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# Occurrence of Three Strains of *Cucumber mosaic virus* Affecting Tomato in Kuwait

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**Three strains of *Cucumber mosaic virus* (CMV) have been found to cause a lethal disease, referred to as fern leaf syndromes and mild mosaic symptoms in tomato (*Lycopersicon esculentum* Mill.) crops grown in Kuwait. CMV strains were detected and identified based on host range, symptomatology, serology, electron microscopy, and ribonucleic acid (RNA) electrophoresis in polyacrylamide gels. A high degree of viral genomic heterogeneity was detected among CMV strains isolated in Kuwait, with no apparent correlation to symptomatology in tomato host plants. Two different virus satellites of "CMV associated RNA 5", designated CARNA 5, were detected in two virus strains that caused both lethal disease and mild symptoms, designated CMV-D1 and CMV-S1 respectively. CARNA5 was not detected in the third CMV strain that caused fern leaf syndromes designated CMV-F. All the three isolated strains were serologically indistinguishable from each other and may belong to one serotype according to Ouchterlony gel diffusion tests. These strains transmitted via aphids (*Myzus persicae* Sulz) in a non-persistent manner. Physical properties of the virus strains were very similar where thermal inactivation test showed that virus withstood heating for 10 min at 70°C, dilution end point was $10^{-4}$, and the longevity *in vitro* at room temperature was less than 5 days for all virus strains. CMV-D1 and CMV-F were the most devastating diseases spreading in both greenhouse and field-grown tomato where aborted flower buds failed on fruit setting due to the viral infection. This is the first report to isolate three different strains of CMV in Kuwait.**

***Keywords*** : CMV, Kuwait, Tomato

Cucumber mosaic virus (CMV) was first discovered as a new infectious mosaic disease of cucumber by Doolittle in 1916 and is one of the most widespread plant virus recorded in more than 775 other species representing 365 genera belonging to 85 families (Douine et al., 1979) including tomatoes (*Lycopersicon esculentum* Mill.). The virus has caused serious total damage to tomato plants in the main production areas in Kuwait where CMV caused an outbreak resulted in high losses in tomato crops over the last five years (Personal communication). In Spain, 50-100% of tomato plants were infected with CMV that had a drastic effect on productivity (Jorda et al., 1992). CMV has been reported in USA (Grogan et al., 1959; Roossinck, 1991) and Australia (Wahyuni et al., 1992). CMV causes systemic mosaic, deformed fruit and poor fruit set of tomatoes. Two disease patterns characteristic for CMV in tomato were observed. The first was the so called shoestring or fernleaf syndrome, and the second pattern was the lethal tomato necrosis disease (Gallitelli et al., 1991). These patterns were similar to the CMV-F and CMV-D1 strains respectively, isolated in Kuwait CMV exists in a number of allied strains which considerably differ in virulence, host range and symptom expression. Symptom expression obtained with CMV on different host ranges from chlorotic to necrotic local lesions, alone or followed by a systemic mosaic disease of varying type and severity. The mosaic symptoms initiated by this virus are frequently associated with leaf distortion and deformity (Kaper, 1993; Reddy and Nariani, 1963). CMV is a highly mechanical transmissible virus by aphids (*Myzus persicae* and *Aphis gossypii*). In Florida, Grogan et al. (1959) indicated that second in prevalence of viruses infecting cucurbits were strains of CMV, and reported their spread by aphids. Physical properties of the virus studied by majority of authors reported that the thermal inactivation point of CMV lied between 65-70°C, dilution end point with different values ranged between $2 \times 10^{-2}$ and $2 \times 10^{-4}$ and longevity *in vitro* lied between 1-4 days at room temperature (Abou-El-Ghar, 1969; Fulton, 1950; Orellana and Quacquarelli, 1968; Reddy and Nariani, 1963; Simons, 1957).

Serological techniques including ELISA, as one of the possible ways to detect plant viruses, and agar gel double-diffusion tests are frequently favored because of their specificity, speed, and the scope they provide for standardization. However, for many important viruses conventional serological techniques cannot be used because of limita-

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tions such as low virus concentration, suitable particle morphology, or the presence in plant extract of virus in activators or inhibitors. These limitations can largely be overcome by the use of ELISA (Clark and Adams, 1977) in detecting a number of morphologically different viruses in purified preparations and in unclarified extracts of infected plants. Antisera of CMV were also used in diagnosis, establishing possible serological relationships between this virus and certain other viruses (Mink et al., 1975; Seth and Raychauthuri, 1973) as well as in surveys of its natural occurrence (Milne et al., 1969). However, CMV strains were serologically indistinguishable from each other and may belong to one serotype according to Ouchterlony gel diffusion tests.

CMV as a member of the cucumovirus group possesses isometric particles about 30 nm in diameter, with a tripartite genome composed of four RNA species. The genome of CMV consists of three single stranded, positive-sense RNAs. By analogy to other tripartite viruses, RNAs 1 and 2 are believed to encode proteins involved in the replication of CMV, while RNA 3 encodes a 30-35 K protein that has been hypothesized to function in promoting the cell-to-cell movement. In 1977, Kaper and Waterworth discovered that some CMV strains contain a fifth RNA molecule, designated CARNA 5 (for CMV-Associated RNA 5), which modifies viral symptoms in the host plant (Kaper et al., 1976) and was used as a biological control agent to control a lethal strain of CMV virus in tomato (Montasser et al., 1991).

The present investigation aimed mainly at providing necessary information about three different strains of CMV affecting tomato crops in Kuwait. Since there is no hard data available about the virus in Kuwait, it was important to search for isolation and characterization of every possible strain that can be found affecting tomato crops in Kuwait.

## Materials and Methods

**Sample collection.** Six hundred thirty nine samples of tomato fruits and foliage including young and fully expanded mature leaves were collected from 19 farms in different locations of farms in Wafra and Abdaly in Kuwait. Attempts were made to collect samples from plants representing a wide range of symptoms, from mild to severe, within each field. Fruit and leaf samples were taken to the laboratory in plastic bags on ice for testing either fresh or stored by desiccation for further studies.

**Mechanical inoculation and virus host range.** One gram of infected tissue sample was triturated in a sterilized mortar and pestle with 9 ml of 0.01 M potassium phosphate buffer pH 7.2, and rub inoculated with a cotton swap on the cotyledons, or young leaves of young test plant seedlings that have been previously dusted with 600-mesh carborundum. Immediately after inoculation, the leaves of test plants were rinsed with distilled water. Test plants were grown and maintained in potting mix (mixture of soil, perlite and peat moss) in an insect proof glasshouse or growth chambers under 16 hr daylight and 8 hr darkness, at temperature ranged between 22-27°C till they were further examined for the presence of virus (Montasser, 1999).

**Insect transmission.** The aphid *Myzus persicae* was obtained from Public Authority for Agriculture and Fish Resources, Kuwait and clones of *M. persicae* were raised on caged healthy eggplants (*Solanum melongena* L.) in greenhouse at temperatures ranging 22-25°C. Test aphids were starved for 30 min. in a Petri dish before the acquisition-feeding period. Aphids were fed on a young virus infected tomato leaf for 2 hrs. Then 100 aphids were transferred by the aid of a camelhair brush and filter paper discs to each of the healthy test plant leaves to feed for another 4 hr. Inoculated plants were sprayed with Dimecron insecticide at the end of the inoculation-feeding period and regularly thereafter.

**Virus purification.** Virus was purified using the method mentioned by Montasser (1999). Infected tissues were blended together with chloroform and 0.5 M Na-citrate-citric acid buffer (pH 6.5) containing 0.1% thioglycolic acid (TGA), in the ratio of 1 g tissue/2 ml chloroform/2 ml buffer. The homogenate was filtered and squeezed through four layers of cheese cloth and clarified by a 10 min. centrifugation at 7,500 rpm. The supernatant volume was measured and stirred with 10% PEG 8,000 (w/v) for 15 min. in cold. The mixture was cooled for 30-40 min. in an ice bath, followed by centrifugation at 7,500 rpm for 20 min. Pellets were re-suspended in a buffer containing 0.05 M Na-citrate-citric acid with pH 7.0, and 2% Triton X 100 (0.05 ml buffer per one gram of original tissue), and submitted to a low speed centrifugation for 5 min at 10,000 rpm, followed by a high speed centrifugation of the supernatant for four hours at 27,000 rpm by Sorval RC-28 S centrifuge. Pellets were resuspended in water over night at 4°C, homogenized with a glass rod, and clarified by low speed centrifugation at 10,000 rpm for 5 min.

**Agar gel double-diffusion test (Ouchterlony gel diffusion tests).** Agar gel double-diffusion tests (Montasser, 1999) or Ouchterlony gel diffusion tests were conducted in 0.7% Noble agar containing 0.85% sodium chloride and 0.25% sodium azide CMV strains were tested against different panels of antisera against a necrotic strain of CMV (CMV-D1), a mild strain of CMV (CMV-S1) and the

fernleaf strain of CMV available in our lab. Antisera were undiluted, and virus antigens consisted of 1:10 crude sap extracted from infected tomato plant leaf tissues as well as from healthy tomato tissues as a negative control. Plates were incubated for 48 hr at 37 °C in a moist chamber for the serological interactions and precipitin lines formation.

**ELISA method.** ELISA was performed as described by Montasser et al. (1991) using specific polyclonal or monoclonal antibodies and alkaline phosphatase conjugate according to the instruction of the manufacturer (Sigma Chemical Company, USA). One gram of infected plant tissues collected from different locations were ground in a sterile mortar and pestle with 9 ml of coating buffer pH 9.6, and centrifuged for 1 min at 5k × g. Then 100 µL of the supernatant sap was micropipetted into each assigned microwell of 96-microwell polystyrene plates that were coated by passive adsorption and incubated for 2 hr at 37°C or over night at 4°C. After three washes of three minutes intervals with phosphate buffer saline containing 0.5% Tween-20 (PBS-T), the plates were blocked with 100 µl of 0.5% bovine serum albumin (BSA) in PBS for 30 min. at room temperature, followed by three PBS-T washes and addition of 100 µL of CMV specific antibody with appropriate dilution. The plates were then incubated followed by three washes with PBS-T and addition of 100 µl goat anti-rabbit alkaline phosphatase conjugate diluted (1:1000) in PBS buffer without Tween, and incubated at 37°C for 2 hours. The plates were then washed three times with PBS-T, and 5 mg para-nitrophenyl phosphate substrate tablets (Sigma Chemical Company, USA) dissolved in substrate buffer pH 9.8 was added. Absorbance values were read at wavelength 405 nm using a Dynatech MR5000 ELISA reader. Absorbance values that exceed twice that of the healthy were considered positive.

**Physical property studies**

**Thermal inactivation point.** One gram of infected tissue was ground in a sterile mortar and pestle with 9 ml of 0.01 M potassium phosphate buffer pH 7.2. Five ml aliquots were heated in a water bath at temperatures of 50, 60, 70, 80, 90 and 95°C, for 10 minutes, then kept on ice. Test plants were inoculated with heat-treated sap extracts by rubbing on leaves powdered with 600-mesh carborundum. Leaves were rinsed after inoculation and test plants were kept under 22-27°C in greenhouse for symptom observation.

**Dilution end point.** One gram of infected tissue was ground in sterile mortar and pestle with 9 ml of 0.01 M potassium phosphate buffer pH 7.2. Different dilutions of crud sap were prepared ranged from $10^{-1}$ to $10^{-8}$. Test plants were inoculated with the diluted preparations by rubbing leaves predusted with 600-mesh carborundum. Leaves were rinsed after inoculation and test plants were kept under 22-27°C in greenhouse for symptoms observation.

**Longevity in vitro.** One gram of infected tissues was ground in a mortar and pestle with 9 ml of 0.01 M potassium phosphate buffer pH 7.2 and kept as 1 ml aliquots at room temperature to be used as an inoculum. One aliquot was inoculated on 10 test plants at the cotyledonary stage were mechanically inoculated daily for ten days. Serial test plants reaching the same age were inoculated daily from the same prepared aliquots. Leaves were rinsed with water after inoculation and test plants were kept at 22-27°C in glasshouse.

**Electron microscopy**

**Leaf dip method and purified virus particles.** Virus particles were detected in tomato leaf samples by using negatively stained leaf dips. Freshly cut edges of the leaf were dipped into a small drop of deionized water placed on a formvar coated grid and left to dry for 5-10 minutes. For purified virus preparations, a drop of purified virus suspension was placed on formvar-coated grid. A drop of 2% phosphotungestic acid pH 7.2 was then added to each grid and allowed to dry for 1-2 min, then the excess of stain was removed with a stripe of a filter paper and the remainder was allowed to air dry completely. The grids were then examined in JEM-1200 EX II electron microscope and micrographs were taken. Three hundred particles in tomato samples were used for particle measurements and for histogram of length distribution.

**Thin sectioning.** Tissue fragments excised from infected leaves were fixed in a drop of 3% gluteraldehyde in 0.01 M potassium phosphate buffer pH 7.0 and were kept in the fixative for three days at room temperature under slight vacuum, followed by four washes with 0.01 M potassium phosphate buffer pH 7.0 for 15 min each. Leaves were kept in pure spurs resin overnight then embedded in labeled capsules. After decapsulation, blocks were ready for sectioning. Thin sections were double stained with uranyl-acetate and lead citrate before examining in the electron microscope (Al-Awadhi et al., 2002).

**Nucleic acid analysis of CMV**

**Extraction of nucleic acid.** Total nucleic acids (TNA) were extracted by powdering 0.25 g of infected tissue in liquid nitrogen in a sterile pre-cooled mortar and pestle (White and Kaper, 1989). With the aid of a sterile brush, the light green powdered tissues were transferred to a 50 ml conical centrifuge polypropylene tube contained 3 ml of each of 1x extraction buffer (0.1 M glycine, 0.01 M EDTA,

0.1 M NaCl, pH 9.0 with 10% SDS and 10% *n*-Lauryl sarcosine), equilibrated water saturated phenol (containing 0.1% 8-hydroxyquinoline) and chloroform. The homogenate were vortexed vigorously for 45 seconds and were centrifuged for 10 min. at 5K × g at 4°C, then kept on ice. The nucleic acids present in the upper aqueous layer were precipitated by adding 3 volumes of 95% ethanol kept at −70°C for 30 min. The precipitate was collected by low speed centrifugation at 8 × g for 10 min. The pellet was resuspended in 400 µl of 1 × extraction buffer in an eppendorf tube then filled with absolute ethanol and left at −70°C for 30 minutes followed by centrifugation in a microfuge. Pellets were washed twice with 70% ethanol then dried in a speedvac and dissolved in 100 µl of 8 M urea containing 0.02% bromophenol blue and 0.02% xylene. The suspension was heated in a 50°C water bath for 5 min, followed by quick cooling in crushed ice, then examined by gel electrophoresis or stored at −70°C.

**Gel electrophoretic analysis of total nucleic acid (TNA).** Polyacrylamide gel electrophoresis (PAGE) was used to detect dsRNAs of specific satRNA molecules. Total nucleic acids or TNA extracts were analyzed by electrophoresis on 6% polyacrylamide gels (39:1 acrylamide:bisacrylamide) in TRIS-borate (TBE) buffer, at 300 V for 1.5 hr. dsRNA forms of satRNAs thus separated were compared to RNA standards electrophoresed on the same gel after staining with ethidium bromide and UV photography.

**Northern blot hybridization.** TNA gels were laid over strips of nylon or nitrocellulose membranes, in such that RNA elutes out of the gel and trapped by the membrane; a Bio-Rad electro blotting apparatus was used for this transfer. The relative positions of the RNA fragments in the gel were preserved during their transfer to the filter and/or membrane. RNA attached to the filter membrane was then immobilized either with UV light for 3-5 min (for nylon) or by baking at 80°C for 30 min (for nitrocellulose). RNA attached to the filter was hybridized to $^{32}$P-labeled nick translated probe (White and Kaper, 1989) in a Hybaid™ mini hybridization oven (National Labnet Co., Woodbridge, NJ,USA), washed and exposed to X-ray film with intensifying screens at −70°C (Hadidi and Yang, 1990). Radioactive signals from hybridized RNA bands on Northern blot membranes were radiographed.

**Dot and squash-blot hybridization.** Leaf disks of plants were excised with a flame-sterilized no. 4-cork borer, and then squashed on a Zetabind nylon membrane with a flame-sterilized glass rod. A known quantity of cloned CMV RNA (1 pg-10 ng) was spotted on the membrane as positive control. The membranes were hybridized with $^{32}$P-radiolabeled probes as described by Montasser et al. (1991). Twenty-five nanograms of the RNA preparations were labeled using a random primer RNA labeling kit (BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Denaturing of the membranes and high-stringency hybridization were performed as described by Montasser et al. (1991).

## Results

Viral diseases were found to be prevalent and were detected in almost all tomato samples collected. Based on the symptoms that occurred on tomato infected with the causal agents and bioassay tests in the laboratory and the greenhouse, we were able to establish the viral nature of these infections. Three viruses have been isolated and then identified as strains of CMV including two strains causing destructive viral diseases: tomato lethal necrosis (CMV-D1), tomato fern leaf (CMV-F) and one mild strain (CMV-S1).

**Symptomatology.** During symptomatology studies in different areas of Kuwait, it was observed that in some locations of greenhouse and fields, tomato plants showed necrotic symptoms, growth reduction and stunting (Figs. 1A and 1B), whereas virus symptoms related to CMV-F were observed throughout the entire growing season in all visited locations (Fig. 1C). This strain of CMV predominantly caused typical mosaic accompanied by yellowing of the interveinal leaf areas. In later stages of infection, leaflets became narrower, slender, and exhibited malformations. Affected leaves became distorted to give a fern leaf type appearance or a tendril-shape (Fig. 1C). It was observed that in some tomato fields, infected tomato plants were less vigorous and showed mild mosaic symptoms.

**Host range studies.** Results of the experimental host range study and the associated symptoms (Fig. 2) are summarized in Table 1. Total 33 host plants belonging to five different families were inoculated with sap extracted from plants showing symptoms of three different CMV strains. As shown in Table 1, two host plants tested from *Chenopodiaceae* family were showing local lesions on the leaves when inoculated with S1, D1 and F strains of CMV. Ironically, no symptoms of these three CMV strains were observed on the test plants, *Brassica oleraceae* of *Cruciferae*, *C. fruticans* var. California of *Cucurbitaceae* and *Vicia faba* of *Leguminosae* families. Total seventeen host plant types of *Cucurbitaceae* were tested and it was noticed that mild mosaic symptoms appeared on maximum number of test plants on inoculating with CMV-S1 in comparison to appearance of mosaics with CMV-D1 and


**Fig. 1.** Systemic symptoms of CMV strains on infected tomato plants grown showing necrotic symptoms on plants grown in a commercial greenhouse (A) and in an open field (B). Fern leaf and severe stunting symptoms occurred on tomato grown in an open field in Kuwait (C).

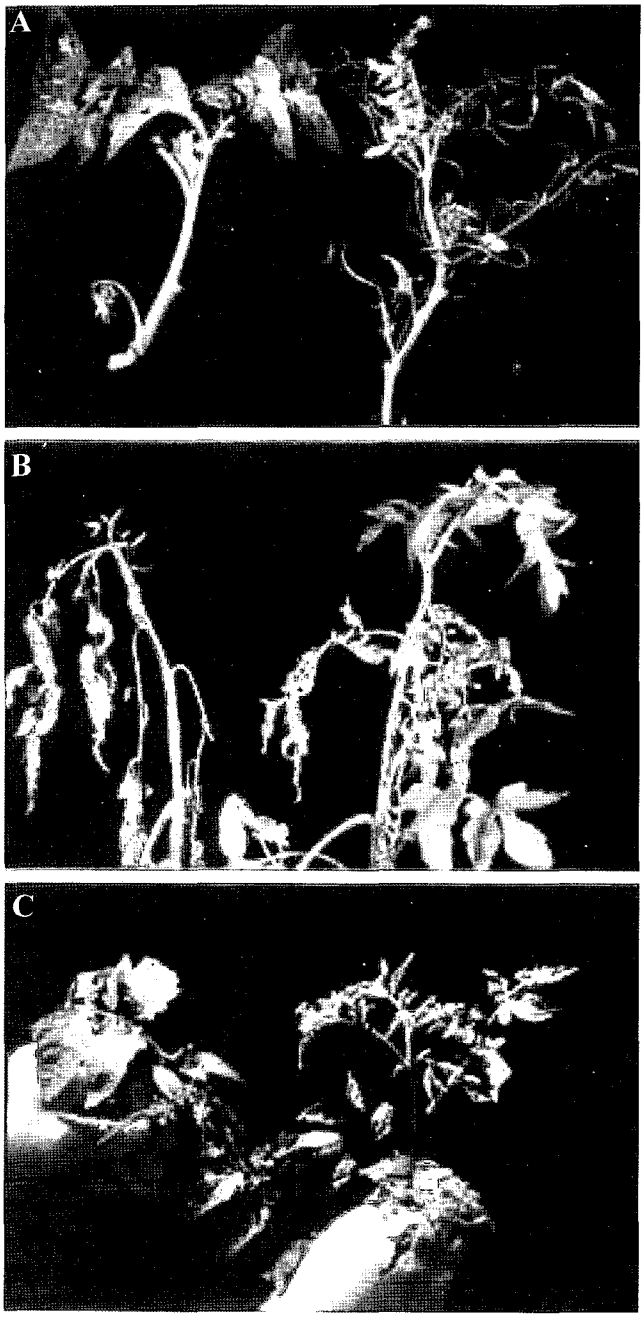


**Fig. 2.** Three strains of CMV isolated and maintained on tomato plants showing the typical symptoms of fern leaf syndrome caused by CMV-F (A), lethal tomato necrosis caused by CMV-D1 (B). Mosaic symptoms and leaf distortion caused by a mixed infection with tobacco mosaic virus TMV) and CMV-S1 (C-right) compared with a mild mottling of CMV-S1 alone (C-left).

mosaics and severe symptoms with CMV-F strains (Table 1). Among total 11 test plants of *Solanaceae* family, maximum plant types were found symptomless with CMV-S1 unlike appearance of necrosis, malformation, leaf distortion like symptoms with CMV-D1 and fern leaf, blistering and leaf distortion like symptoms with CMV-F. Three strains of CMV showed difference in the pattern of their symptoms appearance. Fern leaf like symptoms of CMV-F strain (Fig. 2A), necrotic symptoms of CMV-D1 (Fig. 2B) and mild mosaic symptoms of CMV-S1 strain (Fig. 2C) visualized on tomato plants, clearly differentiated the symptomatology of the three strains of CMV isolated in Kuwait.

**Table 1.** Host range and symptomatology of CMV strains isolated in Kuwait

| Test Plants | CMV-S1 | CMV-D1 | CMV-F |
|---|---|---|---|
| **Chenopodiaceae** | | | |
| *Chenopodium album* Coste & Reyn | NL[a] | NL | CL |
| *C. amaranticolor* Coste & Reyn | NL | NL | NL |
| **Cucurbitaceae** | | | |
| *Cucumis melo* L. var. easy rider | MM | M, VB | B, VB, LD |
| *C. melo L.* var. Honey dew | MM | M | M, VB |
| *C. sativus L.* var. Akhdar | MO | M | B, VB |
| *C. sativus* var. Rawa | MM | M | S, MO |
| *C. sativus* var. Salima | MM | M | S, MO |
| *C. sativus* var. Med1st | MM | M | S, MO |
| *C. sativus* var. Encore | MO | M | S, MO |
| *C. sativus* var. Telegraph | MM | M | M |
| *C. sativus* var. Straight-8 | MO | M | M |
| *C. sativus* var. Hana | MM | B, VB, ST | M |
| *C. sativus* var. Long green ride | MO | M | M |
| *Cucurbita pepo* L. var. Ghada | VC, VB | M | M |
| *C. pepo* var. Diamond | MO | VB, M | M |
| *C. pepo* var. Grise | MO | | |
| *C. pepo* var. Zucchini Elite | MM | M | M |
| *C. maxima* var. Balka | MM | M | M |
| *C. maxima* var. large yellow | MM | M, VB | M, VB |
| **Leguminosae** | | | |
| *Phaseolus vulgaris* | YM | CH | Y |
| *Vicia faba* | NS | NS | NS |
| **Solanaceae** | | | |
| *Capsicum frutescens* L. Var. Yolo Wonder | NS | LD, MF, M | LD, M |
| *C. frutescens* var. Calwonder Early | NS | MF | LD, M |
| *C. frutescens* var. California | NS | NS | NS |
| *Lycopersicon esculentum Mill* var. Montcarlo | NS | N | FL |
| *L. esculentum* var. Diamond | NS | N | Y, S, VB, B, ST, FL |
| *L. esculentum* var. Rutgers | NS | LD, N | LD, FL |
| *L. esculentum* var. UC-82B | NS | LD, N | LD, FL |
| *L. esculentum* var. Marmande | NS | N | FL |
| *L. esculentum* var. Garden Delight | NS | N | LD, FL |
| *Nicotiana tabacum* L. Samsum | MO | CL | MO |
| *N. Tabacum* L. Xanthi | CL | MO | M |
| **Crucifereae** | | | |
| *Brassica oleraceae* var. Capitata | NS | NS | NS |

[a]B, Blistering; CH, Chlorosis; CL, Chlorotic local lesion; FL, Fern leaf; LD, Leaf distortion; M, Mosaic; MM, Mild mosaic; MO, Mild mottling; MF, Malformation; N, Necrosis; NL, Necrotic local lesions; NS, No symptoms; S, Severe; ST, Stunting; VB, Vein banding; VC, Vein clearing; Y, yellowing.

**Insect transmission.** CMV symptom expression (systemic mosaic, leaf distortion, vein banding and raised blistering) monitored in 50 test plants following the feeding period of aphids showed that the characteristic symptoms appeared at 11 days but most of the test plants showed typical symptoms caused by the virus after 30 days. Table 2 explained that for the first 8 days of inoculation, no single CMV strain was transmitted via aphids but the incidence of CMV transmission by all the three strains was found in an increasing order starting from 15 till the 30 days after inoculation. Maximum numbers of plants were infected via aphids by CMV-S1 followed by CMV-D1 and then the F strain of CMV. Among total 50 plants infected via insect transmission, CMV-S1 strain infected 2, 20, 30, 40 and 48

**Table 2.** Number of plants developed symptoms due to CMV transmission *via* viruliferous aphids

| CMV Strains | Days after inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 4 | 8 | 11 | 15 | 22 | 25 | 30 |
| CMV-S1 | 0/50[a] | 0/50 | 0/50 | 2/50 | 20/50 | 30/50 | 40/50 | 48/50 |
| CMV-D1 | 0/50 | 0/50 | 0/50 | 0/50 | 5/50 | 10/50 | 22/50 | 45/50 |
| CMV-F | 0/50 | 0/50 | 0/50 | 0/50 | 8/50 | 9/50 | 17/50 | 39/50 |

[a]All plants were checked periodically for the development of typical disease symptoms.

plants after 11, 15, 22, 25 and 30 days of inoculations respectively. Strain CMV-D1 showed virus transmission in 5, 10, 22 and 45 plants after 15, 22, 25 and 30 days of inoculations respectively and CMV-F infection was found in 8, 9, 17 and 39 plants after 15, 22, 25 and 30 days of inoculations respectively (Table 2).

**Serology.** In agar gel double-diffusion tests, the CMV strains reacted positively with identity against specific antiserum to each viral strain and negatively reacted with healthy control extracts (Fig. 3). Infected samples collected from different locations were tested for CMV using ELISA with virus specific antibodies. ELISA readings (Table 3) revealed that 100% of tomato samples were CMV infected. Absorbance values for tomato plant samples showing mild (CMV-S1), fern leaf (CMV-F) and necrosis (CMV-D1) symptoms were noticed higher than the healthy control plants. Readings are considered significant when the absorbance ratio of diseased to healthy exceeded two. Absorbance of diseased and ratio of diseased to healthy plants was found maximum for CMV-D1 with A1.63 at 405 nm wavelength, and 13.360 values respectively. Minimum absorbance of A1.427 at 405 nm wavelength, and minimum ratio of 2.451 of diseased to healthy was noticed in CMV-F infected samples (Table 3).

**Virus purification.** The yield of purified virus was estimated at 1-1.5 mg/100g of leaf tissue, assuming $A_{260}$=5 for 1 mg/ml of CMV. The ultraviolet absorption spectrum

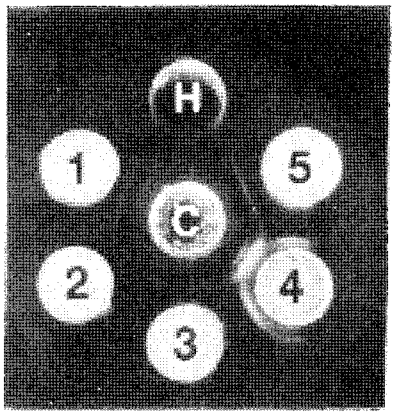


**Fig. 3.** Sodium dodecyl sulphate (SDS) agar double diffusion test (Ouchterlony) with three different isolates of CMV-S1, CMV-D1 and CMV-F from infected tomato tissues. SDS-treated extracts of virus-infected tissues diluted at 1:10 were used as antigens to react with undiluted antiserum of CMV in the center well (C). The peripheral wells were filled with antigens from healthy tomato (H), infected tomato with fern leaf syndromes (1), tomato with mild mosaic symptoms (2, 3 & 5) and tomato with necrotic symptoms (4).

of purified virus showed a typical nucleoprotein curve with a maximum at 260 nm and a minimum at 240 nm. The $A_{260}$ /$A_{280}$ ratio from several purified preparations ranged from 1.60 to 1.65. Purified suspensions proved infectivity when back inoculated on tomato test plants.

**Electron microscopy.** Satisfactory electron micrographs were obtained for CMV. Spherical particles were consistently observed in leaf dip and purified preparations of inoculated tomato plants showing mosaic symptoms (Fig. 4). Ultra-thin sections of infected leaves also showed that virus occur in the cells (Fig. 5). Particle diameters were calculated by measuring 300 particles from different preparations, the particle diameter ranged from 28.9 to 30 nm (Fig. 6).

**Physical properties.** Physical properties of CMV were

**Table 3.** Detection of CMV strains in tomato using enzyme-linked immunosorbent assay (ELISA)

| Plant Samples w/Symptoms | Diseased tissue (Ave) | Healthy tissue Control (Ave) | Diseased/Healthy Ratio | Virus Presence |
|---|---|---|---|---|
| Mild Symptoms (S1) | 1.5327[a] | 0.147 | 10.402[b] | +[c] |
| Fern Leaf (F) | 1.427 | 0.582 | 2.451 | + |
| Necrosis (D1) | 1.63 | 0.122 | 13.360 | + |

[a]Average of absorbance values measured at 405 nm, in three different diseased tomato groups of 250 samples each collected from commercial fields in Kuwait.
[b]Ratios that exceed twice of healthy absorbance values considered significant.
[c]+, Virus is present.

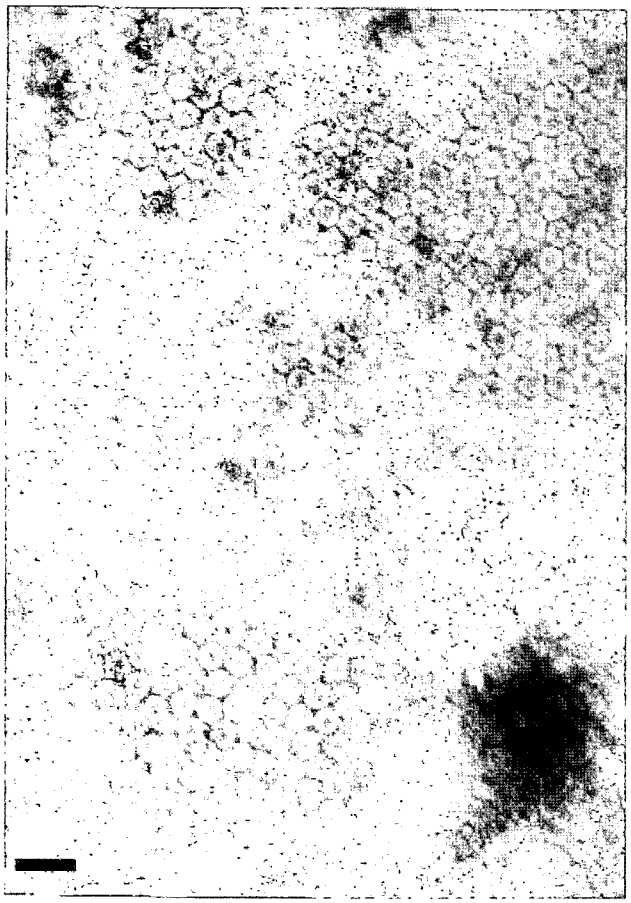

**Fig. 4.** Spherical particles and icosahedrons of the purified CMV stained with 2% phosphotungstate. Bar represents 70 nm.

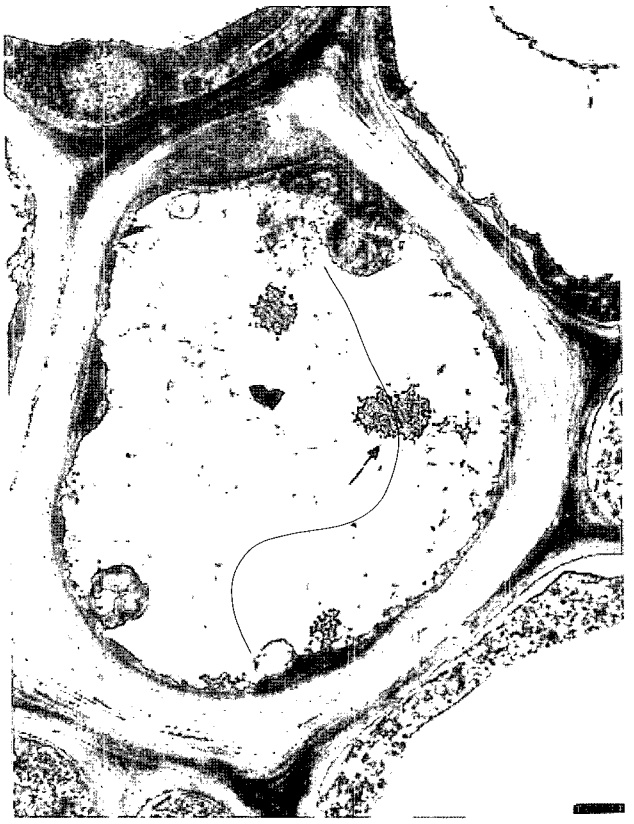

**Fig. 5.** Ultra-thin section of infected plants indicated the presence of CMV double stained with 2% urenylacetate.

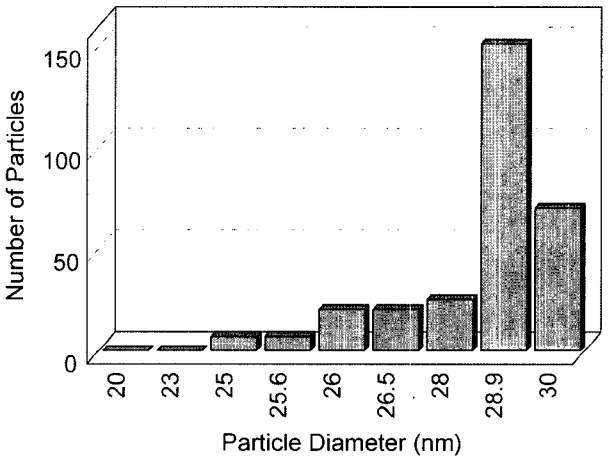


**Fig. 6.** Histogram of length distribution of purified CMV particles using electron microscopy.

studied in leaf extracts of crude sap from CMV-S1, D1 and F infected tomato tissues. It was observed in Table 4 that among the total dilutions of virus made from $10^{-4}$ to $10^{-8}$, dilution end point was found to be $10^{-4}$ with no detectable viral symptoms of three strains on total test plants. The temperature effect on the virus activity was checked ranging from 50°C to 90°C and the three viral strains were noticed to withstand heating for 10 minutes at 70°C with the thermal inactivation point (TIP) of infection as 80°C but (Table 4). Longevity of the three CMV strains checked *in vitro* from 1 to 8 days of inoculation was found inhibited after aging for 4 days at room temperature of 22-27°C.

**Molecular analyses.** Dot blot hybridization (Fig. 7) reacted positively with extracts from infected plants and negatively with healthy tissues. Total nucleic acids (TNA) analyses on 6% polyacrylamide gels resulted in detection of both single and double stranded D1-CARNA 5 of 335 nucleotides and S1-CARNA 5 of 339 nucleotides long (Fig. 8). Northern blot hybridization reacted positively with both single stranded and double-stranded D1-CARNA 5 that caused lethal tomato necrosis (Fig. 9).

## Discussion

The objective of this study was to provide information about detection and identification of the causal agents for

**Table 4.** Physical properties of CMV strains tested on tomato seedlings

| Physical Properties | CMV Strains | No. of plants infected/No. of plants tested | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dilutions | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| Dilution End Point | CMV-S1 | 20/20 | 12/20 | 9/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| | CMV-D1 | 20/20 | 15/20 | 7/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| | CMV-F | 19/20 | 10/20 | 3/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| Temperatures | | 50°C | 60°C | 70°C | 80°C | 90°C | 95°C | | |
| Thermal Inactivation Point | CMV-S1 | 20/20 | 20/20 | 15/20 | 0/20 | 0/20 | 0/20 | | |
| | CMV-D1 | 20/20 | 20/20 | 18/20 | 5/20 | 0/20 | 0/20 | | |
| | CMV-F | 20/20 | 19/20 | 13/20 | 1/20 | 0/20 | 0/20 | | |
| Days | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Aging *in vitro* | CMV-S1 | 18/20 | 5/20 | 2/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| | CMV-D1 | 20/20 | 9/20 | 5/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| | CMV-F | 16/20 | 8/20 | 3/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |

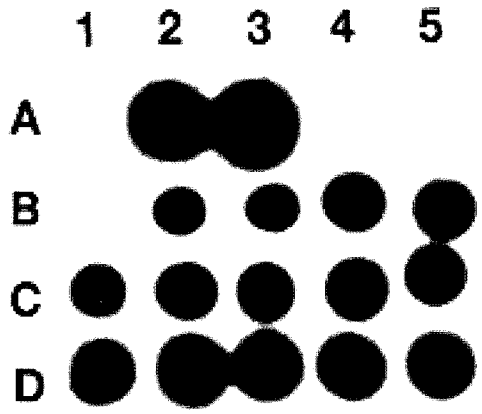


**Fig. 7.** Detection of CMV isolates in tomato tissues using dot blot hybridization. The total nucleic acid samples extracted from both healthy (A1, 4, 5 & B1) as a negative control and infected tomato with mild mosaic symptoms (B2-5); fern leaf syndromes (C1-5) and tomato necrosis (D1-5) extracts were spotted on a nylon membrane and hybridized with $^{32}P$ labeled CMV probe. A positive control of CMV was used in A2 & 3 to confirm probe specificity.

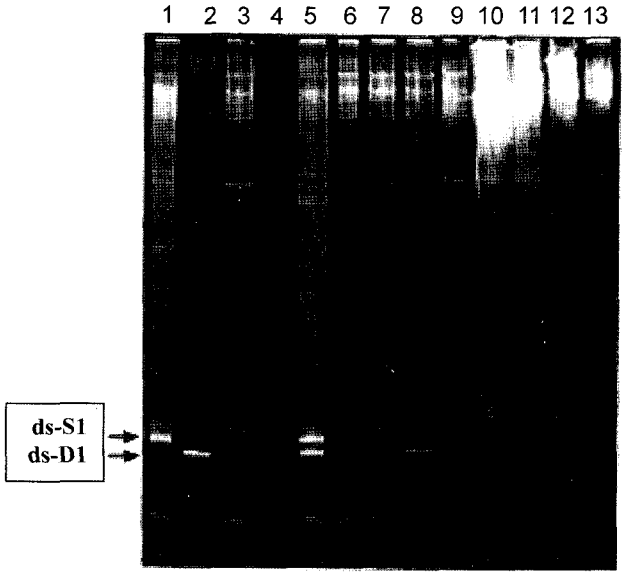


**Fig. 8.** Total nucleic acids (TNA) analyses on ethidium bromide stained 6% polyacrylamide gels to differentiate double stranded S1 and D1-CARNA 5 (CARNA 5 = cucumber mosaic virus-associated RNA 5) in total nucleic acid extracts of field-grown tomato plants. Approximate positions of where ds-S (upper band) and ds-D (lower band) CARNA 5s migrate are indicated. Lane 1, TNA from plants showed mild mosaic symptoms (CMV-S1), lane 2, TNA from plants with necrotic symptoms (CMV-D1), lanes 3, 6, 7, 9-13 TNA from healthy looking tomatoes; lane 4, blank; lanes 5 & 8, natural mixed infections with both CMV-S1 and D1 strains.

the devastating diseases caused by CMV strains affecting tomato crops in Kuwait. Based on the conventional methods and modern technology including electron microscopy, serology, host range and symptomatology, physical property studies and molecular biological analyses for viral genomes, three CMV strains were identified as the causal agents for the diseases occurred on tomato. CMV strains were found to affect plant growth and the quality of crop yield in different locations in Kuwait. Our experimental analyses showed that these three different CMV strains isolated were further found capable of inducing symptoms on test plants in greenhouse experiments. All the three strains showed a wide host range when tested on different host plants of five families and differed in pattern of symptoms appearance. CMV-S1 strain detected was showing mild symptoms whereas the other two detected strains

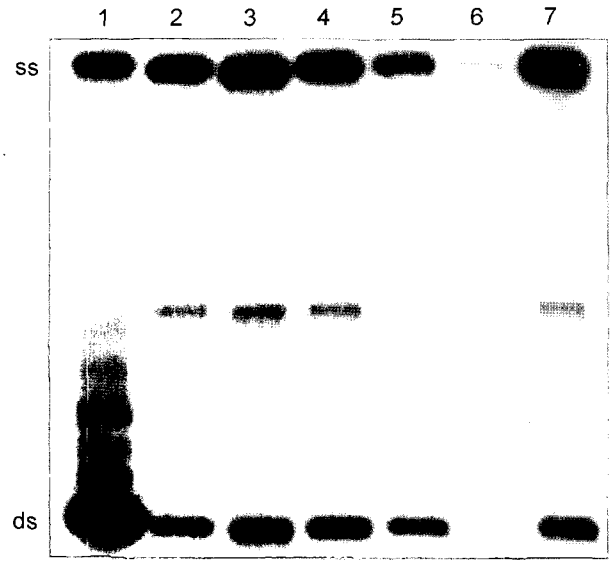


**Fig. 9.** Northern blot hybridization for single stranded (upper bands) and double stranded D1-CARNA 5 (lower bands) causing lethal tomato necrosis. Middle bands represent dimmer molecules of CARNA 5 (CARNA 5 = cucumber mosaic virus-associated RNA 5). Blotted nylon membranes were hybridized with CMV $^{32}P$ labeled probe. Lane 1, total nucleic acids (TNA) extracted from dried necrotic tissues of infected tomato leaves; lanes 2-6, TNA from fresh tomato leaves showing different levels of necrosis from lethal necrosis, severe to mild symptoms. A positive control of purified CMV-D1 was used in lane 7 to confirm probe specificity.

were noticed to give reduced growth, necrosis (CMV-D1) and fern leaf like symptoms (CMV-F). Our analysis of the etiology showed that three diseases were induced by different CMV strains. This conclusion was reached because: (i) there was no consistent association of another virus with fern leaf syndrome or tomato necrosis; (ii) isolated virus was able to induce such symptoms when inoculated on test plants in greenhouse experiments and (iii) the virus was detected easily using serology, electron microscopy and molecular biology techniques. From our observations (data not shown), an endemic disease of tomato, fern leaf syndrome (CMV-F), has been progressing in vegetable farms since 1998, with a serious economic impact on tomato crop. However, the fern-leaf type symptoms were more commonly observed in Kuwait during the winter season, as plants were affected by low light intensity, low temperature and short days in November and February. In some tomato fields infected tomato plants were less vigorous and showed necrotic symptoms, growth reduction, and may become severely stunted caused by another CMV strain of lethal tomato necrosis, similar to CMV-D1, disease occurred suddenly in 1999 and continued to cause total damage and yield loss since then. Similar strain occurred naturally among field tomato in the French Alsace region and caused tomato necrosis in 1972 (Marrou et al., 1973).

Purification procedures described for the three virus strains resulted in infectious virus suspensions. Density gradient centrifugation used as a further purification step for the virus following serial cycles of differential centrifugation and density gradient centrifugation for 2.5 hr at 27K × g gave a light scattering zone with which infectivity was associated. Purified icosahedral particles of CMV strains were found to be 29-30 nm in diameter (Gibbs and Harrison, 1970).Trials to transmit virus strains by insects showed that all the three CMV strains were found transmittable via *Myzus persicae.* Our experiments further suggested that severity of infection increased on infecting the test plant at a very young stage of development and growth at the cotyledonary stage (Daniels and Campbell, 1992). Further the studies done related to the physical properties of CMV virus isolates proved that the tested samples were still active at dilutions of $10^{-3}$, after heating at 70°C for 10 minutes and withstood storage at room temperature for only 4 days (Crescenzi et al., 1993; Daniels and Campbell, 1992; Srivastava et al., 1992).

The current results of the nucleic acid analyses for the isolated strains led to the detection of CARNA 5 in both mild strain of CMV-S1 as well as the necrotic strain of CMV-D1, whereas no viral satellites were detected in the fern leaf strain of CMV (CMV-F).

Another tomato necrosis epidemic in Italy (Kaper et al., 1990) led to description of a necrogenic CMV-satRNA. Waterworth et al. (1978) found that passing a mild strain of CMV without detectable CARNA 5 in tomato plants developed a fern leaf condition without necrosis or death of the plant, while a CMV strain contained CARNA 5 of 335 nucleotide long (Kaper et al., 1988) developed necrosis after 6 transfers in tobacco and that caused tomato plants to collapse and die. This might explain the occurrence of the three different diseases on tomato caused by one pathogen, CMV. In nature the virus undergoes serial transfers naturally in different hosts that might result in the presence of CARNA 5 or determine the amount of associated RNA5 with virions (Kaper and Tousignant, 1984).

This research work carries important information about the detection and identification of CMV strains affecting tomato crops in Kuwait. Since this is the first report regarding isolation of different CMV strains and there is no hard data available regarding this virus in Kuwait could help to provide researchers a strong base for further research studies related to this virus. Further, the CARNA 5 detected in D1 and S1 strains of CMV could help to protect the crops against the devastating effect of these deadly viral diseases (Montasser et al., 1998).

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