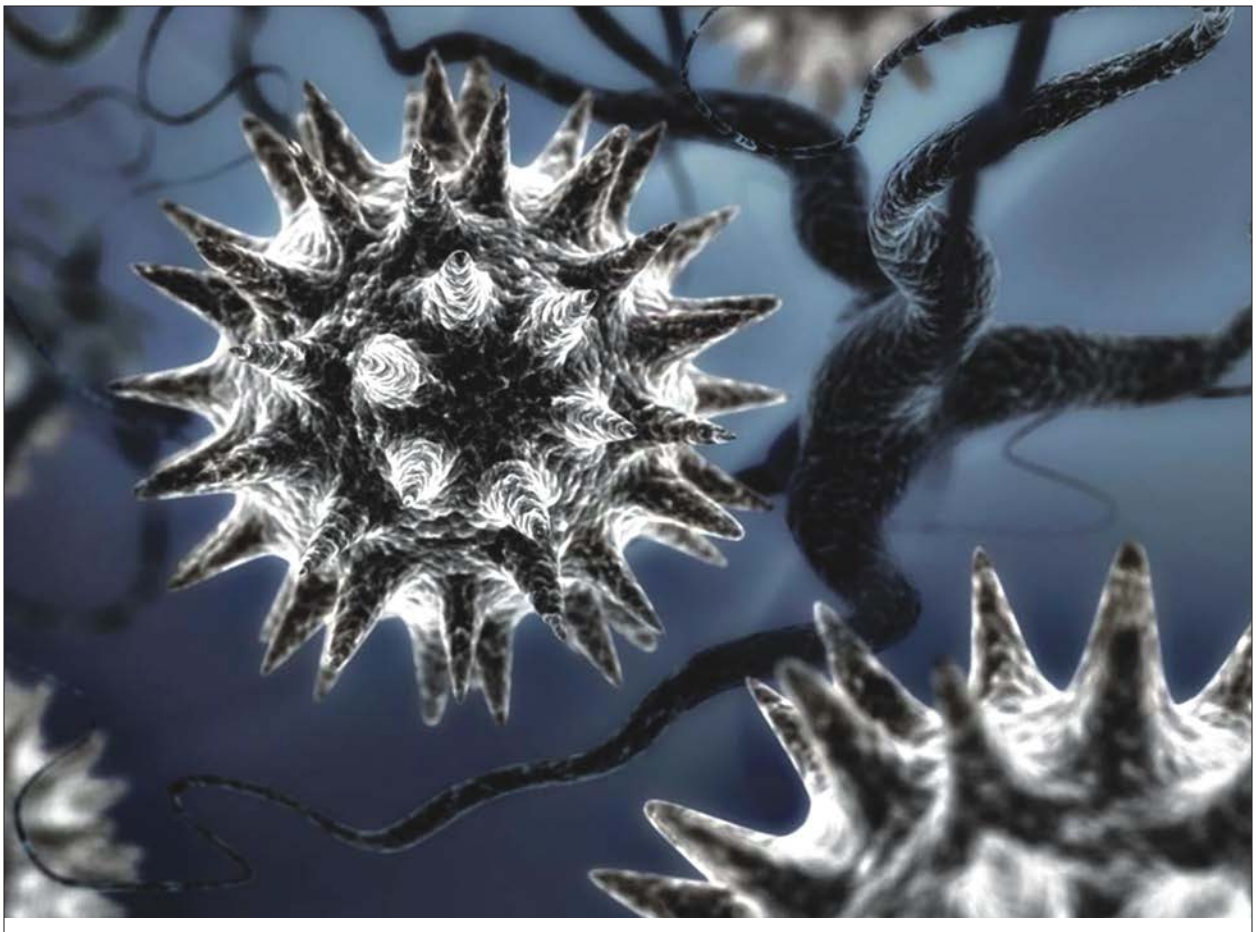

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Tobacco Callus Culture as a Propagating Medium for Cucumber Mosaic *Cucumovirus**

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Abstract: This study was performed on Cucumber mosaic *cucumovirus* (CMV) as a model. The virus was isolated from naturally infected cucumber plants depending on polyclonal antibodies specific for the CMV (subgroup I) and local lesions produced on *Chenopodium amaranticolor* were used as a source of single lesion isolation. CMV isolate was maintained in *Nicotiana tabacum* cv. White Burley. The presence of viral spherical particles was confirmed by electron microscopic examination. Infected callus tissues were prepared by syringe injection with infectious clarified tobacco sap with the aid of Millipore® filter. CMV was purified from both infected tobacco leaves and calli tissues. The purification method was modified to omit the sap clarification step when using calli as a purification source. After spectrophotometry and electron microscopic evaluation purified preparations from both sources were used for rabbit immunization to produce polyclonal antibodies. IgGs were purified and evaluated by determination of their dilution end points using indirect enzyme linked immunosorbant assay (I-ELISA) and their reaction against healthy tobacco sap as a control. The CMV coat protein gene (*cp*) was isolated and amplified from both kinds of infected tissues using immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR). PCR products were analyzed by agarose gel electrophoresis.

Key words: CMV, callus, purification, electron microscopy, antiserum, coat protein gene (*cp*), IC-RT-PCR, I-ELISA

INTRODUCTION

Cucumber mosaic virus (CMV, family: Bromoviridae, genus: *Cucumovirus)* is one of the most widespread plant viruses in the world with extensive host range infecting about 1000 species including cereals, fruits, vegetables and ornamentals (Brunt *et al*., 1996; Roossinck, 1999). CMV is a multicomponent virus with a single stranded positive sense RNA. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. Numerous strains of CMV have been classified into two major subgroups (subgroups I and II) on the basis of serological properties and nucleotide sequence homology (Palukaitis *et al*., 1992; Madhubala *et al*., 2005). The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analysis (Roossinck, 1999). Occurrence of subgroup I was found to be predominant in many countries worldwide (Singh *et al*., 1995; Hord *et al*., 2001; Madhubala *et al*., 2005).

Tissue culture has acquired many practical applications in agriculture, one of the most established uses is an efficient, safe and often economical method of plant propagation (Cooke, 1977). Another

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valuable advantage of tissue culture is to produce virus-free plant materials as most of crop plants especially those propagated by vegetative means are systemically infected with one or more virus. Such infection usually reduces crops yield and quality (Xu *et al*., 1994).

Callus tissues can be used as a source of virus instead of plants growing in greenhouses. The advantage of such application is to protect virus strains from mechanical contamination or by vector insects (McMorran and Allen, 1983; El-Afifi *et al*., 2003 a, b). In addition, concentration of virus is higher in callus tissue compared with infected leaves (PVX: Dhingra *et al*., 1987; SbMV: El-Afifi, 1997; ToMV and PVY: El-Afifi *et al*., 2003 a, b).

Viruses can be easily purify from callus tissues as it is less complicated compared with plant leaf tissues (Duran-Villa *et al*., 1989) and also containing less amount of plant components (Kassanis, 1967). Antisera produced by immunizing rabbits with such purified preparations can be more specific and gave less non specific reactions when used in virus detection within infected plants (El-Afifi *et al*., 2003b).

Therefore, the objective of this study is to propagate CMV in tobacco callus culture, comparative study for using infected tobacco leaves and calli for viral purification and polyclonal antisera production and to evaluate the viral infection level in both sources and produced antisera specificity using IC-RT-PCR.

MATERIALS AND METHODS

Virus Isolation

Fifty samples of naturally infected cucumber plants (Collected from the open field of Faculty of Agriculture, Sohag University, Sohag, Egypt) generally showing mosaic, mottling and malformation were used for virus isolation. According to Koenig (1981) I-ELISA was performed on samples using polyclonal antibodies specific for CMV (Subgroup I) (purchased from Agdia Inc., USA) and zucchini yellow mosaic potyvirus (ZYMV) (purchased from AGERI, ARC, Egypt). Samples giving only CMV positive results were mechanically inoculated on *Ch. amaranticolor*. Local lesions produced were used as a source of single lesion isolation and virus was maintained in *N. tabacum* cv. White Burley under greenhouse conditions (26°C±2).

Preparing of Virus Infected Tissues

Healthy *N. tabacum* cv. White Burley plants (carrying 5 leaves) were mechanically inoculated with CMV isolate and kept under greenhouse conditions. Twenty days later, 200 g leaves (showing symptoms) were collected and kept at -20°C till used.

Healthy *N. tabacum* cv. White Burley callus cultures were prepared using 5×5 mm pieces from upper fully grown leaves as explants. Explants were disinfected (using 0.25% sodium hypochlorite) and cultured on MS medium [containing 1.0 mg benzyl adenine (BA), 2.0 mg naphthalene acetic acid (NAA), 30 g sucrose, 7.0 g agar per liter and pH was adjusted to 5.8] under conditions described by Murashige and Skoog (1962). Twenty days aged calli were inoculated with CMV tobacco infected clarified sap using sterilized syringe connected to Millipore® filter (0.45 μm). Tissues were collected 20 days post inoculation and 200 g was kept at -20°C for usage.

Virus Purification and Evaluation

CMV isolate was purified from both infected leaves and calli tissues (200 g for each) depending on a method described by Lot *et al*. (1972) with some modifications. Clarification step was omitted when callus tissues were used as a source for virus purification. Concerning leaves, chloroform was added to sap for clarification with rate of 1:1 (v/v) and centrifugation was performed at 3000 rpm/15 min. Supernatant was subjected to two cycles of differential centrifugation:

30000 rpm/1½ h and 3000 rpm/15 min at 4°C. Final pellets were resuspended using 2 mL of 0.005 M borate buffer containing 0.005 M EDTA (pH 9.0) for each purification source and kept at -20°C till used. Purified preparations were evaluated spectrophotometrically (using a Shimadzu UV 1201 spectrophotometer). Virus yields were estimated as described by Noordam (1973) using extinction coefficient of 5.0. Preparations were negatively stained with 2% phosphotungastic acid (PTA) according to Griffin (1990) and examined with JEOL JEM 100 CX 11 transmission electron microscope, at Electron Microscope Unite, Assiut Univ., Assiut, Egypt.

Antisera Production and Evaluation

Polyclonal antiserum produced in a New Zealand white rabbit by injecting purified virus preparation from each purification source depending on the method described by Madhubala *et al.* (2005) (1 mg of virus with Freund's incomplete adjuvant, 1:1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected.

Immunoglobulins G (IgGs) purification was carried out as mentioned by Clark and Adams (1977). Final dialyzed proteins were loaded on 10 mL of diethylaminoethyl (DEAE) cellulose column (Whatman Inc., USA), eluted using half strength Phosphate Buffer Saline (PBS) and collected in microtube. Absorption of fractions was measured at 280 nm and concentration of IgGs was adjusted to 1 mg mL^{-1} (OD_{280} = 1.46) using half strength PBS, then stored at 4°C. IgGs dilution end points were determined using I-ELISA against clarified infected tobacco sap and healthy tobacco sap was used as a control.

Confirmation of Antisera Specificity and Evaluating Detection of CMV Using IC-RT-PCR

The IC-RT-PCR was performed depending on infected tobacco leaf and callus tissues collected 20 days post virus inoculation. Immunocapturing and cDNA synthesis was carried out as described by Minafera and Hadidi (1994). The following primers (purchased from Invitrogen Corp., USA) were used for the isolation and amplification of CMV *cp* which were designed depending on CMV (Supgroup I) genes sequences data collected from PubMed (GeneBank) web site (http://www.ncbi.nlm.nih.gov): 5'ATGGACAAATCTGAATCAAC3' (Sense) and 5'TCAAACTGGGAGCACCCCAG3' (Antisense).

PCR was carried out according to Ghosh *et al.* (2002). Five microliter from resulting cDNA were transferred to tube containing 45 µL PCR reaction mixture contained primers (1 µM final concentration each), Taq DNA polymerase (1 unit), 200 µM of each dNTPs, 5 µL 10× PCR buffer (500 µM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.0), completed to 45 µL with Sterile deionized water and the reaction was overlaid with 50 µL sterilized mineral oil. PCR program was 94°C initial melting for 3 min followed by 35 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min and 72°C/10 min final extension (using PerkinElmer cetus thermal cycler, PerkinElmer Inc., USA).

For PCR product analysis, 1.5% agarose gel was used and electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad Lab., USA) at 80V. The gel was photographed under UV-transilluminator using a gel documentation system, band size was determined from the gel photograph using Gel-Pro Analyzer software (Media Cybernetics, USA).

RESULTS

Virus Isolation

CMV of subgroup I was isolated from naturally infected cucumber plants showing mosaic, mottling and malformation (Fig. 1A). Samples gave positive I-ELISA results ranging from 0.946 to 1.079 were mechanically inoculated on *Ch. Amaranticolor* leaves and gave chlorotic local lesions (Fig. 1B). Lesions were extracted and inoculated on *N. tabacum* cv. White Burley for propagation.

Virus Purification and Evaluation

UV absorption data show that A_{260}/A_{280} ratio for virus purified from leaves and calli were 1.10 and 1.25, respectively. Yields were 1.74 and 2.4 mg virus/200 g infected leaves and calli, respectively. Electron micrographs show spherical virus particles of about 30 nm in diameter (Fig. 2A and B).

Antisera Production and Evaluation

The highest titers were 1:128 and 1:512 for antisera produced using virus purified from leaves and calli, respectively (Table 1). It was observed that purified antiserum prepared from healthy leaves gave slightly higher I-ELISA readings with tobacco healthy sap compared with that of virus purified from calli.

Confirmation of Antisera Specificity and Evaluating Detection of CMV Using IC-RT-PCR

By analysis the PCR products on agarose gel, bands with the expected size of 657 bp were observed representing CMV *cp* (Fig. 3). Results indicated that bands produced from callus infected tissues were slightly higher in intensity compared with those of tobacco infected leaves.

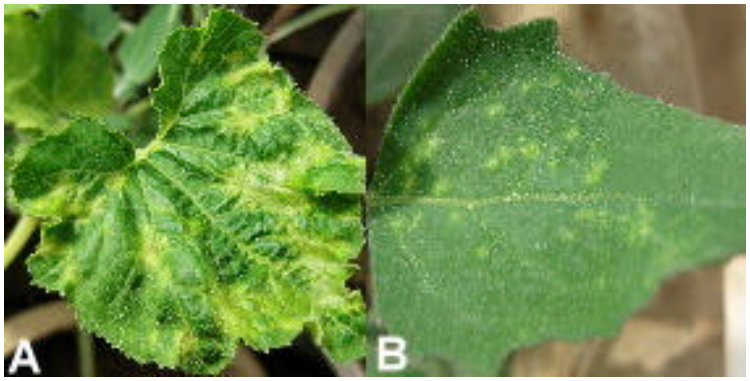

Fig. 1: CMV infected Cucumber leaf showing mosaic, mottling and malformation (A), chlorotic local lesions produced on *Ch. Amaranticolor* leaf 15 days post inoculation with CMV infected cucumber sap (B)

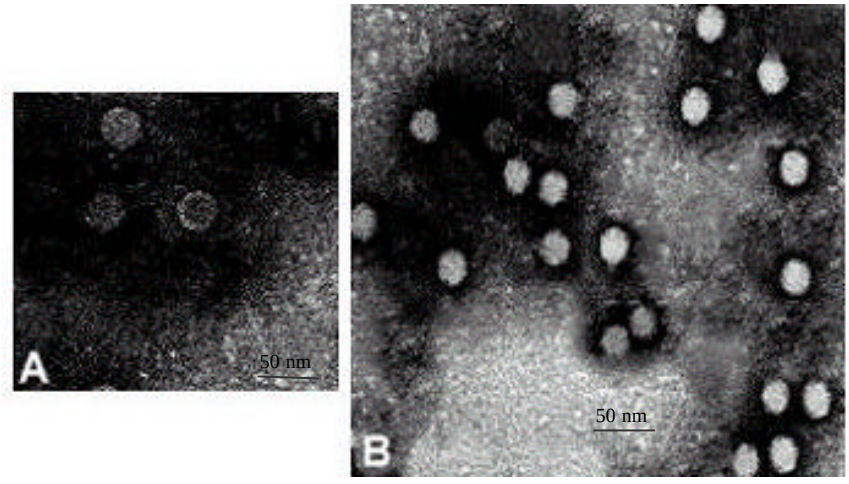

Fig. 2: Electron micrographs of negatively stained purified virus preparations obtained from infected tobacco leaves (A) and calli (B)

Table 1: CMV IgGs evaluation against infected and healthy tobacco sap

	I-ELISA values at 405 nm*							
	$IgGs_1$				IgGs2			
Dilution	I	R	H	R	I	R	H	R
Crude	0.615	+	0.224	-	0.718	+	0.141	-
1:1	0.809	+	0.211	-	0.914	+	0.156	-
1:2	0.764	+	0.312	-	0.900	+	0.091	-
1:4	0.699	+	0.249	-	0.811	+	0.084	-
1:8	0.670	+	0.222	-	0.801	+	0.090	-
1:16	0.600	+	0.081	-	0.711	+	0.077	-
1:32	0.599	+	0.084	-	0.709	+	0.075	-
1:64	0.581	+	0.091	-	0.666	+	0.068	-
1:128	0.499	+	0.066	-	0.658	+	0.055	-
1:256	0.091	-	0.084	-	0.599	+	0.061	-
1:512	0.064	-	0.067	-	0.500	+	0.059	-
1:1024	0.061	-	0.050	-	0.212	-	0.053	-
Purified**	1	0.953	+	0.974	+		+	
	2	1.042	+	1.132	+		+	

*Each ELISA result (R) was the average of three readings.**Reaction of crude purified virus from leaves (1) and calli (2) with crude antiserum. Reaction of infected (I) and healthy (H) tobacco leaves sap with anti-sera produced by rabbit immunization with CMV purified from leaves ($IgGs_1$) and from calli ($IgGs_2$) + Positive reaction, - Negative reaction

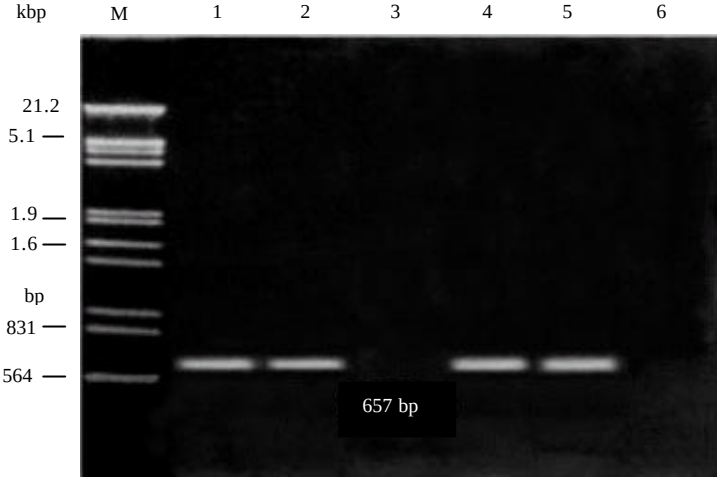

Fig. 3: IC-RT-PCR for amplification of CMV *cp* from tobacco infected leaves (lanes 1 and 2) or callus (Lanes 4 and 5) tissues. Healthy leaves (Lane 3) and callus (Lane 6) as a controls. M: Lambda DNA/*Eco*RI+*Hind*III marker (Promega, USA)

DISCUSSION

CMV has a relatively wide host range, giving systemic symptoms on cucumber plants and can be isolated from single local lesions produced on mechanically inoculated *Chenopodium* leaves (Palukaitis *et al*., 1992; Madhubala *et al*., 2005). Also ELISA and electron microscopy can be used as a helpful tool to confirm isolation success after biological studies (Honda and Matsui, 1973; Raj *et al*., 2002; Madhubala *et al*., 2005).

Tissue culture has one valuable advantage as it can be used for propagation and preservation of plant viruses, avoiding isolates contamination under greenhouse conditions (El-Afifi *et al*., 2003 b).

The virus under study was purified from tobacco leaves and calli, results showed that purified preparation obtained from calli was relatively higher in both purity and yield. It was found that CMV can be purified from tobacco callus tissues without passing with the clarification step representing

another advantage of tissue culture in virus studies. Also less plant components were observed in electron micrographs of calli purified CMV preparations compared with that purified from leaves. Such results were in harmony with those of PVY and ToMV found by El-Afifi *et al*. (2003 a,b).

Callus is a less complicated plant tissue compared with leaves, containing less plant inhibitors and components (Kassanis, 1967). Ikeda *et al*. (1987) found that roots, leaves and stems of *Mirabilis jalapa* showed high inhibitory activity against plant viruses compared with cultured cells. Duran-Vila *et al*. (1989) proved that infected citrus callus is a good host system for virus replication and purification.

Data of antisera production showed that antiserum produced from calli purified viral preparation was highly specific since it gave much weaker reaction with healthy tobacco sap. Antiserum of callus viral preparation has higher titer (1:512) compared with antiserum of leaves viral preparation which has a titer of 1:128. Similar trend was also found by Lyal and Gorbunova (1992) as they succeeded in producing highly specific antiserum for soybean mosaic virus using clarified sap of infected soybean tissue culture. El-Afifi *et al*. (2003b) produced antisera of higher titer and specificity for PVY and ToMV which were purified from infected tobacco callus tissues.

CMV infected tobacco callus culture can gave a clean medium for viral molecular studies, genes isolation and amplification. The *cp* gene of CMV was successfully amplified from infected tobacco callus tissues by IC-RT-PCR. Agarose gel electrophoresis result revealed the expected band size of 657 bp representing CMV *cp* gene. Some investigators detected virus genes in plant tissue culture by means of molecular protocols (Eggenberger *et al*., 1989; Covey and Turner, 1993; Pelah *et al*., 1994).

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