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Limited movement of *Cucumber mosaic virus* (CMV) in yellow passion flower in Brazil

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Symptoms of *Cucumber mosaic virus* (CMV) on yellow passion flower (*Passiflora edulis* f. *flavicarpa*) are characterized by bright yellow mottling on leaves, starting at random points on the vine and diminishing in intensity towards the tip, which becomes symptomless as it grows. To determine whether symptomless portions of vines are CMV-free or represent latent infection, leaves with and without symptoms were collected from infected vines in the field. Biological, serological (plate-trapped antigen enzyme-linked immunosorbent assay, PTA-ELISA), Western blot and dot-blot hybridization assays showed that portions of the vines without symptoms were CMV-free. Vegetatively propagated vines with symptoms showed remission of symptoms on newly developed leaves. One year later, no CMV was detected in the upper leaves of these plants. Mechanically inoculated passion flower seedlings behaved similarly; symptoms were shown by few leaves after inoculation. Afterwards, plants became symptomless and CMV was not detected in the upper leaves or root system, 40 or 85 days after inoculation. The mechanism responsible for remission of symptoms accompanied by CMV disappearance is not known.

Keywords: dot-blot hybridization, PCR, *P. edulis* f. *flavicarpa*, PTA-ELISA, Western blot

Introduction

Cucumber mosaic virus (CMV) is the type member of the *Cucumovirus* genus. It is distributed worldwide and has the largest host range of all plant viruses, causing many diseases in vegetable, fruit and ornamental crops, with severe economic losses. The virus consists of isometric particles containing a single-stranded positive-sense RNA genome of three RNA segments (Palukaitis *et al.*, 1992). RNA-1 and -2 encode proteins 1a and 2a, respectively, which are necessary for viral RNA replication in a single cell (Nitta *et al.*, 1988). RNA-2 also encodes 2b protein, which is suggested to be involved in host-specific long-distance movement of the virus (Ding *et al.*, 1995b). RNA-3 encodes two proteins, 3a and the coat protein (CP). The 3a protein is translated directly from RNA-3, whereas CP is translated from subgenomic RNA-4 and has a molecular weight of 26 000 (Palukaitis *et al.*, 1992). Both proteins are necessary for cell-to-cell movement, whereas CP has also been implicated in long-distance movement of the virus (Ding *et al.*, 1995a; Taliansky & Garcia-Arenal, 1995; Raybov *et al.*, 1999). The virus is naturally transmitted by several species of aphids in a nonpersistent way (Palukaitis *et al.*, 1992).

The occurrence of CMV infection on passion flower (*Passiflora edulis* f. *flavicarpa*) plants was apparently first reported by Magee (1948) in New South Wales, Australia. Since then, the isolation of CMV from *Passiflora* species has been reported in California (Teakle *et al.*, 1963), Japan (Yonaha *et al.*, 1979), Taiwan (Chang *et al.*, 1981) and Brazil (Chagas *et al.*, 1984; Colariccio *et al.*, 1987). In Australia, CMV was considered to be one of the causal viruses of woodiness disease of passion fruit (Taylor & Kimble, 1964). Pares *et al.* (1985) also suggested that tip necrosis of passion fruit was associated with dual infection with CMV and *Passion fruit woodiness virus* (PWV), of the genus *Potyvirus* and family *Potyviridae*.

A recent survey of virus incidence on passion flower in the north-east region of São Paulo showed that the most prevalent and severe virus is PWV, which was found infecting 71·8% of the samples tested. CMV was detected in 40·7% of the samples, followed by an as yet uncharacterized rhabdovirus-like particle causing vein clearing disease (Gioria *et al.*, 2000). In spite of CMV being frequently found in passion flower orchards in São Paulo State, Brazil, since the early 1980s, with high incidence in some areas (Gioria *et al.*, 2000), there is no report of infection being associated with significant detrimental effects on plant development or fruit yield and

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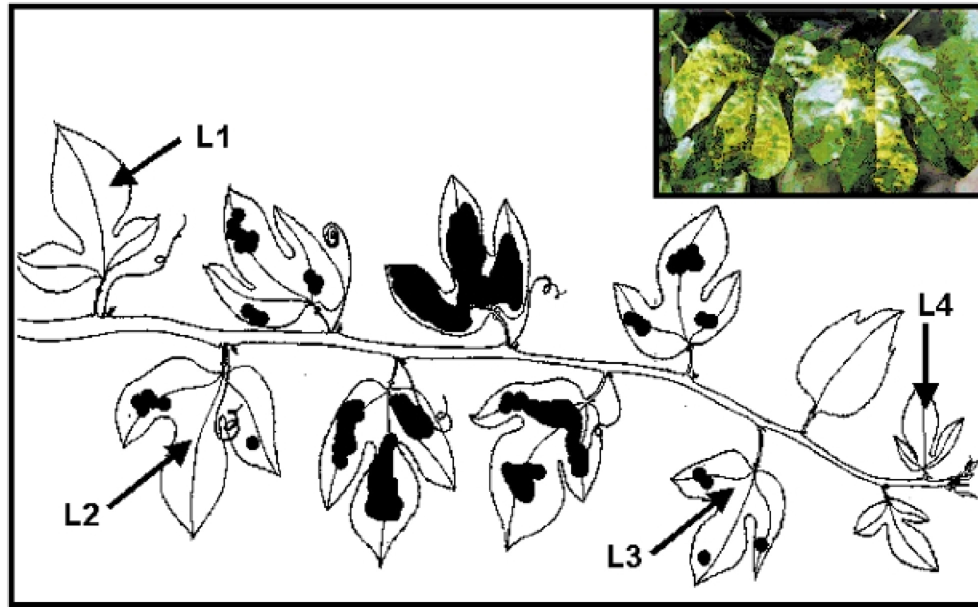

Figure 1 The pattern of symptoms caused by *Cucumber mosaic virus* (CMV) on passion flower vine, showing leaves without (L1 and L4) and with (L2 and L3) symptoms, from which samples were taken for studies. Inset: symptoms of CMV on passion flower leaves.

quality. This is apparently due to the peculiarity of disease development shown by infected plants, in which symptoms caused by CMV are always restricted to small portions of the vines, which are mainly characterized by bright yellow mottling, starting at random points on the vine and diminishing in intensity towards the tip, which becomes symptomless as it grows (Fig. 1). No leaf malformation or woody fruits have been found associated with CMV-infected vines. These observations prompted the current study to determine whether symptomless portions of infected vines are CMV-free or represent latent infections.

Materials and methods

Survey and sampling

Forty-five passion flower orchards, representing nine counties, were randomly chosen in the north-east region of São Paulo, where more than 500 ha are under cultivation with this species. These orchards were visually inspected for the presence of plants showing symptoms characteristic of CMV infection, as described above and shown in Fig. 1. A total of 126 samples, consisting of portions of vines showing symptoms, were collected and kept in plastic bags for further virus isolation, diagnosis and evaluation of the distribution of the virus.

Test plants and environmental conditions

Passion flower, *Chenopodium quinoa*, *Nicotiana tabacum* cv. Turkish and *Cucurbita pepo* cv. Caserta plants were maintained under glasshouse conditions (temperature 18–38°C; photoperiod 12–14 h) for transmission tests.

Virus isolation

Sap from naturally infected passion flower vines was prepared by grinding infected leaves in 10 × volume (w/v) of 0·02 M phosphate buffer containing 0·02 M sodium sulphite, pH 7. This sap was then rubbed on *C. quinoa* (a local lesion host of CMV), dusted with carborundum. A CMV isolate obtained after single local lesion transfer on *C. quinoa* was maintained in *N. tabacum* cv. Turkish.

Serological procedures

Cucumber mosaic virus was purified from infected leaves of *N. tabacum* cv. Turkish following the procedure described by Roossinck & White (1998). Antibodies were raised in a New Zealand white female rabbit by four weekly intramuscular injections, 100 μg each, of intact purified CMV, emulsified with incomplete Freund's adjuvant. The antiserum was used in PTA-ELISA and Western blot assays.

Plate trapped antigen enzyme linked immunosorbent assay (PTA-ELISA) was carried out in microtitre plates (Corning Glass, Corning, NY 14831, USA) coated with sap extracted from plant tissue prepared in 50 mM carbonate buffer, pH 9·6, and incubated at 37°C for 2 h. Antiserum against CMV, diluted 1:1000 in tris-HCl, pH 7·2, was added to the plates and incubated for 2 h at 37°C. Goat-antirabbit IgG conjugated to alkaline phosphatase (Sigma A-3687) was diluted in tris-HCl buffer and incubated for 2 h at 37°C. Substrate reactions (ρ-nitrophenyl phosphate; Sigma N-9389, Sigma, St Louis, MO, USA) at 1 mg mL^{-1} diluted in 10% diethanolamine, pH 9·8 developed at room temperature, and absorbance values (at 405 nm) were measured in a Σ960 ELISA reader

(Metertech, Taipei, Taiwan). A reading was considered positive when its absorbance value exceeded three times the average of the healthy control.

Western blot analysis was carried out as described by Van Regenmortel & Dubs (1993). Total protein was extracted from leaf samples in TE buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred to nitrocellulose membrane and analysed with antiserum against CMV, diluted 1:1000.

Dot-blot hybridization

A cDNA probe was prepared by random incorporation of digoxigenin (DIG-dUTP; Boehringer Mannheim, Indianapolis, IN, USA) using RNA isolated from purified CMV as a template and reverse transcriptase (Superscript II Rnase H; Gibco BRL, Carlsbad, CA, USA). Total RNA was extracted from passion flower samples by the single-step method (Chomczynski & Sacchi, 1987) using the commercial product TRIzol as recommended by the manufacturer (Gibco BRL). One microlitre of each RNA was dotted onto Hybond-N (Amersham Pharmacia Biotech, Piscataway, NJ, USA), air-dried, UV cross-linked and hybridized with the CMV probe. After washing, the hybridization signal was detected by chemiluminescence using CDP-Star™ substrate (Boehringer Mannheim). Films were exposed to the membranes for 10–30 min at 37°C.

PCR analysis

The reverse transcriptase reaction and PCR amplification were performed essentially as described by Wylie *et al.* (1993). The primers 5′-TATGATAAGAAGCTTGTT-TCGCG-3′ (sense) and 5′-GCCGTAAGCTGGAT-GGACAA-3′ (antisense) amplify a DNA fragment of 482–487 bp for subgroup I of CMV, depending on the virus strain. The PCR products were separated on a 1·5% agarose gel in TAE buffer (Sambrook *et al.*, 1989) and stained with ethidium bromide (1 μg mL^{-1}) for 15 min.

Detection of CMV in field-infected vines

Twenty-five vines showing characteristic symptoms of CMV infection, and which tested positive on PTA-ELISA, were divided into four portions as shown in Fig. 1. Portion 1 was represented by one leaf collected from the oldest part of the vine, with no apparent symptoms, just before the first leaf with symptoms; portions 2 and 3 were represented by leaves showing symptoms, while portion 4 was taken from the tip of the vine, where remission of the symptoms had occurred. All four samples from each vine were individually cut into small pieces, homogenized and divided into three aliquots to be tested for the presence of CMV by biological assay, Western blot and dot-blot hybridization. From five vines, additional samples were also taken from the stem at the point of insertion of the petiole of each sampled leaf. These samples were tested for the presence of CMV by biological assay only.

Biological detection of the virus was carried out by mechanical inoculation on test plants of *N. tabacum* cv. Turkish or *C. pepo* cv. Caserta under glasshouse conditions. Inoculation was carried out as previously described. CMV infection was based on symptom expression followed by PTA-ELISA with samples from all inoculated test plants.

Transmission tests of CMV to passion flower seedlings

Mechanical transmission tests were carried out using leaves from CMV-infected *N. tabacum*. The leaves were ground in phosphate buffer and inoculation performed by application to the cotyledons of 10- to 15-day-old-passion flower seedlings. Seven days after inoculation, separate samples were taken from inoculated leaves of all plants and stored at −20°C. Plants were kept in the glasshouse for 40 days, when samples were collected from the upper leaves and root system of each plant, separately. In a second transmission test, samples were collected 85 days after inoculation. All samples were tested for the presence of CMV by means of Western blot analysis and PCR.

Detection of CMV in vegetatively propagated infected vines

Twenty passion flower vines exhibiting symptoms of CMV, and which tested positive for virus infection by PTA-ELISA, were cut into pieces containing two to three axillary buds and placed in pots containing substrate mix, under glasshouse conditions. Rooted vines were allowed to develop further and analysed for the presence of CMV based on symptom expression, biological assay and PTA-ELISA on newly grown leaves. CMV monitoring was performed periodically for 12 months, after plants had established and started new growth. At the end of this period, a final CMV detection test was carried out on new leaves and the root system, using dot-blot hybridization.

Results

Distribution of CMV in field-infected vines

All 126 vines collected in the field with characteristic symptoms of CMV tested positive for the virus by PTA-ELISA. Twenty-five of the 126 vines were tested for the presence of CMV on leaves with and without symptoms by biological assay, Western blot and dot-blot hybridization analyses. Results of these tests are summarized in Table 1. Western blot analysis detected the 26-kDa coat protein of CMV only in the leaves showing symptoms from all 25 infected vines (Fig. 2a). Nineteen of the 25 vines were also tested for virus infectivity, through inoculation of *N. tabacum* or *C. pepo*. Only test plants inoculated with extracts from leaves with symptoms developed systemic mosaic patterning. Infection with CMV was confirmed by PTA-ELISA for all inoculated test plants. Plants inoculated with extracts from symptomless leaves did not show any symptoms and tested negative on

Table 1 Detection of *Cucumber mosaic virus* (CMV) on leaves with and without symptoms from infected vines by biological assay, Western blot and dot-blot hybridization analyses

	No. of tested vines/No. CMV-positive			
Assay	L1	L2	L3	L4
Biological (indicator host)	19/0	19/19	19/19	19/0
Western blot	25/0	25/25	25/25	25/0
Dot-blot hybridization	25/0	25/25	25/25	25/0

Leaf position on vine: L1, leaf collected from the oldest part of the vine, with no apparent symptoms, just before the first leaf with symptoms; L2 and L3, leaves with symptoms; L4, leaves taken from the tip of the vine, where remission of the symptoms had occurred.

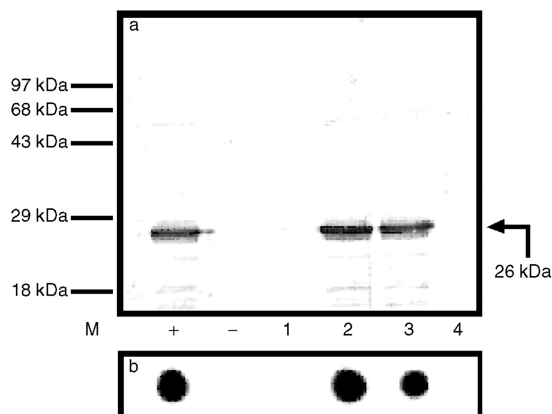

Figure 2 Western immunoblot (a) and dot-hybridization (b) detection of *Cucumber mosaic virus* in leaves with (lanes 2 and 3) and without (lanes 1 and 4) symptoms from one infected vine. Positive (+) and negative (–) controls were included. Lane M, protein molecular weight marker (M).

PTA-ELISA. Furthermore, all 25 vines were also analysed by dot-blot hybridization with a nonradioactive cDNA probe specific for CMV. A signal was only obtained after hybridization with RNA extracts from leaves with symptoms (Fig. 2b).

Five vines out of the 25 were also tested by biological assay for the presence of CMV in the stem at the points of insertion of the petioles of leaves with and without symptoms (Table 2). Only stem extracts from the insertion points of leaves with symptoms produced local lesions on *C. pepo* cv. Caserta, followed by systemic mosaic patterning. CMV infection on *C. pepo* was confirmed by PTA-ELISA.

Transmission and distribution of CMV in inoculated seedlings

Results of two transmission tests of CMV to passion flower seedlings and evaluation of virus distribution in inoculated leaves and new leaves and root systems are shown in Table 3. All 18 seedlings inoculated at the cotyledonary stage developed mild vein clearing on the inoculated and first two true leaves, 7 days after inoculation. Western blot analysis labelled a protein of approximately 26 kDa in all samples from inoculated leaves, corresponding to the coat protein of the virus (Fig. 3). For the second test, PCR analysis amplified a fragment of the expected size using primers specific for RNA-4 of CMV, confirming the presence of the virus in inoculated leaves showing symptoms (Fig. 4). As test plants developed in the glasshouse, new leaves were symptomless. Western blot and PCR analyses carried out with extracts from new leaves and root systems from all plants, 40 or 85 days after inoculation, were negative (Figs 3 and 4).

Table 2 Number of local lesions produced on cotyledonary leaves of *Cucurbita pepo* cv. Caserta inoculated with extracts from leaves with and without symptoms and stems at the points of insertion of the sampled leaves

	Number of local lesions				
Source of inoculum	Stem 1	Stem 2	Stem 3	Stem 4	Stem 5
L1	0	0	0	0	0
S1	0	0	0	0	0
L2	77	70	105	90	128
S2	27	89	14	10	30
L3	77	80	105	90	83
S3	30	7	6	12	14
L4	0	0	0	0	0
S4	0	0	0	0	0

Leaf position on vine: L1, leaf collected from the oldest part of the vine, with no apparent symptoms, just before the first leaf with symptoms; L2 and L3, leaves with symptoms; L4, leaves taken from the tip of the vine, where remission of the symptoms had occurred. S1, S2, S3 and S4, the corresponding portions of stem where the petioles of L1, L2, L3 and L4, respectively, were inserted.

Table 3 Detection of *Cucumber mosaic virus* (CMV) on mechanically inoculated leaves, upper leaves and roots of passion flower test plants by means of Western blot and PCR analyses

Test number	Plant tissue analysed	Days after inoculation	No. of tested plants/ No. CMV-positive: Western blot	PCR
I	Inoculated leaves	7	9/9	NT
	Upper leaves	40	9/0	NT
	Roots	40	9/0	NT
II	Inoculated leaves	7	9/9	9/9
	Upper leaves	85	9/0	9/0
	Roots	85	9/0	9/0

NT, not tested.

Perpetuation of CMV in vegetatively propagated infected vines

More than 100 portions of vines showing symptoms caused by CMV were placed in substrate mix for rooting. For unknown reasons, the majority died. Only six portions

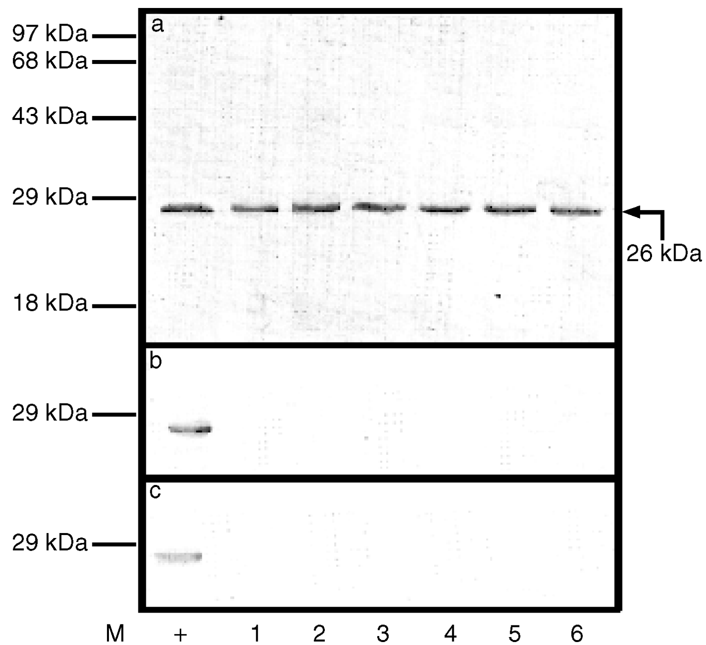

Figure 3 Western immunoblot detection of the coat protein of *Cucumber mosaic virus* in (a) inoculated leaves with symptoms from six seedlings (lanes 1–6) of passion flower, (b) upper leaves and (c) root systems from the same seedlings, collected 40 days after inoculation. A protein molecular weight marker (M) and positive (+) control were included.

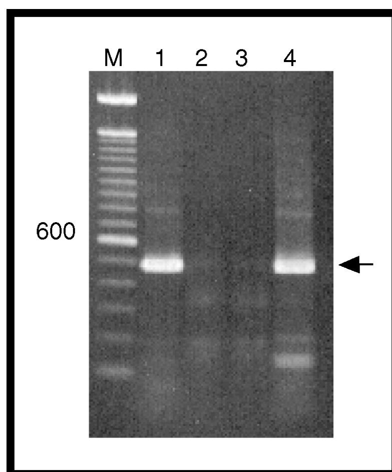

Figure 4 A representative PCR detection of *Cucumber mosaic virus* (CMV) in an inoculated leaf with symptoms (lane 1), an upper leaf (lane 2) and the root system (lane 3) from one inoculated passion flower seedling. Lane 4, positive control using CMV genomic RNA as a template. The arrow indicates the product of amplification. M is the molecular weight marker.

rooted and developed into new plants, which showed characteristic symptoms of CMV infection. The presence of CMV on leaves with symptoms on these plants was confirmed by PTA-ELISA. As plant development continued, all the leaves with symptoms fell off. Three months after plants were well established, with vines approximately 150 cm long, CMV was no longer detected on the top leaves by biological assay or Western blot analysis. The plants remained symptomless and were re-evaluated when they were 1 year old. CMV was not detected by PTA-ELISA on new leaves. Also, dot-blot hybridization failed to detect the virus in new leaves and root systems of these plants (data not shown).

Discussion

The symptoms caused by CMV infection in *P. edulis* f. *flavicarpa* are very characteristic in commercial orchards in São Paulo, Brazil (Colariccio *et al.*, 1987; Gioria *et al.*, 2000), as well as in other states where this species is grown (Barbosa *et al.*, 1999). Infected plants always display a bright yellow mottling limited to a few leaves, as shown in Fig. 1. The same pattern of symptoms was also apparently observed by Taylor & Kimble (1964) in CMV-infected passion flower in Australia. The limited symptoms shown by this virus–host combination prompted the current investigation into the accumulation of the virus in different parts of field-infected vines and mechanically inoculated seedlings.

Biological, Western blot and dot-blot hybridization analyses on CMV accumulation in leaves with and without symptoms, from field-infected vines, gave similar results. The virus was only detected in leaves with symptoms or portions of the stem corresponding to the insertion points of the petioles of these leaves. No CMV was detected in symptomless portions of the vines (Table 1). Based on the number of local lesions produced by extracts from leaves and stems of infected vines on *C. pepo* cv. Caserta, it is suggested that CMV accumulation in leaves with symptoms was higher than in the stem (Table 3). Furthermore, Western blot analysis on CMV accumulation within leaves with symptoms showed that the virus was unevenly distributed (data not shown).

Mechanical transmission tests of CMV to passion flower seedlings was highly effective, even though difficulties in transmitting this virus to seedlings of this species

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were reported by Teakle *et al*. (1963) and Colariccio *et al*. (1987). In the present work, all inoculated plants showed symptoms 7 days after inoculation and CMV accumulated in the inoculated leaves, as detected by Western blot and PCR analyses (Table 3, Figs 3 and 4). The symptoms persisted for approximately 15 days, but then they disappeared and the plants recovered completely, and no further symptoms were observed during the next 40–85 days. At the end of this period, no CMV was detected in the upper leaves or root systems of these plants. The same pattern of remission of symptoms was reported by Taylor & Kimble (1964) and Colariccio *et al*. (1987) after CMV inoculation of passion flower seedlings.

Vegetatively propagated vines which tested positive for CMV infection at the time of harvesting also showed remission of symptoms on new growth in the glasshouse. With the exception of one plant that was reinfected with CMV, the others remained symptomless for more then a year and tested negative for CMV by PTA-ELISA. Reinfected plants showed characteristic symptoms of CMV infection and afterwards exhibited the same pattern of remission of symptoms shown by other plants.

These results suggest that after infecting the plants, CMV is able to move from cell to cell and establish systemic movement through a couple of leaves in infected seedlings and through a few leaves in field-infected vines. After that, the virus–host relationship is dramatically altered, further systemic movement stops and the virus is no longer detected in the plant.

Three hypotheses were raised to explain this phenomenon. The first involves a change in the virus, which gradually loses the ability to replicate and/or move systemically in the plant. As all CMV isolated from field-infected passion flower plants caused systemic infection in *C. pepo* cv. Caserta or *N. tabacum* cv. Turkish, which continued to show symptoms until discarded, the virus did not appear to change in those hosts. The second hypothesis involves a resistance mechanism in the plant, which is induced as infection progresses. Yazawa *et al*. (1996) reported that *Capsicum annuum* cv. Af-5 plants inoculated with CMV produced symptomless vigorous lateral shoots about 2 years after infection. These newly developed lateral shoots were virus-free and resistant to infection with CMV, in spite of a high concentration of CMV in the infected shoots. The resistance mechanism involved in this virus–host relationship is still unclear. For the passion flower–CMV combination, no apparent resistance was observed in the new growth of recovered vines. In a very few instances, CMV infection on two distant parts of the same vine was observed under field conditions, suggesting that the recovered vine was still susceptible to infection. Also, one out of six vegetatively propagated vines, grown in the glasshouse, was reinfected with CMV, but remission of symptoms, followed by complete disappearance of the virus, was later observed. The third hypothesis involves some sort of inhibitory activity in CMV-infected passion flower vines, which progressively impairs virus replication and consequently diminishes its movement throughout the vines. The presence of a virus inhibitor has been suggested in several virus–host combinations. Loebenstein *et al*. (1977) suggested that green areas of mosaic-affected leaves of CMV-inoculated tobacco plants were virus-free and resistant to reinoculation with CMV due to the presence of a virus inhibitor. However, considering that CMV replicates in the inoculated leaves, and often in just one or two newly formed leaves, the inhibitor may be affecting the 2b protein and/or the coat protein, which may be involved in host-specific long-distance movement of the virus (Ding *et al*., 1995a,b; Taliansky & Garcia-Arenal, 1995; Raybov *et al*., 1999).

Whatever mechanism is involved in the limited movement of CMV in infected passion flower vines, this apparent genetic trait should be maintained in any breeding programme for this species. Any change in this trait which would allow the virus complete and permanent invasion of passion flower plants would certainly turn CMV into a very destructive pathogen for this crop. Systemic mosaic disease caused by CMV has been reported on *P. caerulea* and *P. alato-caerulea*, which are mainly grown as ornamentals in California (Teakle *et al*., 1963). Additional studies are necessary for better understanding of this phenomenon on CMV-infected passion flower.

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