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Characterization of Cucumber Mosaic Virus (CMV) Causing Mosaic Symptom on *Catharanthus roseus* (L.) G. Don in Malaysia

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ABSTRACT

A cucumber mosaic virus (CMV) isolate, causing leaf mosaic and distortion, malformed flowers or colour-breaking on the petals of *Catharanthus roseus* in Serdang, Selangor, Malaysia, was identified and designated as Malaysian periwinkle isolate (CMV-MP). The virus was spherical in shape with the size of 28.6 ± 0.48 nm in diameter with a central core. It was mechanically transmitted to various test plants which produced typical symptoms of CMV infection. The coat protein (CP) gene of the virus was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned in *Escherichia coli* using TOPO-TA vector. A single open reading frame of 657 nucleotides, potentially encoding for 218 amino acids was sequenced. A comparison with the CP genes of other CMV isolates indicated that CMV-MP shared 100% sequence homology to the CP gene sequence of *C. roseus* isolate of CMV in India. This is the first aetiology report on *C. roseus* in Malaysia showing natural mosaic disease symptoms supported with the nucleotide sequence analysis of the causal virus.

Keywords: ***Catharanthus roseus*, cucumber mosaic virus, mosaic disease, nucleotide sequence analysis, coat protein gene**

INTRODUCTION

Catharanthus roseus (L.) G. Don or periwinkle, which is also known as 'kemunting cina' in Malaysia is widely used as an ornamental plant to decorate gardens and landscapes. The plant is also famous for its medicinal properties, particularly as anti-cancer (Manganey *et al*., 1979; Svoboda, 1983; Cragg & Newman, 2005), anti-diabetic (Ghosh & Gupta, 1980; Chattopadhyay *et al*., 1991; Singh *et al*., 2001; Wiart, 2002) and antihypertensive remedies (Van de Heijden *et al*., 2004). Two important *Catharanthus* alkaloids, namely vinblastine and vincristine, have been developed into cancer chemotherapy agents since 1960s and also marketed as vinblastine sulphate (Velbe®) and vincristine sulphate (Oncovin®) (Van de Heijden *et al*., 2004). In Malaysia, *C. roseus* has long been used in traditional medicine and one of the popular and potential medicinal plants for both cultivation and conservation (Loh, 2008; Musa *et al*., 2009).

As a medicinal plant, tremendous research efforts have been given to study the bioactive compounds of *C. roseus* compared to its phytopathological aspect. Due to very little

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emphasis on the diseases of the plant, very limited records of virus infections are available. Among other, Espinha and Gaspar (1997) reported cucumber mosaic virus (CMV) infection in *C. roseus,* showing mild mosaic, chlorosis and plant distortion. Meanwhile, tomato spotted wilt virus (TSWV) has also been reported in *C. roseus* with black spots, systemic mosaic, leaf deformation and browning of larger leafs at the bottom part of the plant (Chatzivassiliou & Livieratos, 2000). Samad *et al*. (2008) recently reported the natural infection of *C. roseus* with an isolate of CMV in India.

In Malaysia, CMV has been reported to be present in many important economic crops (Mohamad Roff & Anang, 1989; Sidek & Sako, 1996; El-Sanousi, 1997) and weeds (Sidek *et al*., 1999). Although CMV infection on *C. roseus* has been mentioned elsewhere (Ong & Ting, 1977; Inon *et al*., 1999), information associated with the viral disease and its characterization has not reported. This paper describes the morphology, symptom and molecular characterization of CMV as the causal agent of mosaic disease on *C. roseus*.

MATERIALS AND METHODS

DAS-ELISA

Leaf extracts from the diseased and symptomless *C. roseus* plants growing under natural conditions in Serdang, Selangor were tested by DAS-ELISA as described by Clark and Adams (1977), following the procedure recommended in the diagnostic kit by the manufacturer (Bioreba, Switzerland). Antisera against cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV) and impatien necrotic spot virus (INSV) were used in the assays to determine the presence of CMV, TMV, TSWV and INV. Briefly, each well was initially coated with 200 µl anti-virus IgG diluted in coating buffer (20 µl anti-virus IgG in 20 ml coating buffer). Plates were covered tightly and placed in humid boxes and incubated at 30ºC for 4 h. The contents of the wells were discarded and washed 3-4 times with washing buffer. The plates were blotted on paper towels to remove any liquid residue. The leafs were homogenized in an extraction buffer (0.05 g tissue in 1 ml buffer). Two hundred µl of the crude sap was loaded into each well and the plates were incubated at 4ºC overnight. The plates werethen subjected to washing before the addition of 200 µl enzyme conjugates (20 µl enzyme conjugate in 20 ml buffer) to each well. After incubation at 30ºC for 5 h, the plates were washed and loaded with substrate (p-nitrophenyl phosphate at 1 mg/ml in substrate buffer). The plates were incubated at room temperature in the dark. The ELISA reactions were read at 405 nm by using an ELISA reader (Thermolab System, USA) after 30-120 min incubation. All the samples were tested in duplicates and the average A_{405} values of more than twice compared to that of the healthy controls were considered as positive for virus detection.

Virus Isolate and Maintenance

C. roseus var. rosea plant, which exhibited mosaic symptoms and showed positive CMV detection in DAS-ELISA, was used as the source of virus isolate. The symptomatic leafs were ground in chilled phosphate buffer (0.01 M phosphate, pH 7.0, containing 0.25% DIECA) and carborundum (600 mesh) and the extract was rubbed on the leafs of healthy *Chenopodium amaranticolor* to obtain pure virus culture through three serial single-lesion transfers and the inoculum was maintained on *C. roseus*, *Nicotiana tabacum* and *N. glutinosa* for subsequent studies.

Virus Purification

The virus was isolated and purified from the primary leafs of the inoculated *N. tabacum* cv. Coker 254 harvested 15-20 days post-inoculation and kept at -80ºC prior to purification. The procedures of Scott (1963) were used with major modifications. Briefly, 100 g of infected leaf tissues were homogenized in 0.5 M sodium citrate buffer (pH 7.5) containing 0.005 M EDTA and 0.5% thioglycolic acid and filtered with 2 layers of muslin cloth. The filtrate was mixed with an equal volume of chloroform, stirred for 40 min before it was centrifuged at 9500x *g* for 10 min at 4ºC. The aqueous phase was collected and mixed with 10 % polyethylene glycol (PEG 6000). The mixture was then centrifuged at 8000x *g* for 20 min and the pellet was resuspended in 10 ml of 0.005 M sodium borate buffer (pH 9.0). The suspension was centrifuged at 9500x *g* to collect supernatant, followed by centrifugation at 139,000x *g* for 3 h at 4ºC to obtain the pellet. Suspension of the pellet in 2 ml of borate buffer was centrifuged at 9500x *g* for 15 min and the supernatant was layered onto a 10-50% sucrose density gradient in 0.5 M sodium citrate buffer prior to centrifugation at 185,000x *g* for 3 h. The virus band was collected and pelleted through high speed centrifugation at 139,000x *g* for 2 h and resuspended in 2 ml of borate buffer. The virus preparation was analyzed spectrophotometrically and the UV spectra values of A_{260}:A_{280} and A_{max}:A_{min} were determined. The virus yield per 100 g leaf tissues was calculated by assuming the extinction coefficient at 260 nm for CMV to be 5 (Francki *et al*., 1979). The purified virions were used for symptomatological studies of the test plants, morphological determination, virion RNA extraction and RT-PCR.

Morphological Determination of the Virions

A formvar-carbon coated copper grid was floated on a drop of purified virus for 5 min and subsequently stained with 2% uranyl acetate adjusted to pH4.2. The grids were examined under a transmission electron microscope (Phillips HMG 4000). The mean virion diameter was determined from the measurements of 138 virus particles at a magnification of 100,000x.

Symptomatological Studies

Four different plant families, grown in pots under insect proof condition, were mechanically inoculated with the purified virus at 1.2-1.5 mg/ml: *Solanaceae* (*N. tabacum* cv. White Burley, *N. glutinosa*, *N. benthamiana*, *Datura metel*, *Capsicum annuum* cv. MC11, *Lycopersicon esculentum* cv. MT1), *Chenopodiaceae* (*C. amaranticolor*), *Leguminosae* (*Vigna sesquipedalis* cv. MKP5, *Phaseolus vulgaris* cv. MKB1) and *Cucurbitaceae* (*Cucumis sativus* cv. local). Five plants were inoculated for each species and kept under observation for 2 months. Both the symptomatic and symptomless plants were recorded and checked for the presence of virus by back inoculation onto *C. amaranticolor* and *C. roseus*. The CMV infection on the test plants were confirmed by DAS-ELISA using the CMV antiserum.

Viral RNA Extraction and RT-PCR

Viral RNA was isolated from the purified virus using proteinase K and phenol-SDS procedures, as described by Sambrook *et al.* (1989). The virus was incubated at 50ºC for 20 min with an equal volume of RNA extraction buffer (0.02M Tris-Cl, pH 7.4, 0.03M KCl, 3 mM $MgCl_2$, 0.01M SDS) and 50 µl of proteinase K (2 mg/ml). After the addition of 80 µl of 1 M NaCl, the mixture was subjected to 2 times phenol extractions (50ºC) and 3 times chloroform: isoamylalcohol (24:1) extractions, and this was followed by the precipitation of the RNA with 3 M NaAc (pH 5.2) and cold 100% ethanol. The RNA was air-dried prior to resuspension in TE buffer (pH 8). Meanwhile, RT-PCR was performed using degenerate primers which were designed based on the conserved regions of CMV coat protein (CP) genes available in the GenBank. The upstream primer CMVF1 (5'-TAGACAT/ACTGTGACGCGA-3') and the downstream primer CMVR2 (5'-GTAAGCTGGATGGACAAC-3') were designed to amplify a region of about 1000bp in length covering complete CP region of CMV. The synthesis of cDNA was carried out with Reverase™ M-MuLV reverse transcriptase (BIORON, Germany), following the protocol recommended by the manufacturer. One µg of the viral RNA or 2.0 µg virions and 10 pmole of the downstream primer CMVR2 were used for reverse transcription reaction at 42ºC for 90 min. For PCR, 5 µl of the cDNA was used as a template and this was proceeded to PCR using the following conditions: one cycle of denaturation at 94ºC for 3 min, 35 cycles of 94ºC for 1 min; 60ºC for 1 min; 72ºC for 1 min followed by one cycle of elongation at 72ºC for 10 min. The PCR product was electrophoresed on a 2.0% agarose gel in TAE buffer, stained with 0.5 µg/ml ethidium bromide, and viewed on a UV-transilluminator. One hundred bp extended DNA blue ladder (BIORON) was used as a standard marker.

Cloning and Sequencing of the Amplified PCR Product

The amplified product was gel-purified, ligated into T&A cloning vector and transformed into competent *E. coli* cells using TOPO TA Cloning kit (Invitrogen). The recombinant clones were identified using PCR and three clones were selected for sequencing. The obtained sequences were compared with the sequences from the GenBank through the BLAST programme of the National Centre for Biotechnology Information (NCBI) (Altchul *et al*., 1990).

RESULTS AND DISCUSSION

DAS-ELISA

All *C. roseus* plant samples used in the assays at flowering stage. Two kinds of viruses were detected throughout the assays (Table 1). Amongst 100 plant samples of *C. roseus* tested, 25 symptomless plant samples were determined to be positive for TMV, one plant sample with mild mosaic symptoms was CMV positive and one plant sample with severe leaf mosaic and deformed flowers were found positive for both TMV and CMV infections. 73 other samples reacted negatively against all antisera tested. For TSWV and INSV, the absorbance readings of the tested samples were as similar as the healthy controls, suggesting no occurrence of the viruses in the assays.

Table 2 shows the results of rescreening for CMV and TMV in the original diseased *C. roseus* and the inoculated plants. Rescreening on 5 original *C. roseus* plants which had initially detected positive TMV failed to detect the virus. Forty *C. roseus plants* inoculated with the crude leaf extract from the TMV positive plants also exhibited negative reaction against TMV antibody, whereas CMV was consistently detected in the original diseased *C. roseus* as well

TABLE 1
DAS-ELISA detection (absorbance at 405 nm) of viruses in *C. roseus* grown wild or cultivated in pots in Serdang, Selangor.

Total number of sample	Visual observation	Antisera[1]			
		CMV	TMV	TSWV	INSV
25	SL	0.131-0.151	0.339-0.523*	0.137-0.160	0.135-0.156
65	SL	0.114-0.151	0.138-0.310	0.132-0.232	0.132-0.157
1	MM, CB	0.276*	0.150	0.149	0.131
1	SM, DF	0.635*	0.538*	0.123	0.140
8	MM	0.114-0.216	0.170-0.309	0.139-0.175	0.084-0.144
PC	-	3.510	3.131	2.172	3.56
NC	-	0.114	0.165	0.134	0.177
BC	-	0.115	0.165	0.130	0.136
HS	-	0.114	0.156	0.141	0.150

[1]Asterisk (*) indicates positive reactions (greater than 2x negative mean). SL: symptomless; MM: mild mosaic; CB: colour breaking on petals; SM: severe mosaic; DF: deformed flowers; PC: positive control; NC: negative control; BC: buffer control; HS: healthy sap.

TABLE 2
Rescreening assay (absorbance at 405 nm) of CMV and TMV using DAS-ELISA in original diseased *C. roseus* grown under natural conditions and the inoculated plants grown in a glasshouse in Serdang, Selangor.

Total number of sample	Visual observation	Antisera[1]	
		CMV	TMV
7	SM, DF, CB	2.447-3.328*	0.195-0.396
1[a]	MM, CB	3.039*	NT
17[b]	SM, DF	2.447-3.483*	0.173-0.396
5[c]	SL	NT	0.310-0.374
40[d]	SL	NT	0.252-0.422
1[e]	SM, DF	3.102*	0.198
10[f]	SM, DF	2.233-3.324*	0.190-0.235
Positive control		3.076	3.158
Negative control		0.549	0.263
Buffer control		0.202	0.206
Healthy sap		0.222	0.212

[1]Asterisk (*) indicates positive reactions (greater than 2x negative mean). NT: not tested; SL: symptomless; MM: mild mosaic; CB: colour breaking on petals; SM: severe mosaic; DF: deformed flowers.
[a] Original diseased plant detected CMV positive in the first assay.
[b] Plants inoculated with leaf extract from original CMV positive *C. roseus* in the first assay.
[c] Original diseased plants detected TMV positive in the first assay.
[d] Plants inoculated with leaf extract from original TMV positive *C. roseus* in the first assay.
[e] Original diseased plant detected positive of TMV and CMV infection in the first assay.
[f] Plants inoculated with leaf extract from original TMV and CMV positive *C. roseus* in the first assay.

as in the inoculated *C. roseus* plants. For the *C. roseus* plant with mix infection, only CMV was consistently detected during the rescreening of the original plant and the inoculated plants. Very high absorbance readings of CMV detections were observed (>2.0) throughout rescreening DAS-ELISA, and this revealed the occurrence of the virus in very high concentration in the plants. The results of rescreening also confirmed the prevalence of CMV in diseased *C. roseus,* showing mosaic symptoms with malformed flowers or slight colour breaking on the petals. Failure to detect CMV in the plants with mosaic symptoms, as indicated in the first screening, was probably due to very low concentration of the virus, which was below the detection limit of the assays used. Based on the DAS-ELISA results, aetiology studies only focused on CMV. The naturally diseased *C. roseus* exhibited leaf mosaic and distortion, malformed flowers or colour-breaking on the petals (see *Fig. 1*).

Purification of the Virus

The virus was banded as single light-scattering zone at 2.4 cm depth from meniscus in a sucrose density gradient. The purified virus preparation exhibited a typical nucleoprotein absorption spectrum with a maximum and a minimum absorption at 258-260 nm and 240-245 nm, respectively. Meanwhile, the A_{260}:A_{280} and A_{max}:A_{min} ratios were calculated as 1.5 and 1.2, respectively. The values of A_{260}:A_{280} and A_{max} : A_{min} ratios for the purified virus were close to the values reported for other CMV isolates (Noordam, 1973; Srivastava *et al*., 1992; Sarma *et al.*, 2001). The differences in the values may be due to the impurities which present in the purified preparations. The virus concentration as calculated spectrophotometrically using an Extinction coefficient ($E^{0.1\%}$ 1 cm) at 260 nm = 5 (Francki *et al.*, 1979) varied from 1.2 mg/ml to 5.0 mg/ml per 100 g leaf sample. The purified virus preparation at 1.3-1.5 mg/

ml was found infectious when tested on *N. tabacum* and *C. sativus,* as well as on *C. roseus*. The symptoms developed on *C. roseus* were identical to the natural diseased *C. roseus*. The reproduction of the disease by inoculating healthy plants with plant sap and purified virus preparations confirmed the pathogenicity of the virus according to Koch's postulates (Rivers, 1937).

Morphological Determination of Virions

Plenty isometric particles with a central core were observed in negatively stained purified preparations diluted to 0.2 mg/ml (*Fig. 2*). No other virus particles were observed in the preparation. For size determination, the histogram represents the diameter distribution of the virus particles in the purified preparation

Fig. 1: Mosaic symptoms on leafs (A and B), flowers of the deformed shape (C) and slight colour breaking on the petals (D) of naturally-infected C. roseus were detected positive for CMV infection.

TABLE 3
Host range and symptomatology of cucumber mosaic virus isolated from *C. roseus*.

Test plants	Reactions	Days required for for symptom expression
Chenopodiaceae		
Chenopodium amaranticolor	LL	5-7
Cucurbitaceae		
Cucumis sativus	SM	4
Leguminosae		
Phaseolus vulgaris cv. MKB1	Neg.	Neg.
Vigna sesquipedalis cv. MKP5	NLL	3-5
Solanaceae		
Capsicum annuum cv. MC11	Neg.	Neg.
Datura metel	Neg.	Neg.
Lycopersicon esculentum cv MT1	Neg.	Neg.
Nicotiana benthamiana	SM, M, LD	10-13
Nicotiana glutinosa	C, SM	7-10
Nicotiana tabacum cv. White Burley	SM	10-15

SM: systemic mosaic; M: mottling; LD: leaf deformation; C: chlorosis; NLL: necrotic local lesion; LL: local lesions; SL: symptomless; Neg: negative reaction.

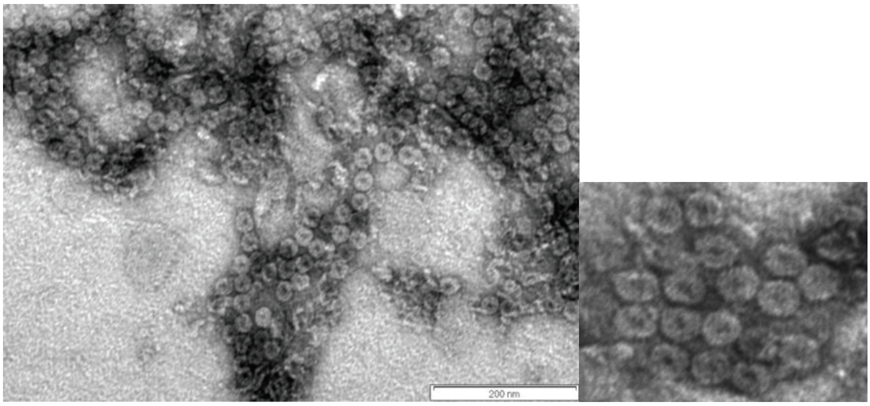

Fig. 2: Electron micrograph of negatively stained virus particles with 2% uranyl acetate. The inserted picture shows the particles. Bar = 200 nm.

TABLE 4
Percentage identity of nucleotide (nt) and predicted amino acid (aa) sequence between Malaysian CMV isolate (GenBank: EU726631) extracted from *C. roseus* and other published CMV isolates.

GenBank Accession number	Natural host	Sub-group	Location	% identities at the level of	
				nt	aa
EU310928	*Catharanthus roseus*	IB	India	100	100
EF593025	*Rauvolfia serpentine*	IB	India	98	99
EF593023	*Amaranthus tricolor*	IB	India	97	96
EF153733	*Chrysanthemum morifolium*	IB	India	97	95
AY545924	*Piper nigrum*	IB	India	95	99
AY965892	*Capsicum* sp	IB	China	93	97
AJ810264	*Cucumis sativus*	IB	Thailand	93	96
DQ070746	*Beta vulgaris*	IB	China	93	95
AM183119	*Lycopersicon esculentum*	IB	Spain	92	95
AY380533	*Chrysanthemum* sp	IA	Brazil	92	95
DQ295914	*Gladiolus* sp	IA	India	91	95
AJ810258	*Cucurbita* sp	IA	USA	91	94
AB109908	*Capsicum annuum*	II	Korea	76	78
EF202597	*Lycopersicon esculentum*	II	China	76	78
AJ242585	*Nicotiana* sp	II	China	76	78
EU642567	*Daucus carota*	II	India	76	76
EF424777	*Catharanthus roseus*	Not known	China	92	97
EF424778	*Catharanthus roseus*	Not known	China	92	97
AY376840	*Catharanthus roseus*	Not known	Brazil	92	95

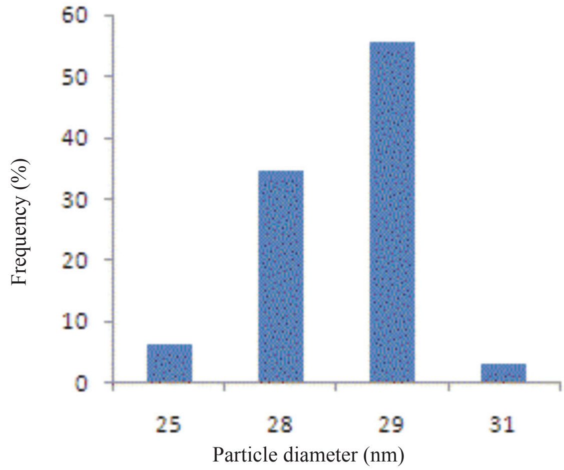

Fig. 3: Histogram showing the particle diameter distribution of C. roseus CMV in purified preparation observed under EM.

(*Fig. 3*) which shows the maximum number of particles with a modal diameter between 28 to 29 nm. The mean diameter of virions, which was determined from the measurements of 138 particles, was found to be 28.6 ± 0.48 nm. Meanwhile, the shape and size of the virions resembled CMV as described by Francki *et al.* (1979).

Symptomatology of the Test Plants

The virus was easily transmitted by mechanical means to selected test plants, while the symptoms induced varied according to plant species (*Fig. 4*). Details of the host range and symptomatology of the virus are given in Table 3. Inoculated *N. tabacum* cv. White Burley showed a systemic mosaic within 10-15 days post-inoculation. The top leafs of the inoculated *N. glutinosa* exhibited chlorosis within 7-10 days of post-inoculation, and this was frequently followed by mosaic symptoms. Systemic mosaic, mottling and leaf deformation were noted on *N. benthamiana* within 10-13 days post-inoculation. Severe leaf mosaic was induced by the virus four days after inoculation on *C. sativus* plants. Local lesions were observed on the inoculated leafs of *V. sesquipedalis* cv. MKP5 and *C. amaranticolor* within 3-5 days and 5-7 days post-inoculation, respectively, suggesting the plants as local lesion hosts for the virus. On the contrary, no symptom was observed on *C. annum* cv. MC11, *L. esculentum* cv. MT1, *D. metel* and *P. vulgaris* cv. MKB1. When back inoculated to the healthy seedlings of *C. roseus,* the leaf extracts of the symptomatic test plants produced similar mosaic symptoms as the natural diseased host. Back inoculation to *C. amaranticolor* from the test plants showed only susceptible hosts reproduced the symptoms on *C. amaranticolor.* Positive reactions with CMV antiserum in DAS-ELISA confirmed the CMV infection in the symptomatic test plants. The symptoms induced by the virus on the susceptible test plants were identical to those induced by a number of CMV isolates (El-Sanousi *et al*., 1997; Madhubala *et al*., 2005), even though no symptom was exhibited on *D. metel, L. esculentum* cv. MT1*, C. annuum* cv. MC11 and *P. vulgaris* cv. MKB1. Factors such as temperature, age of the test plants and the source of the virus inoculum may also greatly influence the symptomatology of the test plants studied.

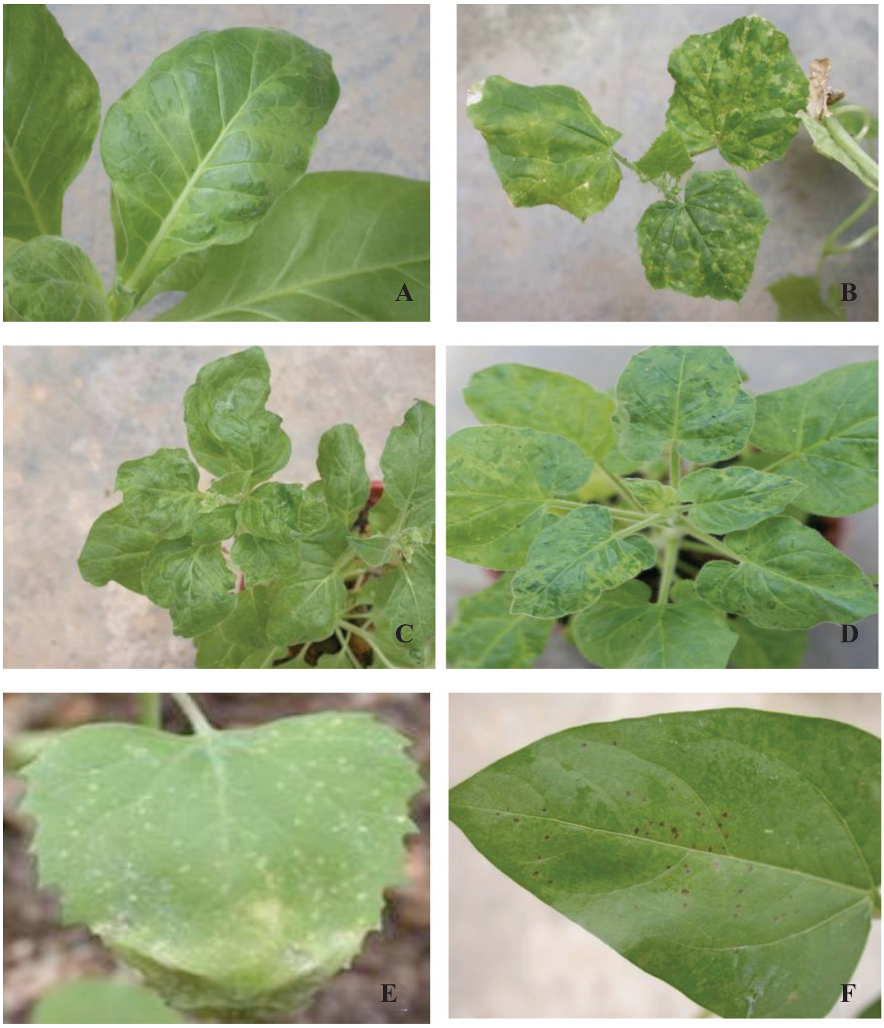

Fig. 4: The response of test plants following mechanical inoculation with the virus isolate. A) N. tabacum cv. White Burley; B) C. sativus; C) N. benthamiana; D) N. glutinosa; E) C. amaranticolor; F) V. sesquipedalis.

Sequence Analysis of CP Gene

The amplification of the CP gene of the virus isolate was successfully performed using RT-PCR on the viral particles and its RNA. A DNA fragment of 1000bp was amplified using the primers, CMVF1 and CMVR2 (*Fig. 5*). No amplicon was obtained in water control. The sequenced region was analyzed and confirmed to have a single open reading frame which comprised of 657 nucleotides potentially coding for 218 amino acids. The sequence obtained showed 92-100% sequence homology to the CP sequences of CMV isolates in the Genbank, confirming the identity of the virus. The local CMV CP gene sequence data was submitted to the GenBank (Accession number EU726631) and the database search was also performed. The sequence data revealed 100% nucleotide and amino acid identity to a CP gene of the CMV isolated from *C. roseus* in India (GenBank

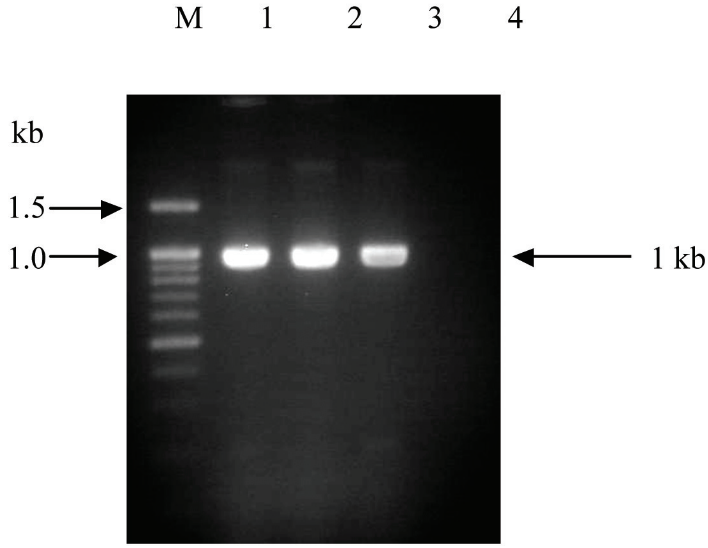

Fig. 5: Gel photograph of RT-PCR amplicons of 1000 bp using CMV RNA and its particle as a template. Lane 1, CMV RNA; lane 2, CMV partial purified particles; Lane 3, CMV purified particles; lane 4, water control; M, I00 bp blue extended DNA ladder (Bioron).

accession EU310928) which is a member of the subgroup 1B (Samad *et al*., 2008).

Meanwhile, the sequence analysis clearly indicated that the Malaysian CMV isolated from *C. roseus* belonged to sub-group 1 (with >90% sequence identity at nucleotide and >93% at amino acid levels) compared to sub-group II (<79% at both nucleotide and amino acid levels) (Table 4). A further analysis revealed that the Malaysian CMV isolate possesses higher sequence identity with subgroup IB strains, with nucleotide percent identity ranging between 92 and 100%. It has only 91-92% sequence identity with subgroup IA. At the amino acid level, the percentage of identity of the local isolate with IB members was higher (95-100%) as compared to those of IA members (95%).

Alignment of the deduced amino acids of the CP for this isolate with four other CMV of *C. roseus* from abroad showed unique differences at five positions (*Fig. 6*). The CP of this isolate and an Indian isolate (EU310928) are unique as they have threonine, arginine, lysine, valine and threonine residues at position 31, 76, 82, 172 and 193, respectively, compared to asparagine, lysine, arginine, alanine and alanine residues which were conserved in all other sequences. The existence of CMV isolates that are genetically related but occur in geographically distinct areas, as noted in this work, suggests that they may move together with infected plant materials between the countries.

CONCLUSIONS

The causal agent of the *C. roseus* mosaic symptom consists of virions (28.6 ± 0.48 nm in diameter) which are spherical in shape with a central core. It induces typical symptoms of CMV infection on various test plants and shows a positive reaction to CMV antiserum in DAS-ELISA. The coat protein (CP) gene sequence analysis revealed 100% sequence identity to the CP gene of *C. roseus* CMV isolated from India. The results of this study have revealed that the causal agent that induces mosaic symptoms on local *C. roseus* was an isolate of CMV. Meanwhile, the highest homology

AY376840 Brazil	MDKSESTSAG RNRRRRPRRG SRSAPSSADA NFRVLSQQLS RLNKTLAAGR PTINHPTFVG
E F424778 China	. . . . S . . . . . N . . . .
E F424777 China	. . . . S . . . . . N . . . .
EU726631 Malaysia	. . . . S . . . . . **T** . . . .
EU310928 India	. . . . S . . . . . **T** . . . .
	61
AY376840 Brazil	S E RCRPGYTF TS I TLKPP KI DRGSYYGKRL LLPDSITEYD KKLV SRI Q IR VNPLPKFDST
E F424778 China	. . . . K. . . . . K. . . . . R . . . . .V. . F . . . .
E F424777 China	. . . . K. . . . . K. . . . . R . . . . V. . F . . . .
EU726631 Malaysia	. . . . K. . . . . **R**. . . . . **K** . . . . V. . F. . . . .
EU310928 India	. . . . K . . . . **R**. . . . . **K** . . . . V. . F. . . . .
	121
AY376840 Brazil	VWVTVRKVPA SS DLSVTAI S AMFADGASPV LVYQYAASGV QANNKLLYDI SAMRADIGDM
E F424778 China	. . . . T . . . . A . . . .
EF 424777 China	. . . . A . . . . A . . . .
EU726631 Malaysia	. . . . S . . . . **V** . . . .
EU310928 India	. . . . S . . . . **V** . . . .
	181 218
AY376840 Brazil	RKYAVLVYSK DDALETDELV LHVDIEHQRI PTSGVLPV
E F424778 China	. . . . A . . . .
EF 424777 China	. . . . A . . . .
EU726631 Malaysia	. . . . **T** . . . .
EU310928 India	. . . . **T** . . . .

Fig. 6: Amino acid sequence alignment of the coat protein gene of five CMV C. roseus isolates. The Malaysian CMV isolate from C. roseus is in bold. Identical residues are denoted as a dot. Five positions of amino acid sequence unique to Malaysian and Indian isolates are in bold and highlighted.

scored for both the nucleotide and predicted amino acid sequences of the CP region of the local and Indian CMV isolates of *C. roseus* suggest a similar virus origin. To the best of the researchers' knowledge, this is the first aetiology report of a natural mosaic disease symptom of *C. roseus* in Malaysia, which is supported with the nucleotide sequence analysis of the causal virus.

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