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Characterisation of a geminivirus infecting cassava in Sri Lanka

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Abstract

A geminivirus, related to Indian cassava mosaic virus (ICMV) was detected in fifteen samples of cassava collected from different areas of the island. The virus was transmitted by sap inoculation to herbaceous hosts and graft inoculation to cassava. The isolates tested showed a narrow host range. The virus was detected in many varities of ***Manihot esculenta*** and ceara rubber (wild cassava) by enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). The virus was purified from systemically infected ***Nicotiana benthamiana*** leaves. Geminate particles of 18-20x 30 nm in size were observed by electron microscopy. An atiserum was prepared and the titre was found to be 1/32 as determined by gel double diffusion. This polyclonal antiserum detected geminiviruses of ***Acalypha*** mosaic disease and ***Ageratum*** yellow vein disease but not the geminivirus of ***Hibiscus*** yellow vein disease in indirect ELISA.

Key words : ***Acalypha, Ageratum,*** **geminiviruses,** ***Hibiscus,*** **Indian cassava mosaic virus**

1. Introduction

Cassava is reported to be infected by several viruses belonging to potyvirus, geminivirus, and potexvirus groups (Allan *et al.,* 1990). The Indian cassava mosaic virus (ICMV), belonging to begomovirus genus is a member of whitefly transmitted geminivirus which causes a serious yield loss of cassava in India (Mathew and Muniyappa 1992). Two other closely

related viruses, African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) are the causes of a severe mosaic diseases of cassava grown in Africa (Zhou *et al.,* 1997). All three viruses are transmitted by whitefly ***Bemisia tabaci.*** Although the occurrence of an isolate related to ICMV in diseased cassava in Sri Lanka has been reported by Harrison *et al.,* in 1990, there are no details available on the disease. A survey carried out in 1997, evidenced the sporadic occurrence of a mosaic disease in wild and cultivated varieties of cassava in Sri Lanka (Bandumala and Salim, unpiublished results). The work reported here describes the isolation, identification and characterisation of a geminivirus infecting cassava mosaic disease in Sri Lanka.

2. Matrials and methods

Virus isolates

Isolates were collected form cassava showing virus like symptoms during a survery carried out between January to August in 1997. Cuttings were taken from diseased cassava plants (variety not known), and propagated in plastic pots of 30 cm diameter. Same isolates were also maintained in mechanically inoculated ***N. benthamiana*** in a greenhouse at 28±3^{0}C. Unless otherwise stated, the isolates designated as CV1 and CV2 were chosen for experiments as those were contrasting in their symptom expression under greenhouse conditions.

Sap transmission and host range

Young cassava leaves with clear virus-like symptoms were ground with a pestle and mortar using cold phosphate buffer (1ml/g leaf material) at concentration of 0.01 M with EDTA and sodium sulphite (1% and 0.1% respectively). The crude extracts were inoculated to 'healthy' cassava and seedlings of 15 species of test hosts, previously dusted with carborundum and kept in an insect-protected glasshouse for symptom development. Five plants from each host were inoculated and observed for symptoms for about five weeks. Infection was confirmed by TAS (tripple antibody sandwich) ELISA (Muniappa *et al.,* 1991) test.

Graft transmission :

Infected scions collected from diseased plants were wedge grafted to healthy 'stock' plants growing in pots (30 cm in diameter) and maintained in the greenhouse at 28±3^{0}C until a union is established. Newly formed lateral shoots were examined for characteristic symptoms. Wild cassava, MU51, Kirikavadi and CARI 555 were evaluated for their susceptibility to the virus.

Polymerase Chain Reaction (PCR)

Total DNA was prepared from cassava leaves as described by Method A of Harrison *et al.,* (1997). The two degenerate primers used were exactly similar to those described by Hong *et. al.,* (1993). The sequences of these primers have been derived from highly conserved regions in published sequences of WTG (whitefly-transmitted geminiviruses) genome (Stanley and Gay 1983). The PCR was done as described by Harrison *et al.,* (1997).

Purification

Systemically infected ***N. bethamiana*** leaves, harvested 35 days after inoculation were used. At first, a method described by Sequeira and Harrison (1982) to purify ACMV was employed but the yield was found to be very low. Therefore a method described by Muniappa *et al.,* (1991) to purify tomato leaf curl geminivirus was followed with some modifications to purify the CV2 isolate. The preparation was examined in electron microscope (EM) after differential centrifugation, and found to be in good quality to produce antiserum. Therefore, the sucrose gradient step was omitted.

Preparation of antiserum

After a per-immune bleed, a white New Zealand rabbit was injected subcutaneously with 0.5ml virus preparation mixed with an equal volume of Fruend's complete adjuvant. Further two intra-muscular injections were given 2 and 3 weeks after first injection respectively, mixed with an equal volume of Fruend's incomplete adjuvant. The rabbit was bled three times at weekly intervals starting the third week after first injection. Serum containing antibodies was collected and stoted at - 20^{0}C after adding 50% glycerol and 0.02% sodium azide.

Serological tests TAS ELISA was used to detect virus in the samples collected from field as described by Muniappa *et al.,* (1991). The wells were coated with gamman globulin from polyclonal antiserum to ACMV (Thomas *et al.,* 1986). The monoclonal antibody used was SCR 60, prepared against an ICMV Indian islates (Aiton and Harrison, 1989). The polyclonal antiserum prepared for Sri Lankan isolate was tested by both the standard gel double-diffusion test and by the indirect PTA ("plate trapped antigen"/"anigen coated") ELISA test using antibodies from two different animal species (Bar-Josaph and Malkinson, 1980).

Electron microsocopy Crude sap and virus preparations of various stages at the purification was examined under EM after staining with a negative stain, 2% urinyl acetate.

3. Results

Thirteen isolates showing virus-like symptoms and two isolates which did not show any symptoms of virus infection, collected form the field showed positive results for geminivirus in TAS ELISA test. All these isolates were from wet zone and produced severe mosaic symptoms when grafted to cassava varieties MU 51, Kirikavadi and CARI 555 and to wild cassava. All isolates were somewhat similar in their symptom expression except one, which produced general yellowing symptoms accompanied by severe leaf curl in grafted plants under greenhouse conditions. This isolate designated as CV2 (from Narahenpita) and another isolate designated as CV1 (from Maharagama) were selected for host range studies.

Host range and symptomatology Both CV1 and CV 2 had a narrow host range that limited to Euphobiaceae and Solanaceae (Table 1). Both the isolates have same host range but considerably differed in symptom expression in ***N. benthamiana.*** In this host CVI showed severe mottle, leaf curling and dark green blisters on leaves but no general yellowing of the leaves which was characteristic for CV2. It took 4-5 weeks of inoculation to produce symptoms in all those species which were positive for virus infection. However, ***N. occidentalis***, the only sp. in which local lesions were produced, the chlorotic lesions (3-4 mm in dimeter) appeared in 6 days of inoculation. However, systemic symptoms were porduced only after about 3 weeks of inoculation (Plates 1-4).

In contrast, graft-inoculated cassava plants developed symptoms in a relatively shorter time as 7-8 days of inoculation. All cassava varities and the wild cassava, ***M. glaziovii*** produced mosaic and severe leaf curl symptoms. However, none of the cassava varieties were infected when sap from infected cassava or ***N. benthamiana*** was inoculated mechanically.

Table 1. Host range of ICMV isolates, CV1 and CV 2

Host species	Symptoms
1. ***Nicotiana glutinosa***	S, M, Lc
2. ***N.occidentalis***	L, S, M
3. ***N. debneyi***	S,M, Lc
4. ***N. benthamiana***	S,M, Lc,* Y
5. ***N. tabacum*** cv. "Xanthy"	0
6. cv. "White Burley"	0
7. cv. "Samsun'	0
8. ***N. clevelandii***	S, M, Lc, Y
9. ***Chenopodium quinoa***	0
10. ***C. amaranticolor***	0
11. ***C. murale***	0
12. ***Cucurbita sativus***	0
13. ***Cucurbita maxima***	0
14. ***Phaseolus vulgaris***	0
15. ***Vigna unguiculata***	0
16. ***Pisum sativum***	0
17. ***Ricinus communis***	0
18. ***Manihot esculentus*** var. MU 51**	S, M, Lc, B*Y
19. var. CARI 5555**	"
20. var. "kirikavadi'**	"
21. ***Manihot glaziovii*****	"

L = local lesions Lc = leaf curling and puckering
S = systemic symptoms Y= yellowing
B = dark green blisters * = only by CV 2
** = graft inoculation 0 = no infection
M = mosaic

1-11 = seeds were obtained from Horticulture Research International/UK
12-16 = from commercial packets (variety not known)
17&21 = collected from a private garden.
18-20 = from Regional Agricultural Research & Development Centre/ Makandura

TAS ELISA detected the presence of geminivirus in all the test host that became infected by mechanical/graft inoculation. Further confirmation of the virus identity of only CV2 was done at the Scottish Crop Research Institute (SCRI), Scotland, using panels of monoclonal antibodies produced for ACMV and ICMV. The CV2 reacted with SCR 18, 20, 52, 53, 54, 55, 60 and 66 produced against ICMV Indian isolate, but not with SCR 14, 16, 17, 21, 25, 27, 29, 32 produced against ACMV. This epitope profile is typical for ICMV except for no reaction with SCR 62 and therefore, it confirmed that CV2 is not an isolate of ACMV but an siolate related to ICMV.

PCR. Geminivirus specific PCR product was always obtained for CV1 isolate but no product was obtained for CV2 isolate in more than 15 trials but in only one occasion, gave a faint product. (Plates 5a and 5b).

Purification. Partially purified preparations of CV2, after precipitated with PEG contained particles of 20x30 nm and shape characteristic of geminiviruses. Particles were also seen in the preparations after one cycle of differential centrifugation (Plate 6). However, when the clarified suspension was layered on sucrose gradients which were then centrifuged, no virus specific band could be seen, but virus particles were recovered form throughout the top part of the gradient. The partially purified preparation after differential centrifugation was found to be clean enough under EM (Plate 6) to produce antiserum, therefore it was used directly to immunise the rabbit.

Serology : In gel double diffusion test, the polyclonal antiserum had a titre of 1/32 (Plate 7). No precipitin bands were produced when crude leaf sap of cassava was used. Only the partially purified sap from infected **N. *benthamiana*** gave precipitin lines in gel diffusion test. PTA ELISA readily detected virus in both the crude sap and partially purified sap from **N. *benthamiana*** but not in the crude sap from cassava (Table2). Geminiviruses infecting ***Ageratum conyzoydes*** and ***Acalypha indica*** also were readily detected in PTA-ELISA by the polyclonal antiseum produced against CV 2 but not the geminvirus infecting okra ***(Hibiscus esculentus)***.

Table 2. Detection of geminivirus by Indirect PTA ELISA using the ploYclonal antiserum to CV2.

Source/isolate	Sample no.	A_{405} average values	
		Healthy	infected
Cassava/CVI	1	0.148	0.267
	2	0.189	0318
	3	0.179	0.297
	4	0. 178	0.308
	5	0.168	0.326
Cassvava/CV2	1	0.148	0.226
	2	0.189	0.224
	3	0.179	0.294
	4	0.178	0.206
	5	0.168	0.216
N. benthamiana/CVI	1	0.035	0.162
	2	0.048	0.165
	3	0.039	0.187
	4	0.056	0.196
	5	0.033	0.165
N.benthamiana/CV2	1	0.035	0.142
	2	0.048	0.167
	3	0.039	0.129
	4	0.056	0.196
	5	0.033	0.155
****N. benthamiana***/CV 2	1	0.035	>3
	2	0.048	>3
	3	0.039	>3
	4	0.056	>3
	5	0.033	>3

Dilution of crude sap antigen = 1/10

Period of plate incubation = 30 min at room temperature

Antiserum dilution = 1/1000

* Partially purified sap (dilution 1/32000) as antigen and 'healthy' crude sap as negative control

Value greater than twice the healthy control was considered as positive.

Table 3. Comparison of TAS ELISA and PTA ELISA methods in detecting geminivirus infecting cassava using Mab, SCR 60

Source and isolate	A405 average vaalues			
	PTA ELISA		TAS ELISA	
	H	I	H	I
Cassava/CVI	0.062	0.722	0.012	>3
	0.064	0.666	0.014	>3
	0.038	0.701	0.024	>3
	0.038	0.711	0.024	>3
	0.019	0.909	0.025	>3
Cassava/CV2	0.062	0.169	0.012	0.081
	0.064	0.134	0.014	0.108
	0.038	0.189	0.024	0.092
	0.038	0.188	0.024	0.102
	0.019	0.189	0.025	0.088
N. benthamiana /CV2	0.010	>3	0.071	>3
	0.012	>3	0.074	>3
	0.011	>3	0.064	>3
	0.010	>3	0.071	>3
	0.010	>3	0.081	>3

Dilution of crude sap antigen = 1/10

Period of plate incubation= 1h at room temperature, over night at 4^0C for PTA ELISA, 15 min at room temperature for TAS ELISA

Mab dilution = 1/100 for both types of ELISA

Gama globulin dilution (For TAS ELISA) =1/1000

Value greater than twice the healthy control was considered as positive.

4. Discussion

A geminivirus was detected readily by TAS ELISA in all 13 samples of cassava showing mosaic symptoms in the field. The Mabs used in this study (SCR 60) has been produced against an isolate of ICMV. However, a

preliminary study using an antiserum to squash leaf curl virus also detected the geminivirus in all the 13 cassava samples tested by PTA ELISA. As serological relationships are common among geminiviruses, the identity of virus can not be confirmed by polyclonal antibodies used in indirect ELISA tests. The Mabs have proved valuable for the identification and separation of geminiviruses (Muniappa *et al.,* 1991). In this study only CV2 was checked for epitope profile and found it is closely related to ICMV.

TAS ELISA was very satisfactory in the detection of geminivirus isolates infecting cassava in Sri Lanka. In comparison, the PCR readily detected only CV 1 among the two contrasitng isolates tested in the present study. The geminivirus general primers used here seems to amplify part of the CVI genome but not that of CV2. This suggests that although the two isolates are serologically related they are distinguished variants. This was also reflected in symptoms induced by these two isolates in graft-inoculated cassava and in ***N. benthamiana*** under greenhouse conditions. An Indian isolate of ICMV has infected ***N. tabacum*** var. White Burley, Xanthi and Samsung (Mathew and Muniappa, 1992). In contrast, none of these varieties of tobacco were infecte by both the isolates CVI and CV2. However, in further study of molecular characterisation, it was found that CV2 is a distinct virus although it is serologically related to ICMV, and therefore, suggested to name it as Sri Lankan cassava mosaic virus (SLCMV, Keith *et al.,* unpublished results).

Two apparently healthy plants from the field which were also positive for geminivirus in TAS ELISA could have been freshly infected so that it was too soon to observe any visible symptoms. Even under greenhouse conditions it was noticed that all hosts in which the symptoms developed took about 4-5 weeks of inoculation to do so. Such plants without showing symptoms in the field however, create serious problems as these could be easily overlooked by the farmers when collecting stakes for propagation.

There is no generally applicable method for purifying geminiviruses. Sequeira and Harrison (1982) have used Tris-HCI buffer to extract ACMV whereas Bock *et al.,* (1978) used borate buffer for the same. Malathi *et al.,* (1989) were succeeded in purification of ICMV using the method described by Sequiera and Harrison (1982). However, we could not get a good yield using this method in the present sutdy. We found that the particles were unstable in Tris-HCI buffer. The method described by Muniappa *et al.,* (1991) using citrate buffer was very satisfactory in purifying CV2 which gave a good quality product of reatively high yield. This suggests that best procedure for purifying geminiviruses varies with diffierent isolates/strains.

Gel diffusion was not successful in detecting the geminivirus isolates in crude extracts from infected cassava or ***N.benthamiana*** probably due to low concentration of virus in the extracts. Similar results have been reported by Mathew and Muniappa (1992) for their isolates. Only the partially purified preparation from infected ***N. benthamiana*** produced precipitin bands in gel diffusion tests.

The polyclonal antiserum produced against CV2 detected the Sri Lankan isolates of geminivirus in PTA ELISA in the extracts of infected ***N. benthamiana, N. debneyi, N. occidentalis*** *and* ***N. clevelandii*** but not in the infected sap of cassava. Further, the polyclonal antibodies were also reacted in PTA-ELISA with leaf extracts form plants infected with geminiviruses of ***Acalypha*** mosaic and ***Ageratum*** yellow vein but not with that of ***Hibiscus*** yellow vein. These observations suggest either the latter is not serologically related to geminivirus of cassava or the PTA ELISA is not suiitable to detect the geminivirus infecting ***Hibiscus esculentus*** as in the case of cassava.

However, the polyclonal antiserum to ICMV used in DAS ELISA have detected the virus in crude sap from infected cassava (Malathi *et al.*, 1989 and Mathew and Muniappa, 1991). Therefore, it is important proceeding with the polyclonal antiserum to CV2, to establish a DAS ELISA test, as indirect PTA ELISA was not suitable to detect geminivirus in the extrcts from infected cassava.

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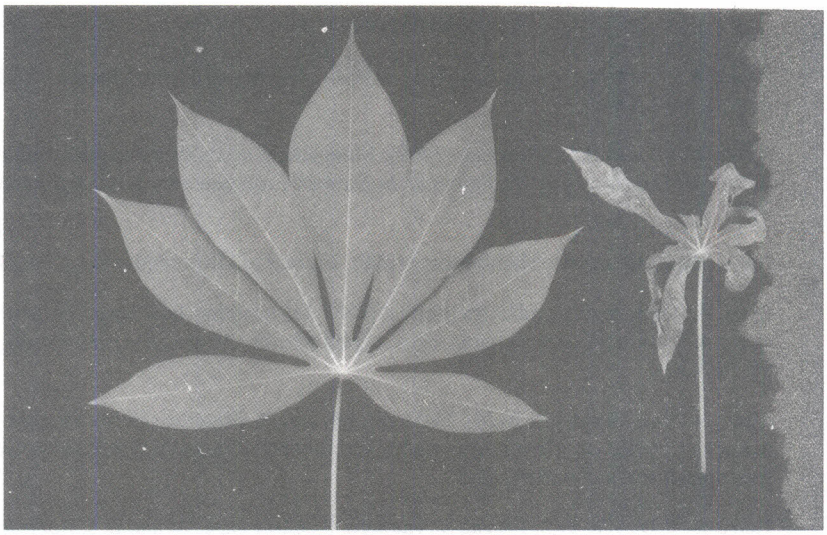

Plate 1. Cassava leaves infected with CVI showing mosaic and leaf curl symptoms (right); healthy leaf (left)

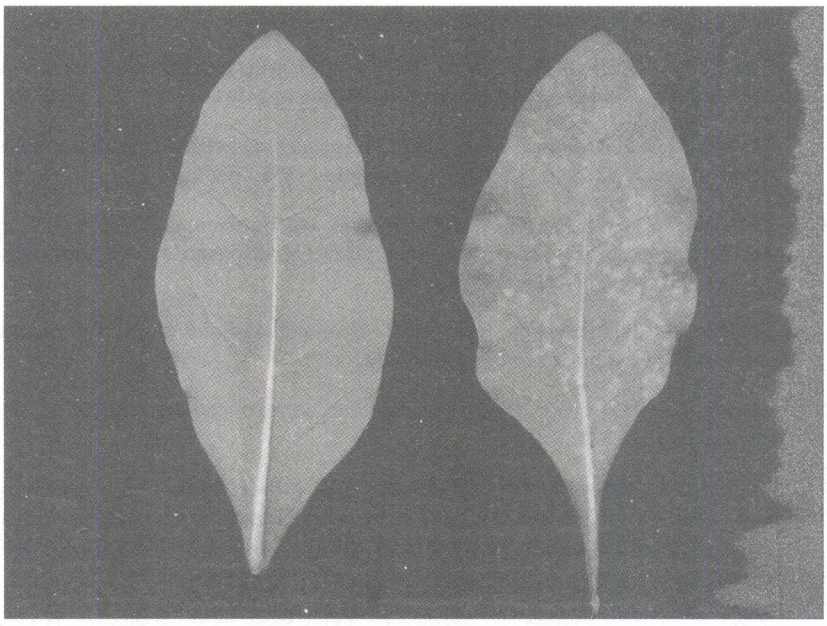

Plate 2. *N. occidentalis* infected with CV2 showing local chlorotic lesions (right) and healthy (left)

Plate 3 ***N.clevelandii*** infected with CV2 showing mosaic, leaf curl and stunting symptoms (right); healthy (left)

Plate 4. *N. debneyi* infected with CV2 showing severe leaf curl and mosaic symptoms

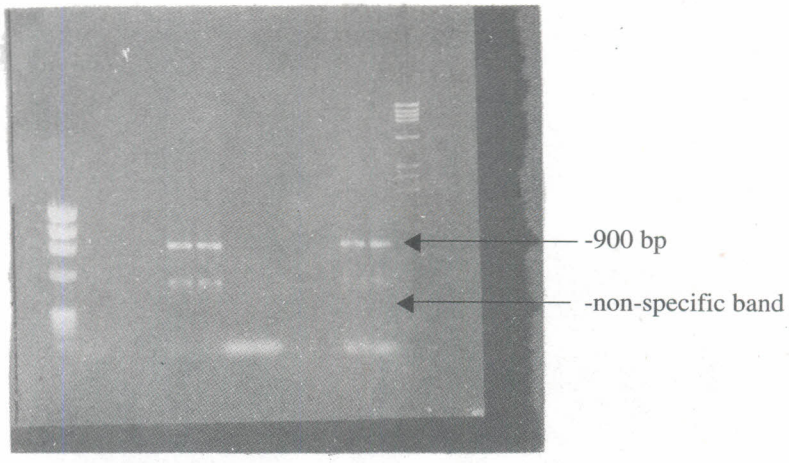

Plate 5 (a) PCR amplification usnig general primers of geminivirus

Lane 1	-	Marker 1
Lanes 5, 6,11 & 12	-	CVI
Lanes 2, 9, & 10	-	Control (water)
Lanes 7&8	-	CV 2
Lanes 3&4	-	Control ('healthy' sample)
Lane 13	-	Marker 2

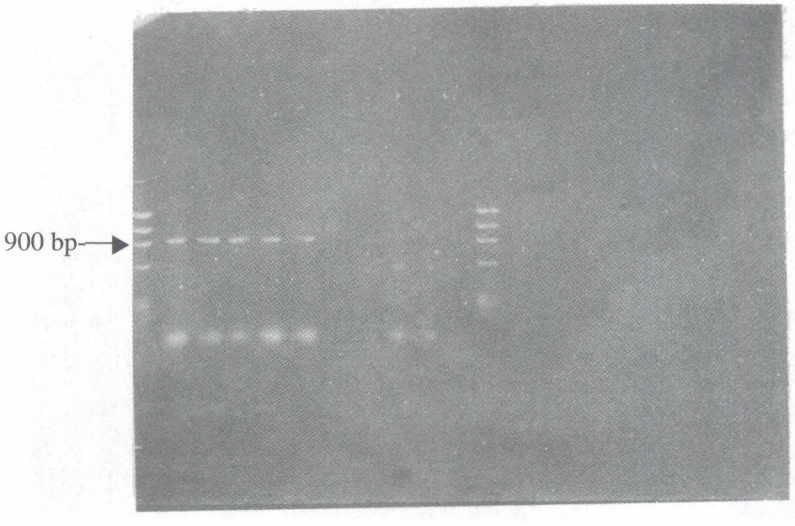

Plate 5 (b). CV 2 showing faint product (Lanes 1 & 12 - Marker, Lanes 2 - 6 CVI, Lanes 7-10 - CV2, Lane 11- healthy control).

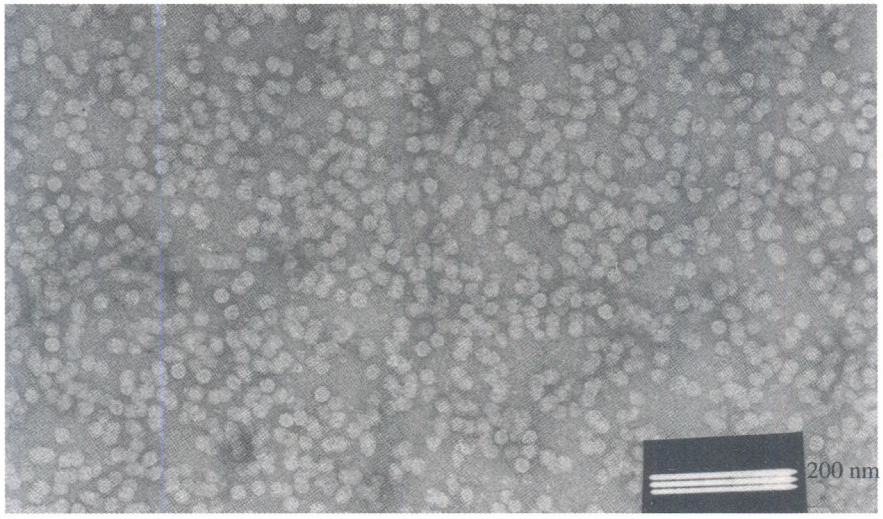

Plate 6. Electron micrograph showing geminate particles in partially purified preparation of CV 2.

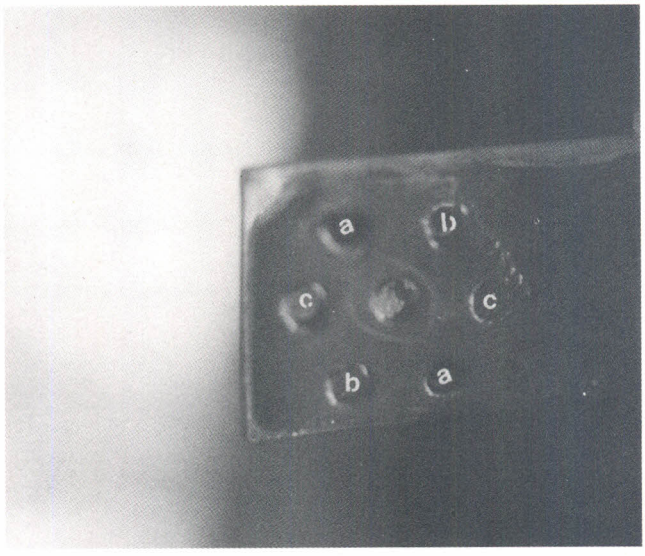

Plate 7. Gel - double diffusion (Centre well, polyclonal antiseum to CV 2, a, heaalthy control, b & c, partially purified CV 2 from *N. benthamiana*