 x *g*, 90 min at 4 C
Supernatant	:	7) Pellet; resuspended in 25 ml 0.05 M phosphate buffer (pH 7.6)
	:	8) Centrifuge at 1000 x *g*, 2 min at 4 C
Pellet	:	9) Supernatant; layered on a 5 ml 30% sucrose pad
	:	10) Centrifuge at 100,000 x *g*, 90 min at 4 C
Supernatant	:	11) Pellet; resuspend in 2 ml 0.05 M phosphate buffer (pH 7.6)
	:	12) Layer on a 0-30% Cesium sulphate linear density gradient
	:	13) Centrifuge at 190,000 x *g*, 1 hr at 4 C
	:	14) Fractionate gradient, collect virus containing fractions;
	:	15) Centrifuge at 100,000 x g, 90 min at 4 C
Supernatant	:	16) Pellet; resuspended in 200 µl 0.01 M phosphate buffer (pH 7.6)
	:	17) Dialyse in l/2 strength phosphate buffer (pH 7.6)

and tubules (T) separated in cesium sulphate linear gradients. The antisera were designated: AC (capillovirus), AL (long flexuous particles) and AT (tubules). These antisera were used for the detection of CRSV-I in ELISA (4,14) and ISEM (7). Serological relationship among CRSV-I components was done in ISEM tests. The individual components associated with CRSV-I were also tested in ISEM with the antisera of CRSV (K. S. Derrick), apple chlorotic leafspot (J. Dunez) and CTV (S. M. Garnsey; ACPV) garlic mosaic, henbane mosaic, papaya ringspot, PVY, PVX, TMV (ACPV), garlic latent (D. E. Lesemann), shallot latent (L. Bos), and sweet potato feathery mottle (J. W. Moyer).

RESULTS

Transmission and host range. Mosambi sweet orange plants inoculated by side grafting from CRSV-I glasshouse isolate showed typical symptoms of the disease 2-4 months after inoculation (Fig.1A, B). CRSV-I was also transmitted by dodder to Mosambi sweet orange and Kinnow mandarin. The host range and symptoms on various citrus and hybrids are listed in Table 2. However, CRSV-I could not be transmitted to any of the herbaceous hosts tested. It was neither transmitted by four aphid species nor by seed or soil.

Purification. The procedure mentioned in Table 1 provided maximum yield of virions as compared to other methods. Three bands containing different types of particles were separated after cesium sulphate gradient centrifugation. The major band contained particles with the morphology of capilloviruses designated as (C) while long flexuous particles (L) and tubules (T) were observed in the other two bands (Fig. 2) with modal length of 640 x 15 nm, 690 x 9 nm and 2250 x 40 nm, respectively. The spectrum of purified C and L particles had maximum absorbance at 260 nm and minimum at 250 nm with a small peak at 280 nm, which is the characteristic of a nucleoprotein. The A 260/ 280 ratios were 1.216 and 1.197 respectively. Preparation of purified tubules had maximum absorbance at 275 nm and minimum at 255 which is characteristic of proteins. The A 260/ 280 ratio was 0.932.

Undigested and DNase treated nucleic acid preparations from C particles showed a distinct nucleic acid band upon agarose gel electrophoresis whereas, no band was observed in preparations treated with RNase. RNA content of C particles was estimated as 6%. Particles C and T contained a single protein with an estimated molecular weight of 29 kDa (Fig. 3).

Serology. The optimum conditions determined for ELISA were: 1:20 (w/v) extract of symptomatic tissue in PBST, pH 7.4, 1 μg/ml purified IgG in carbonate buffer, pH 9.6, a 1:1000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) and 0.6 mg/ml of the substrate (p-nitrophenyl phosphate) diluted in diethanolamine substrate buffer. The three types of particles reacted in indirect PTA-ELISA and also in ISEM with the homologous and heterologous antisera prepared. CRSV-I components did not react in ISEM with any of the other antisera assayed including that obtained to CRSV from Florida.

Distribution and incidence of CRSV. The incidence of the disease was recorded on the basis of ringspot symptoms on old leaves or by indexing symptomless field trees in ELISA. Results of the survey are summarised in Table 3. It is evident that CRSV-I is distributed in five states of North India with an incidence up to 100%.

DISCUSSION AND CONCLUSION

During the last few years, there have been various reports on the causal agent(s) of citrus psorosis and ringspot diseases but it is not clear whether the two diseases are caused by one or more different disease agents. The relationship of the two diseases have been discussed by some workers (9,12,20,21). A common feature emerging from these and other reports (8,10,16,19) is that the most ringspot isolates are associated to

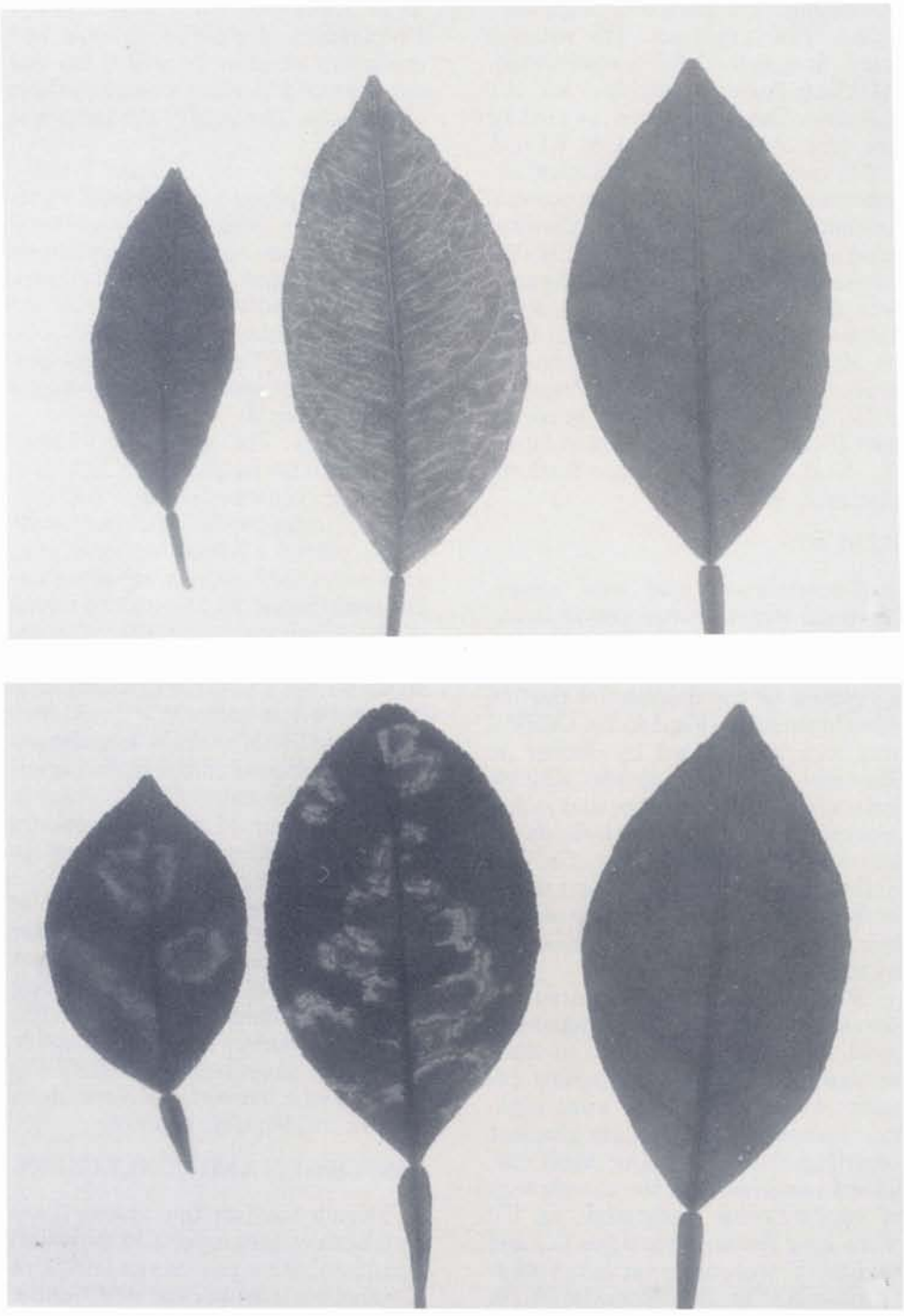

Fig. 1. Leaf symptoms induced by CRSV-I on sweet orange. A) young leaf symptom (top), B) ring pattern on mature leaves (bottom). The leaves on the right are from healthy plants.

a two component ssRNA virus that is mechanically transmissible and produces local lesions on inoculated leaves of *Chenopodium quinoa*. Most of these isolates produce bark scaling in field trees. The CRSV-I isolates collected in India are not mechanically transmissible to *C. quinoa* and produce no bark

TABLE 2
HOST RANGE AND SYMPTOMS INDUCED BY A CRSV-I ISOLATE

Cultivar	No. of plants infected out of ten inoculated	Symptoms[z]
Calamondin	7	CS, RS
Darjeeling orange	9	CS
Etrog citron	4	CS
Grapefruit	6	LI
Citrus indica	5	CS
Kagzi kalan (a selection of acid lime)	8	CS
Kagzi lime (Key lime)	7	LF, CS
Kinnow mandarin	9	CS, RS
Lemon	6	LI
Malta sweet orange	10	SR, LF, CS, RS
Mosambi sweet orange	10	SR, LF, CS, RS
Nagpur mandarin	7	CS
Pummelo	6	LI
Rough lemon	5	LI
Satgudi sweet orange	8	SR, LF, CS, RS
Sour orange	7	VB
Sweet lime	3	LI

[z]SR-shock reaction; LF-leaf flecking; VB-vein banding; CS-chlorotic spots; RS-ringspots; LI-latent infection.

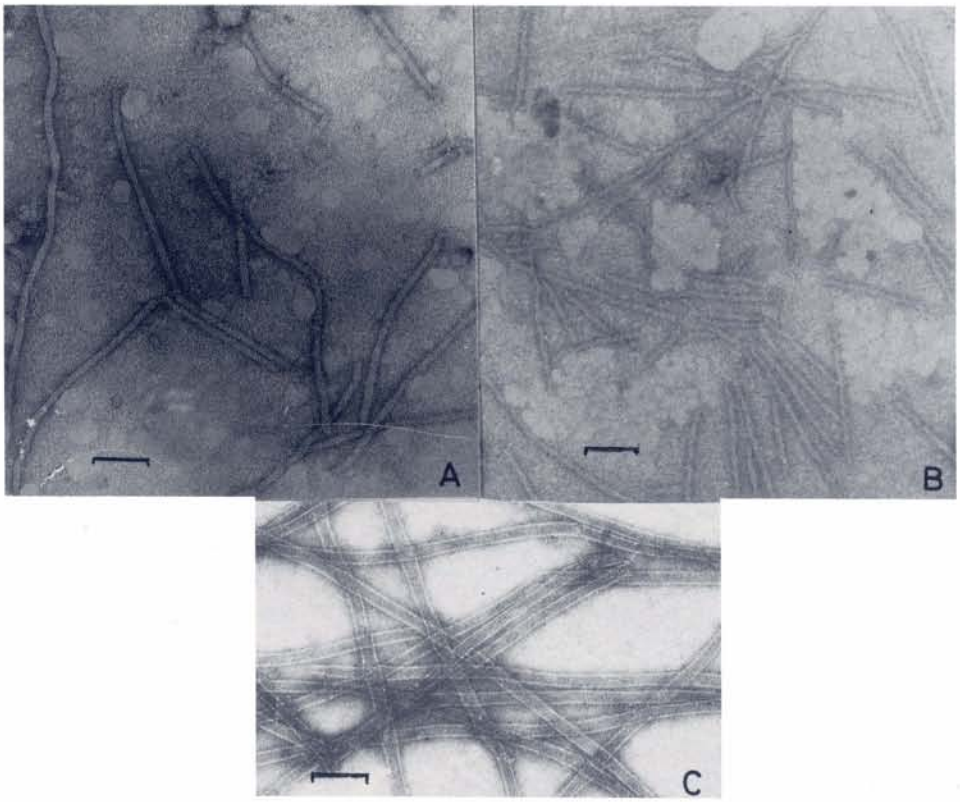

Fig. 2. The particles associated with Indian ringspot disease of citrus: A) Capillovirus-like particles; B) Long filamentous particles; C) Tubules. Bar = 100nm.

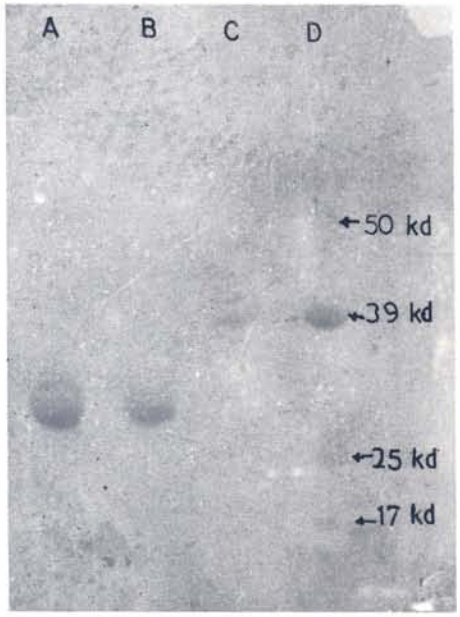

Fig. 3. Polyacrylamide gel electrophoresis (12% acrylamide in resolving gel) of protein preparations from tubules (lane A) and C particles (lane B). Lanes C and D: size markers. Capsid protein of Henbane mosaic virus (40kDa) (lane C) and ovalbumin (50kDa), carbonic anhydrase (39kDa), soybean trypsin inhibitor (25kDa) and lysozyme (17kDa) (lane D). The gel was stained with coomassie blue R-250.

scaling on field infected or glasshouse inoculated plants.

Derrick *et al.* (8) reported the presence of two types of flexuous particles (short and long) containing ssRNA in preparations from plants inoculated with citrus ringspot and both types were required for infectivity on *C. quinoa*. Similar results have also been reported from a Spanish isolate of citrus ringspot (17). Levy and Gumpf (18) showed the association of flexuous particles, 660-665 x 12 nm (possibly a carlavirus) to a mechanically transmissible psorosis isolate. The CRSV-I isolates were associated with three components of variable size. The size of C particles (640 x 15 nm) is somewhat similar to the one reported by Levy and Gumpf (18) but its helical morphology seems to be different from carlaviruses and is entirely different from spiral form particles reported elsewhere (8,17). The other type of particles (L) remains to be characterised. The presence of tubules (T) in high concentration in CRSV-I infected plants has not been previously reported. The absence of mechanical and insect transmission of CRSV-I makes it difficult to establish if all the three components are required for infectivity.

TABLE 3
INCIDENCE OF CRSV IN DIFFERENT PARTS OF INDIA

Place	No. of orchards surveyed	Cultivar	No. of trees infected/tested in ELISA	Incidence (%)
PUNJAB				
Bhatinda	2	Kinnow mandarin	10/50	20
	2	Malta	35/50	70
Ludhiana	1	Kinnow	25/33	75
	1	Kagzi Kalan	72/95	76
Abohar	1	Malta	2/20	10
RAJASTHAN				
Ganganagar	3	Kinnow	45/150	30
	2	Malta	0/50	00
HARYANA				
Hasanpur	1	Kinnow	6/6	100
	1	Malta	6/6	100
Karnal	1	Kagzi Kalan	25/150	17

TABLE 3 (CONTINUED)
INCIDENCE OF CRSV IN DIFFERENT PARTS OF INDIA

Place	No. of orchards surveyed	Cultivar	No. of trees infected/tested in ELISA	Incidence (%)
UTTAR PRADESH				
Modipuram	1	Kagzi Kalan	20/198	10
DELHI				
IARI	1	Kinnow	25/25	100
	1	Kinnow	123/330	37
	1	Mosambi	0/25	00

Particles C and T contained a 29-kDa protein. A 48-kDa protein was found associated to different CRSV isolates (5,6,8,16). Garcia *et al.* (10) found a 50-kDa protein associated to an Argentine psorosis isolate. Navas-Castillo *et al.* (16) reported the presence of a 38-and 48-kDa protein associated with a ringspot isolate. The presence of 29-kDa protein associated to CRSV-I and the absence of a 48-kDa protein clearly differentiates this disease from the other ringspot diseases.

Based on particle size and the presence of a 29-kDa protein the CRSV-I might be close to psorosis isolate P-203-m from California (18) than to other CRSV isolates reported. However, CRSV-I differs from P-203-m in its inability to infect herbaceous hosts including *C. quinoa*, particle morphology and presence of tubules. It is, therefore, concluded that CRSV-I is not related to any of these viruses and is a new citrus virus different from other CRSV isolates. Antisera prepared against individual components cross reacted to all components, suggesting the presence of similar epitopes in three components. Particle L and tubules T, therefore could be a degradation product of the C particles associated with the Indian citrus ringspot disease.

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