**Detection, characterization and host range studies of *Pepino mosaic virus* in Cyprus**

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**Abstract**

*Pepino mosaic virus* (Genus *Potexvirus*, Family *Flexiviridae*) is a mechanically transmitted viral disease that has emerged as a significant problem of greenhouse tomato crops in Europe and around the world. Although previous studies in Cyprus suggested that the virus was not present on the island, in 2009 tomato fruits from two major tomato production areas exhibited symptoms of yellow mosaic and discoloration, similar to those induced by PepMV. Consequently, an extensive survey was conducted in all tomato producing areas of the island to identify the incidence and prevalence of PepMV on protected and open field tomato crops. Approximately 3500 samples of tomato plants and weeds were analyzed using serological and molecular methods. A real-time RT-TaqMan PCR assay was developed and used for the detection of the virus. The assay was optimized to allow the use of simple template preparation methods, eliminating the need for laborious nucleic acid extraction procedures. Results showed that PepMV was rapidly spreading on the island, infecting both protected and open field tomato plants, as well as a number of weed species. Molecular characterization and phylogenetic analysis of two isolates collected from Cyprus showed that they clustered together with the isolates of the Chilean CH2 strain. The host range of the Cypriot isolates showed that they could infect other cultivated and weed plants including *Vigna sinensis*, *Solanum melongena*, *Nicotiana tabacum*, *Malva parviflora*, *Sonchus oleracea*, *Solanum nigrum*, *Convolvulus arvensis*, *Chrysanthemum segetum* and *Calendula arvensis*.

## Introduction

During the past ten years, *Pepino mosaic virus* (PepMV) has emerged as an important viral disease of greenhouse tomato crops worldwide. PepMV was first described in Peru during the 1970s infecting *Solanum muricatum* Ait. (Jones, 1980), and since then the virus has been reported to cause significant losses to tomato crops (*Solanum lycopersicum* L.) in many regions around the globe. PepMV was first observed on the European continent in 1999, on glasshouse tomatoes in the UK (Mumford and Metcalfe, 2001) and the Netherlands (van der Vlugt et al., 2000). Later on, the disease spread rapidly to many European countries leading EU plant health authorities to enforce legislation against the introduction of the virus from third countries and the further spread of the virus within Europe (EU Decision 2004/200/EC).

PepMV is a positive sense single-stranded RNA virus classified in the genus *Potexvirus* of the *Flexiviridae* family. The virus is highly contagious and can easily be transmitted by mechanical means such as contaminated hands, tools, clothing, packaging, glasshouse equipment and by direct plant-to plant contact (Wright and Mumford, 1999). Bumblebees, often used as pollinators in commercial tomato production, have been shown experimentally to contribute to the virus spread (Lacasa et al., 2003; Shipp et al., 2008). In addition, PepMV was reported to be transmitted through the nutrient solution in a closed recirculated system (Schwarz et al., 2010) and its spread is enhanced by the root-infecting fungal vector *Olpidium virulentus* (Alfaro-Fernández et al., 2010a). Recent findings confirmed that PepMV is a seed-borne pathogen that can be efficiently transmitted from contaminated tomato seeds to young seedlings (Córdoba-Sellés et al., 2007; Ling, 2008; Hanssen et al., 2009). The virus is relatively stable at room temperature and can survive without losing its infectivity for several weeks in plant debris and on contaminated surfaces (van der Vlugt, 2009).

In general, infected tomato plants show symptoms of mosaic, nettled heads, bubbling and yellow spots on the leaves as well as yellow mottling, mosaic, discoloration, marbling and uneven ripening of fruits, which cause a significant reduction in fruit quality (Spence et al.,

2006). PepMV tomato infection is characterized by a high variability in symptom development; however, for the moment, no correlation has been found between different symptomatology and genotypes of PepMV. Four different strains are currently recognized (the European tomato, the Peruvian, the US1/CH1 and the Chilean CH2), with an intergenotype RNA sequence identity of at least 80% (van der Vlugt, 2009; Hansen and Thomma, 2010).

Epidemiological and host range studies have shown that, in addition to tomato, PepMV can infect other cultivated plants including eggplant, potato and tobacco (Jones et al., 1980; van der Vlugt et al., 2002; Verhoeven et al., 2003; Pospieszny et al., 2008). In addition, several weed species neighboring infected tomato fields, the majority of which exhibited no symptoms, were found to be naturally infected with the virus. Infected weeds may influence the epidemiology and spread of PepMV by acting as virus reservoirs in crop free periods (Jordá et al., 2001b; Córdoba et al., 2004).

In Cyprus, the first PepMV-like symptoms were observed in January 2009 in greenhouse tomato crops, located in the areas of Parekklisia and Pyrgos (Lemesos District) on the southern part of the island (Fig. 1, Table 1). The virus was identified using serological and molecular methods on tomato fruit samples which showed severe yellow discoloration and mild leaf mosaic. Consequently, an extensive survey was performed in 2009 and 2010 in protected and open field tomato crops in order to identify the disease incidence on the island. This paper presents the results of the 2009-2010 survey on the occurrence of PepMV in Cyprus and information on host range, molecular detection and characterization of the reported isolates.

**Materials and Methods**

**Survey and sample collection**

The survey was carried out during 2009-2010 in the main tomato growing areas of Lefkosia, Ammochostos, Larnaka, Lemesos and Pafos districts (Fig. 1, Table 1). More than 2500 samples were collected from 22 open field crops and 47 greenhouse tomato crops at 24 locations. In each field or greenhouse, plants were inspected and samples from those showing PepMV-like symptoms were collected. Each sample consisted of the youngest fully developed leaf from tomato plants exhibiting both leaf symptoms such as mosaic, mottle, or yellow patches as fruit symptoms such as abnormal discoloration, yellow-green mosaic, uneven ripening and shape distortion. In cases that no symptoms occurred, plant samples were randomly collected. In addition, the presence of PepMV was assessed on 964 weed samples belonging in 12 different families. The weed samples were randomly collected from a greenhouse in Parekklisia (Lemesos) and an open field tomato production area in the village of Odou (Larnaka) known to be infected with PepMV (Fig. 1, Table 2). Tomato and weed samples were placed in plastic bags, stored at 4°C and tested for virus infection either serologically (DAS-ELISA) or by RT-PCR (conventional and real-time) within 1-3 days following collection. Stock material was stored at -80°C for further studies.

**Serological assays**

Serological tests were carried out using the standard double-antibody sandwich enzyme linked immune-sorbent assay (DAS-ELISA) using commercial polyclonal antibodies (Bioreba, AG, Switzerland). Tissue samples (approximately 0.5 g of plant leaves or tomato fruit skin) were homogenized using Homex 6 semi-automated homogenizer (Bioreba, AG, Switzerland) in 5 ml of extraction buffer and processed according to the suppliers instructions. Positive and negative controls were included in all tests. Absorbance values were measured at 405 nm using a Jenway 640 S UV/Vis Spectrophotometer. Samples were considered infected (virus positive) if $A_{405}$ nm value was greater than three times the average of the corresponding healthy plant extract.

**Template preparation using total RNA and crude plant extracts**

Total RNA was extracted using MagMAX™ Total Nucleic Acid Isolation Kit, suitable for automated RNA extraction from plant tissues using MagMAX™ Express Magnetic Particle Processor (Applied Biosystems Inc., CA, USA) according to the manufacturer instructions. To avoid laborious and expensive nucleic acid extraction methods (Dovas and Katis 2003a; Dovas and Katis 2003b; Papayiannis et al., 2010a; Papayiannis et al., 2010b), a previously reported template preparation procedure, suitable for large scale surveys, was also applied. Briefly, hybond N+ membranes (Roche Diagnostics GmBH, Mannheim, Germany) were cut in small pieces (5 x 5 mm) and placed in the bottom of a sterile 0.5 ml microfuge tube using forceps. Samples of 0.2 g of plant tissue were macerated in an extraction buffer containing 20 mM Tris-HCl pH:8, 140 mM NaCl, 2% PVP-40, 1% Bovine serum albumin (BSA), 0.5% $Na_2SO_3$ and 0.05% Tween 20 at a 1:10 dilution using plastic micropestles. An aliquot of this extract was centrifuged at 2000 g for 2 minutes and 5 µl of the supernatant was carefully spotted onto the membrane into the microfuge tube and allowed to dry at room temperature for 30 min. Release of viral template was achieved by boiling the spotted membrane in 50 µl of a virus release buffer (0.1M Glycine pH:9, 50mM NaCl, 1mM EDTA, 0.5% Triton X and 0.2 g per ml Betaine) at 94°C for 5 min, followed by vigorous vortexing and placement on ice. Alternatively, 10 µl of the prepared supernatant were placed into a sterile 1.5 ml microfuge tube containing 90 µl of virus release buffer, boiled at 94°C for 5 min, vortexed and chilled on ice.

**Conventional RT-PCR assays**

One microlitter of RNA extracts was analyzed by one step conventional reverse transcription (RT)- polymerase chain reaction (PCR) with universal primers KL05-13 and KL05-14, designed to amplify a 200 bp product from all PepMV genotypes (Ling et al., 2008) (Table 3)

using SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen Life Technologies, Carlsbad, USA). Amplification reactions were performed in a PTC-200 thermal cycler (MJ Research, Inc. USA). Amplified products were analyzed by gel electrophoresis on 1.5% agarose gels in TAE buffer, stained with ethidium bromide and photographed under UV light.

**Real-time RT-PCR analysis**

In addition to conventional RT-PCR, a TaqMan RT-PCR was developed and used to speed up the procedure. A multiple sequence alignment of different PepMV isolates from different geographical regions available in the GenBank database (FJ940223, AM491606, AJ438767, AF484251, AJ6066359), AJ606360), FJ940224, AY509926, FJ940225, AY509927, DQ000984, FJ212288, DQ000985, FJ612601, EF408821) was performed using the Clustal X programme (Thomson et al., 1997). Two forward primers, one dually labelled probe and a reverse primer (Microsynth-Biotech AG, Ebersberg, Germany) were designed for a TaqMan® RT-PCR assay to amplify an 85bp fragment of the triple gene block (TGB1) of different PepMV isolates. The probe was designed to have the $T_m$ value approximately 9-10°C higher (69-70°C) than that of the primers (59-60°C), with its length not exceeding 30 nt and its % GC content higher than 40% (Table 3).

To determine the optimum primer concentration for each TaqMan® RT-PCR assay, a range of primer concentration titration was carried out using a 5X5 matrix of 600, 500, 300, 100 and 50 nM. The optimum primer concentration that resulted in the highest recorded fluorescence and the lowest threshold cycle (the cycle at which a significant increase of fluorescence occurs, CT) was selected. The TaqMan® reaction assay was validated and optimized in a final volume of 25 µl containing 12.5 µl of EXPRESS One-Step Superscript® qRT-PCR Universal (Invitrogen, Carlsbad, CA), 0.3 µM of each primer, 0.1 µM TaqMan® probes and 1 µl purified RNA extract or 2 µl crude or membrane spotted extract. Cycling parameters were 50°C for 30

min and 4 min at 94°C followed by 35 cycles of 10 sec at 94°C and 30 sec at 60°C. The assays were developed and evaluated on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers and probe were further tested using PepMV isolates collected from Cyprus, Spain, UK and the Netherlands. Number of *Potato virus X* (PVX) isolates were included in the assays to verify the specificity of the reaction.

**Biological and molecular characterization of PepMV isolates from Cyprus**

Two isolates collected from infected tomato plants (CY-Parekklisia and CY-Odou) were selected for biological and molecular characterization assays. For the biological characterization, leaf samples were ground in 0.1 M phosphate ($Na_2HPO_4/KH_2PO_4$) buffer pH 7.2 in a ratio of 1/10. Mechanical inoculations were performed to at least five plants of different cultivated plant species by gentle rubbing the third or fourth truly developed leaf with 200 µl of the extracts pre-dusted with Carborundum. Five plants of different weed species that were previously identified as virus hosts, were also mechanically inoculated. The complete list of test plant species used in the bioassay is shown in Table 4.

For the molecular characterization of CY-Parekklisia and CY-Odou PepMV isolates, total nucleic acid extraction was performed from 0.1 g of dried leaf tissue using the silica capture extraction protocol (MacKenzie *et al.,* 1997). In order to identify the specific isolate of PepMV from the infected samples, a multiplex RT-PCR assay was performed as described by Alfaro-Fernández *et al.* (2009). Later, a RT-PCR reaction was performed using the SuperScript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen Life Technologies. Barcelona, Spain) with three different pairs of primers listed in Table 3. The following primer sets were used: Pep3/Pep4 and CP-D/CP-R which amplify a fragment of the RNA dependent RNA polymerase (RdRp) gene and the complete coat protein (CP) gene, respectively (Pagán *et al.,* 2006), and set PepTGB-D/PepTGB-R which amplify the

complete triple gene block (TGB) genes (Alfaro-Fernández *et al.,* 2008). The amplified PCR products were analysed on 1.2% agarose/TAE gels stained with ethidium bromide and visualized under UV light. Amplified products of each group were purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced.

The obtained sequences were deposited in GenBank and compared with PepMV isolates retrieved online from the National Centre of Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The identity of the obtained nucleotide and the deduced amino acid sequences were calculated with MatGAT version 2.01 (Matrix Global Alignment Tool; Campanella *et al.,* 2003; http://bitincka.com/ledion/matgat). Phylogenetic analyses based on the nucleotide and amino acid sequences were performed with MEGA version 3.1 (Kumar *et al.,* 2004), using the neighbour-joining algorithm. The statistical reliability of the constructed tree was assessed with 10,000 bootstrap pseudoreplicates.

## Results

### Incidence of PepMV on tomato crops and weeds in Cyprus

More than 2500 tomato plants were collected from all major tomato production areas of Cyprus and tested using serological and molecular methods. PepMV was found to infect tomatoes in three districts of Cyprus (Lemesos, Larnaka and Ammochostos). The virus incidence in Lemesos district was higher than that in Larnaka (69.7% and 25.6% of the positive samples were detected, respectively), whereas only a few infected samples (4.7% of the positives) were detected in Ammochostos district (Table 1). In Larnaka, most of the positive samples were found in open field cultures in the area of Odou and sometimes in mixed infection with *Tomato yellow leaf curl virus* (TYLCV) and *Tomato spotted wilt virus* (TSWV) (data not shown). A total of 964 weed samples from 12 families occurring in PepMV infected tomato crops from greenhouses and open fields were randomly collected and

identified in Weed Science Laboratory of Agricultural Research Institute (Table 2). These species were tested by DAS ELISA, conventional and real-time RT-PCR. Sixty-three weed plants belonging to 20 species in the families of *Amaranthaceae*, *Chenopodiaceae*, *Compositae*, *Convolvulaceae*, *Malvaceae*, *Plantaginaceae* and *Solanaceae* were infected with PepMV (Table 2). Most of the infected weeds (48) were collected outdoors, where weed flora was abundant, whereas the remaining 14 virus-infected weeds were collected in a tomato greenhouse.

**Virus detection with real-time RT-PCR, conventional RT-PCR and serological assays**

The concentration of primers and probes that yielded the highest reporter fluorescence and the lowest threshold cycle was 300 nM for primers and 100 nM for fluorogenic probes on all tests (data not shown). The results showed that the assays were specific for PepMV and did not cross react with any of the PVX isolates. Primers PepMV F1/PepMV F2/PepMV R and the probe PepMV-TAQ were able to amplify and produced increased fluorescence in all tested isolates belonging to strain Chile-2 (CH-2) including the Cypriot isolates. In addition, the assay reacted positively with European strains from Spain, Nethelands and the UK.

The sensitivity of the real-time RT-PCR was compared with that obtained by conventional RT-PCR and DAS-ELISA for infected tomato samples. In a direct comparison, the TaqMan® RT-PCR assay detected the virus down to a dilution of $1:10^6$. In contrast, the dilution end-point for the conventional assay that gave a visible band in gel electrophoresis was $1:10^3$. Serology tests gave a clear positive reaction of two times the optical density of the negative value at a dilution of 1:100.

**Influence of template preparation method on the detection of viral RNA with real-time RT-PCR**

Results showed that real-time RT-PCR assay using TaqMan® chemistry was able to detect the virus target, either using positively charged nylon membranes spotted with sap, or by the direct addition of crude plant extract into the real-time reaction cocktail. In general, the recorded cycle threshold (CT) values were lower when total RNA was used as template in the reaction. The resulted CT for PepMV infected tomato were approximately 18-20 when total RNA was used as template, 23-25 for diluted crude plant extracts and 24-28 for extracts spotted onto membrane (Fig.2). Other possible hosts, such as infected weeds were also tested using the three template preparation types. Results indicated that both crude and spotted preparations from alternative infected hosts can work in real-time RT-PCR. Comparisons of end point analyses revealed that the pathogen target RNA was preserved for at least 6 months at both room temperature and fridge storage, without significant threshold cycle differences. Deep freezer storage of membranes at -20°C, allowed detection for up to one year after application to the membrane.

**Biological and molecular characterization of PepMV isolates from Cyprus**

Both CY-Parekklisia and CY-Odou infected systemically two different tomato (*S. lycopersicum*) varieties that are widely used in Cyprus (Table 4). In addition, both isolates infected systematically black-eye bean (*Vigna sinensis*), eggplant (*Solanum melongena*) and tobacco (*Nicotiana tabacum*) plants. Symptoms of mild mosaic were recorded on tomato and tobacco plants, whereas eggplant and bean plants appeared to be symptomless. The virus could not infect cucumber, melon, watermelon, zucchini, pepper, bean and potato plants (Table 4). In addition to cultivated plants, seven weed species belonging to different families that were found to be naturally infected with PepMV (Table 2) could be artificially infected without the appearance of any symptoms (Table 4).

Partial sequences of 2406 nt from isolates CY-Parekklisia (GenBank Accession number GU119903) and CY-Odou (GenBank Accession number GU119904) were obtained

comprising partial polymerase gene, complete TGB and CP genes. BLAST analyses revealed 98% to 99% nt identity with sequences from the CH2 strain of PepMV published in GenBank (GenBank accession nos FJ612601 and DQ000985, respectively). The two Cyprus isolates shared a 99.9% nt identity, with the only difference being a nucleotide substitution in TGBp1 which did not cause an amino acid substitution. Analysis performed with the MatGAT software showed that the Cypriot isolates shared nucleotide identities of 98.0% for the RdRp partial gene, 99.2% for the CP, and 98.0-98.2%, 97.8 and 98.0% for the TGBp1, TGBp2, and TGBp3, respectively with the CH2 isolate (Accession no. DQ000985; Table 5). Phylogenetic analyses of the two PepMV isolates from Cyprus grouped both isolates within the CH2 genotype of PepMV (Fig. 3).

**Discussion**

Since its first appearance on protected tomato crops in Europe in 1999, PepMV has displayed a great potential to adapt and spread, and within a few years the virus became established in several geographical regions and many different environments around the globe. Cyprus was considered to be free of PepMV for a long period of time, and the virus was not detected in any of the large scale surveys conducted between 2005-2008 in response to EU directives (Papayiannis et al., 2008). The first evidence of PepMV in Cyprus was a severe outbreak reported in the winter of 2009, on greenhouse tomato crops planted during September 2008 in two major producing areas. The results of this study showed that the virus has rapidly spread and established in several tomato production areas in Cyprus, as expected by the easy mechanical transmission of this virus.

The sudden appearance of PepMV in Cyprus is still under investigation and possible virus pathways include contaminated seeds, as well as imported infected tomato fruits (Hanssen et al., 2010). After pathogen introduction, an important transmission route could have been through the sharing of plastic package boxes between farmers, a local practice in tomato crops

in Cyprus. The rapid spread of the disease in different tomato production areas in Cyprus could have been facilitated by the proximity of tomato cultures, and farmers' unawareness for PepMV hygiene protocols.

PepMV-infected tomato fruits in Cyprus showed severe discoloration, yellow patches and uneven ripening that negatively affected yield quantity and quality. On rare occasions a mild mosaic appeared on newly-developed leaves. The symptoms occurred between January to mid March 2009, when temperatures were below 20°C. The same symptoms appeared from December to March in 2010, indicating that high temperatures in spring, summer and autumn which are above 22°C, and sometimes can reach up to 40°C during summer, have a negative effect on symptom appearance as reported before for Spanish tomato crops (Jordá et al., 2001a).

PepMV was mainly reported as a glasshouse/greenhouse disease as it was found to infect protected tomato crops, however the virus has been identified in natural infections of wild tomato species in Perú (Soler et al., 2002) and in some weed species grown in or around tomato greenhouses in Spain (Jordá et al., 2001b; Córdoba et al., 2004). In this study, the virus was detected in open field tomato crops and several different weed species, demonstrating that PepMV can easily infect outdoor fields. Furthermore, new weed species were identified as PepMV hosts in this study, including *C. arvensis* and *O. cyprium* (*Compositae*). In addition, mixed infections of PepMV with other economically important viruses, fungal and bacterial diseases such as *Tomato yellow leaf curl virus* (TYLCV), *Tomato spotted wilt virus* (TSWV), *Potato virus Y* (PVY), *Phytophthora infestans*, *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas corrugata* were recorded (L.C. Papayiannis, unpublished, Davino et al., 2008; Alfaro-Fernández et al., 2010b).

Real-time RT-PCR assays for the detection of PepMV were previously described and proved to be effective and reliable (Ling et al., 2007; Gutiérrez-Aguirre et al., 2009). In this study, a real-time RT-PCR assay was developed and optimized for the detection of PepMV based on TaqMan® chemistry, and was used to support serological assays. The results of this work

showed that the real-time RT-PCR assay allowed detection of the virus at far lower dilutions than conventional RT-PCR or serological assays.

The high sensitivity of real-time RT-PCR permits the use of a simple and quick template preparation of crude tomato plant extracts and eliminates the need for RNA extraction procedures. Template preparation can be accomplished either by spotting the crude extracts on a nylon membrane and elution into the reaction mix, or by the direct addition of crude plant extract into the real-time RT-PCR reaction mixture. This alternative template preparation procedure is simple, fast and cheap, as it avoids the expensive and time-consuming RNA purification step. This kind of template preparation procedures have been widely used for the detection of several vegetable and fruit-tree virus pathogens (Rowhani et al., 1995; La Notte et al., 1997; Dovas and Katis 2003a; Dovas and Katis 2003b; Osman and Rowhani 2006; Papayiannis et al., 2010a; Papayiannis et al., 2010b). The results of this study showed that this method can be applied for large scale surveys for PepMV detection in both tomato and weed hosts without compromising the reliability of the diagnosis. Furthermore, it enables detection of the virus in templates preserved at room temperature for up to 6 months, which allows field collection of samples and shipping to remote laboratories for testing.

Biological and molecular analyses showed only slight differences between the two selected PepMV isolates CY-Parekklisia and CY-Odou. Both isolates belonged to the CH2 genotype of PepMV, which is the predominant genotype in several countries of Europe. However, other PepMV genotypes and mixed infections between genotypes have been reported from European countries (Gómez et al., 2009; Hanssen and Thomma, 2010). Results of experimental host range analysis of the two Cypriot PepMV isolates were similar to those performed with other PepMV isolates belonging to EU and CH2 genotypes (van der Vlugt et al., 2002; Davino et al., 2008; Pospieszny et al., 2008). The Cypriot isolates of PepMV were efficiently transmitted to two tomato varieties widely used on the island, as well as to black-eye bean, eggplant and tobacco plants, but did not infect cucumber, melon, watermelon, zucchini, pepper, bean and potato plants. Plants not infected by PepMV could be used as

alternative cultures in virus control strategies. Although the host range of PepMV is considered to be quite narrow and mainly restricted to the *Solanaceae* family (van der Vlugt et al., 2002; Verhoeven et al., 2003), this work and other studies have shown that the virus can infect nonsolanaceous weeds which may play an important role in virus epidemiology by acting as virus reservoirs in crop free periods (Jordá et al., 2001b; Córdoba et al., 2004).

Efforts have to be undertaken to implement strict hygiene protocols during the growing season and at the end of the crop inside the greenhouses and outdoors to avoid further introductions and spread of the virus. Control measures must include using virus-free seeds and planting material, periodic monitoring of tomato crops and strict hygiene measures, since no resistant varieties are available at present.

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**Table 1** Characteristics of tomato crop surveys carried out in Cyprus in 2009 and 2010: map code, geographic location, number of samples tested and results of PepMV detection for all tomato isolates collected from Cyprus (areas with PepMV infection are indicated in bold)

| **Map Code** | **Area** | **District** | **No of samples tested** | | **PepMV postive** | |
|---|---|---|---|---|---|---|
| | | | **Glasshouse** | **Open field** | **Glasshouse** | **Open field** |
| 1 | **Parekklisia** | Lemesos | 415 | 115 | 182 | 58 |
| 2 | **Pyrgos** | | 326 | 25 | 139 | 12 |
| 3 | Kellaki | | 82 | 0 | 0 | 0 |
| 4 | **Zakaki** | | 54 | 0 | 15 | 0 |
| 5 | **Odou** | Larnaka | 0 | 218 | 0 | 112 |
| 6 | **Melini** | | 0 | 64 | 0 | 16 |
| 7 | Kiti | | 52 | 0 | 0 | 0 |
| 8 | **Zygi** | | 127 | 0 | 21 | 0 |
| 9 | Maroni | | 0 | 26 | 0 | 0 |
| 10 | Kalavasos | | 82 | 0 | 0 | 0 |
| 11 | Kokkinotrimithia | Lefkosia | 0 | 41 | 0 | 0 |
| 12 | Akaki | | 0 | 18 | 0 | 0 |
| 13 | Farmakas | | 0 | 134 | 0 | 0 |
| 14 | Deftera | | 0 | 41 | 0 | 0 |
| 15 | Lempa | Pafos | 72 | 0 | 0 | 0 |
| 16 | Mandria | | 79 | 0 | 0 | 0 |
| 17 | Kissonerga | | 55 | 0 | 0 | 0 |
| 18 | Chlorakas | | 61 | 0 | 0 | 0 |
| 19 | Argaka | | 64 | 0 | 0 | 0 |
| 20 | Gialia | | 68 | 0 | 0 | 0 |
| 21 | Agia Marina | | 81 | 0 | 0 | 0 |
| 22 | **Sotira** | Ammochostos | 92 | 55 | 21 | 0 |
| 23 | **Paralimni** | | 46 | 51 | 6 | 0 |
| 24 | Derynia | | 26 | 0 | 0 | 0 |
| **Total** | | | 1782 | 788 | 384 | 198 |

**Table 2.** Number of PepMV-infected annual (A) and perennial (B) weed species collected in greenhouses or open fields during the surveys conducted in 2009 and 2010.

| **Family** | **Species** | **Life span** | **Number of plants infected/tested** | **Greenhouse (G)/ Open field (OF)** |
|---|---|---|---|---|
| Amaranthaceae | *Amaranthus retroflexus* L. | A | 2/41 | 0/2 |
| | *Amaranthus viridis* L. | A | 3/18 | 0/3 |
| | *Amaranthus graecizans* L. | A | 5/51 | 1/4 |
| Chenopodiaceae | *Chenopodium album* L. | A | 0/21 | - |
| | *Chenopodium murale* L. | A | 2/30 | 0/2 |
| Compositae | *Calendula arvensis* L. | A | 6/58 | 0/6 |
| | *Chrysanthemum coronarium* L. | A | 0/12 | - |
| | *Chrysanthemum segetum* L. | A | 1/19 | 0/1 |
| | *Matricaria recutita* L. | A | 0/12 | - |
| | *Onopordum cyprium* Eig. | A | 2/21 | 0/2 |
| | *Sonchus asper* (L.) Hill | A | 6/28 | 2/4 |
| | *Sonchus oleraceus* L. | A | 2/31 | 0/2 |
| | *Sonchus tenerrimus* L. | A | 1/19 | 0/1 |
| Convolvulaceae | *Convolvulus arvensis* L. | B | 4/27 | 3/1 |
| | *Convolvulus humilis* Jacq. | B | 1/11 | 1/0 |
| Cruciferae | *Hirschfeldia incana* L. | A | 0/5 | - |
| | *Raphanus raphanistrum* L. | A | 0/9 | - |
| | *Sinapis alba* L. | A | 0/15 | - |
| | *Sinapis arvensis* L. | A | 0/22 | - |
| Euphorbiaceae | *Chrozophora tinctoria* L. | A | 0/8 | - |
| | *Euphorbia helioscopia* L. | A | 0/14 | - |
| | *Mercurialis annua* L. | A | 0/12 | - |
| Geraniaceae | *Erodium ciconium* (L.) L' Hérit. | A | 0/11 | - |
| | *Erodium cicutarium* L. | A | 0/6 | - |
| Malvaceae | *Malva cretica* Cav. | A | 0/16 | - |
| | *Malva neglecta* Wallr. | A | 1/29 | 0/1 |
| | *Malva nicaeensis* All. | A | 5/92 | 0/5 |
| | *Malva parviflora* L. | A | 7/71 | 2/5 |
| | *Malva sylvestris* L. | A | 2/54 | 0/2 |
| Plantaginaceae | *Plantago lagopus* L. | A | 1/18 | 1/0 |
| | *Plantago major* L. | A | 1/16 | 1/0 |
| Solanaceae | *Datura innoxia* Mill. | A | 2/25 | 0/1 |
| | *Datura stramonium* L. | A | 0/31 | - |
| | *Solanum nigrum* L. | A | 9/55 | 3/6 |
| | *Solanum villosum* Mill. | A | 0/12 | - |
| Umbelliferae | *Scandix pecten-veneris* L. | A | 0/5 | - |
| Urticaceae | *Urtica urens* L. | A | 0/39 | - |
| TOTAL | | | 63/964 | 14/48 |

**Table 3.** Primers and probes used in the conventional and real-time RT-PCR assays

| Names | Primer sequences (5′-3′) | Length | TM | Position | Product size | Reference |
|---|---|---|---|---|---|---|
| PepMV F1 | TGAACACAAGATTAACACTGAAGG | 24 | 60 | 4472-4495 [a] | 85 bp | This study |
| PepMV F2 | CGAGCCAAAACTCAGTGTTGAA | 22 | 60 | 4476-4497 [b] | | |
| PepMV TAQ | FAM-ATTGTTGTGCACGGAATTGCTGGAACTG-BHQ1 | 28 | 69 | 4500-4527 [a] | | |
| PepMV R | AAAGTCCTAAGYAATGTGGTTTTC | 24 | 60 | 4552-4529 [a] | | |
| KL05-13: | GTCCTCACCAATAAATTTAG | 22 | 52 | 5374-5393 | 201 bp | Ling et al., 2008 |
| KL05-14: | AGGAAAACTTAACCCGTTC | 21 | 53 | 5557-5575 | | |
| Pep3 | GAGGTTGTCTGGTGAAAGGTCC | 22 | 55 | 3899-3919[b] | 624 bp | Pagán et al., 2006 |
| Pep4 | CAACTTCCGTGCACAACTATGA | 22 | 55 | 4525-4502[b] | | |
| Cp-D | CACACCAGAAGTGCTTAAAGCA | 22 | 57 | 5522-5543[b] | 845 bp | Mumford and Metcalfe, 2001 |
| Cp-R | CTCTGATTAAGTTTCGAGTG | 20 | 57 | 6367-6348[b] | | |
| TGB-D | GATGAAGCTGAACAACATTTC | 21 | 55 | 4298-4318[a] | 1435 bp | Alfaro-Fernández et al., 2008 |
| TGB-R | GGAGCTGTATTRGGATTTGA | 20 | 55 | 5737-5717[b] | | |

[a] GenBank Accession no. DQ000985 (Pepino mosaic virus, CH2 strain), [b] GenBank Accession no. FJ940223 (Pepino mosaic virus, EU strain)

**Table 4.** Host range studies and symptoms caused by two PepMV isolates from Cyprus on different cultivated and weed plant hosts

| | **PepMV isolate** | | | |
|---|---|---|---|---|
| | **CY-Parekklisia** | | **CY-Odou** | |
| | **Symptoms** | **RT-PCR** | **Symptoms** | **RT-PCR** |
| **Cultivated crops** | | | | |
| *Lycopersicon esculentum* FA179 | mm | + | mm | + |
| *Lycopersicon esculentum* Petra | mm | + | mm | + |
| *Vigna sinensis* | sl | + | sl | + |
| *Capsicum annuum* | - | ND | - | ND |
| *Phaseolus vulgaris* | - | ND | - | ND |
| *Solanum tuberosum* | - | ND | - | ND |
| *Solanum melongena* | sl | + | sl | + |
| *Cucumis sativum* | - | ND | - | ND |
| *Citrullus lannatus* | - | ND | - | ND |
| *Cucumis melo* | - | ND | - | ND |
| *Cucurbita pepo* | - | ND | - | ND |
| *Nicotiana tabacum* | mm | + | mm | + |
| **Weeds** | | | | |
| *Malva parviflora* | sl | + | sl | + |
| *Solanum nigrum* | sl | + | sl | + |
| *Sonchus oleraceus L.* | sl | + | sl | + |
| *Convolvulus arvensis L.* | sl | + | sl | + |
| *Chrysanthemum segetum L.* | sl | + | sl | + |
| *Calendula arvensis* L. | sl | + | sl | + |

+= positive, -= negative, ND= Not Detected, Mm= mild mosaic, sl= symptomless

**Table 5.** Percentage of nucleotide sequence identity based on partial RNA-dependent RNA polymerase (RdRp), and complete triple gene block protein 1 (TGBp1), triple gene block protein 2 (TGBp2), triple gene block protein 3 (TGBp3), coat protein (CP) genes among Cypriot isolates CY-PepMV-Parekklisia, CY-PepMV-Odou and selected PepMV isolates from the GenBank database.

| **Isolate** | **RdRp partial** | **TGBp1** | **TGBp2** | **TGBp3** | **CP** |
|---|---|---|---|---|---|
| SM.74 (Accession no. 109896) | 78.7 | 79.7-79.9 | 83.2 | 85.6 | 78.7 |
| LP-2001 (Accession no. AJ606361) | 79.0 | 79.7-79.9 | 83.2 | 85.6 | 78.7 |
| Sp-13 (Accession no. AF484251) | 77.7 | 78.7-78.9 | 81.9 | 85.2 | 78.3 |
| Fr (Accession no. AJ438767) | 77.9 | 79.1-79.3 | 82.7 | 86.8 | 78.2 |
| LE-2002 (Accession no. AJ606360) | 77.7 | 78.3-78.4 | 83.0 | 86.8 | 78.2 |
| CH1 (Accession no. DQ000984) | 75.9 | 78.2 | 84.9 | 84.8 | 80.5 |
| US1 (Accession no. AY509926) | 75.7 | 78.4 | 84.6 | 84.8 | 80.7 |
| US2 (Accession no. AY509927) | 75.7 | 90.6-90.8 | 98.6 | 97.6 | 99.6 |
| CH2 (Accession no. DQ000985) | 98.0 | 98.0-98.2 | 97.8 | 98.0 | 99.2 |
| PepMV-Pa (Accession no. FJ612601) | 99.1 | 99.0-99.1 | 98.9 | 98.4 | 99.4 |
| PepMV-PK (Accession no. EF408821) | 99.1 | 99.0-99.1 | 98.9 | 98.4 | 99.4 |

**Fig. 1.** Map of Cyprus showing the tomato samples collection sites. Locality codes are given in Table 1.

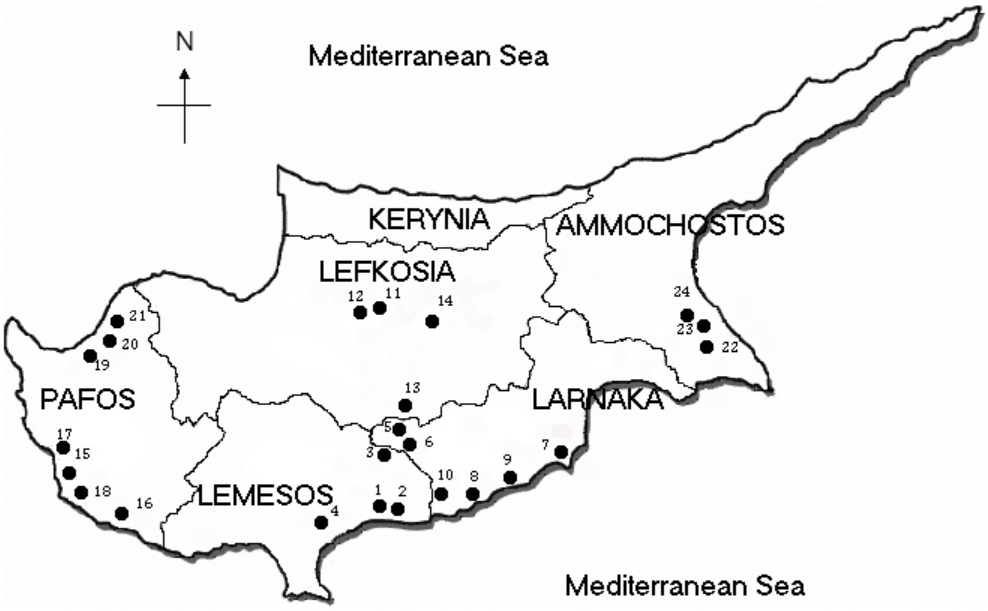


**Fig. 2.** Results of the comparison of different template preparation methods on end point real time RT-PCR: purified RNA (A), samples prepared by dilution extracts (B), samples prepared by spotted nylon discs (C), no template control (X)

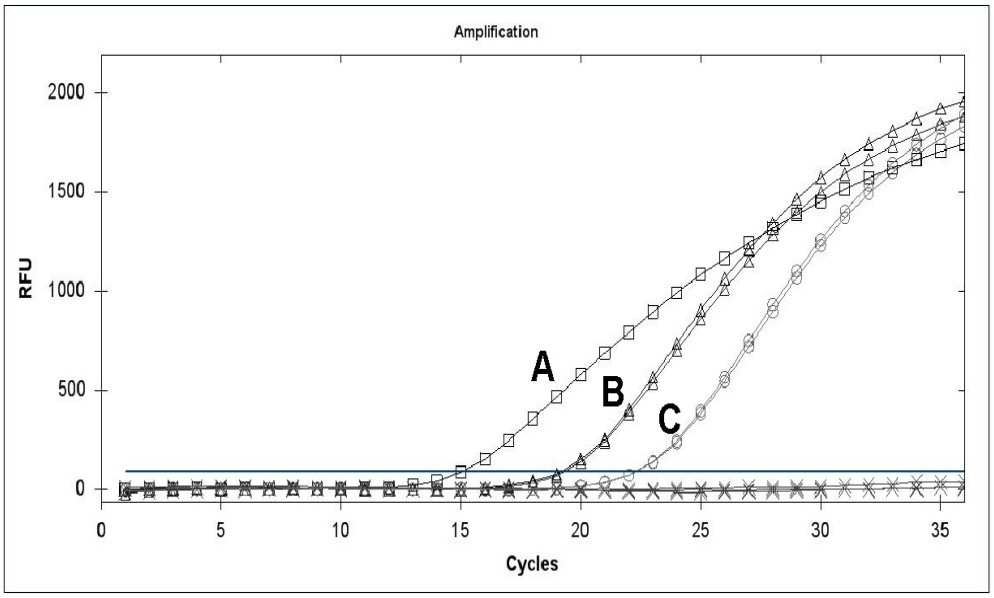
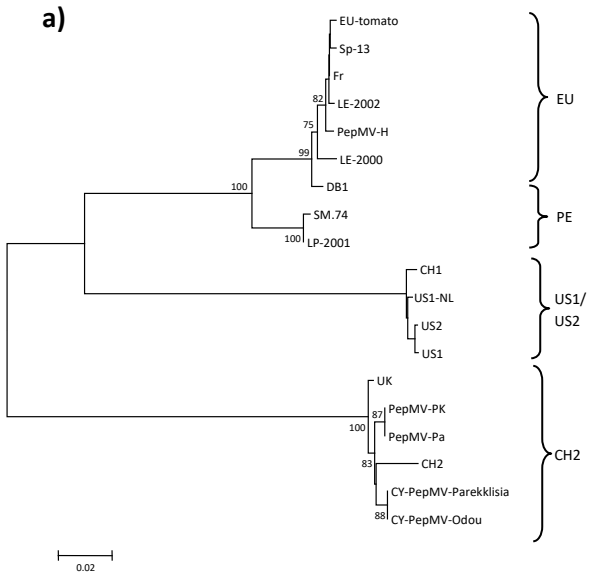
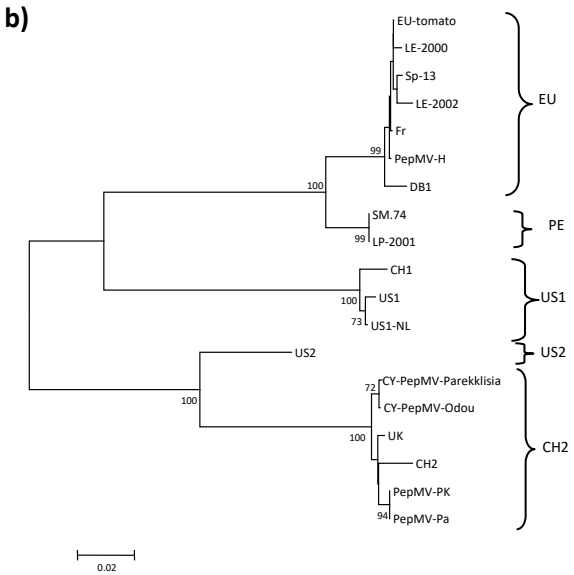


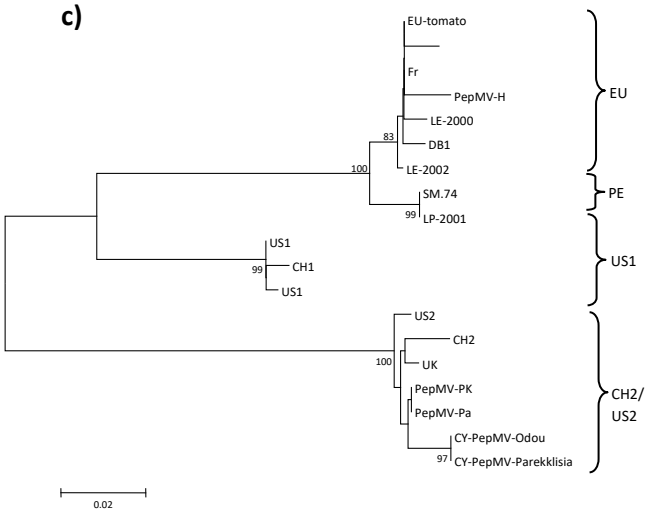
**Fig. 3.** Neighbour-joining trees based on the nucleotide alignments for five genomic regions of Cypriot isolates CY-PepMV-Pareklissia (Accession No. GU119903) and CY-PepMV-Odou (Accession No. GU119904) of *Pepino mosaic virus* (PepMV): RNA-dependent RNA polymerase partial gene (RdRp), the triple gene block-TGBp1, TGBp2, TGBp3 and coat protein gene (CP). Sequences of other PepMV isolates obtained from GenBank database were included as isolates from tomato: EU-tomato (Accession no. FJ940223), PepMV-H (Accession no. AM491606), Fr (Accession no. AJ438767), Sp13 (Accession no. AF484251), LE2000 (Accession no. AJ6066359), LE2002 (Accession no. AJ606360), DB1 (Accession no. FJ940224), US1 (Accession no. AY509926), US1-NL (Accession no. FJ940225), US2 (Accession no. AY509927), CH1 (Accession no. DQ000984), UK (Accession no. FJ212288), CH2 (Accession no. DQ000985), PepMV-Pa (Accession no. FJ612601), PepMV-Pk (Accession no. EF408821). Peruvian (PE) isolates were from *Lycopersicon peruvianum*, LP2001 (Accession no. AJ606361) and *Solanum muricatum*, SM74 (Accession no. AM109896). The numbers at each branch point indicate bootstrap values, branch values shown are also >70%. The scale bar for horizontal branch lengths represents genetic distances of 0.02.



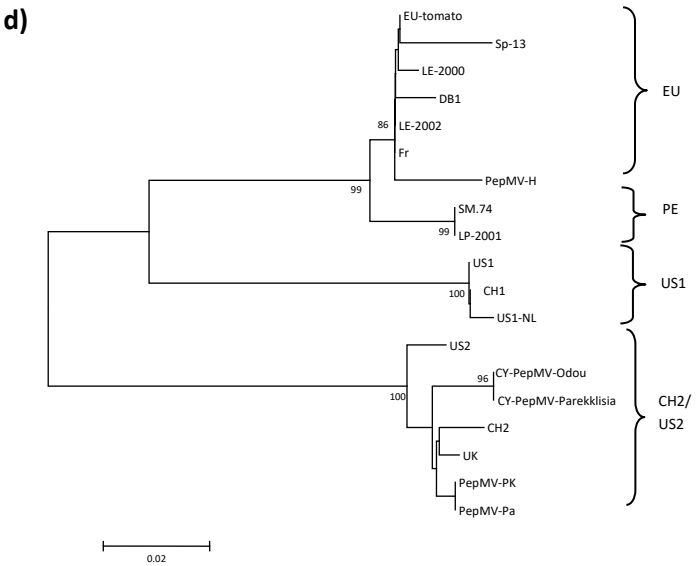

**RdRp partial**



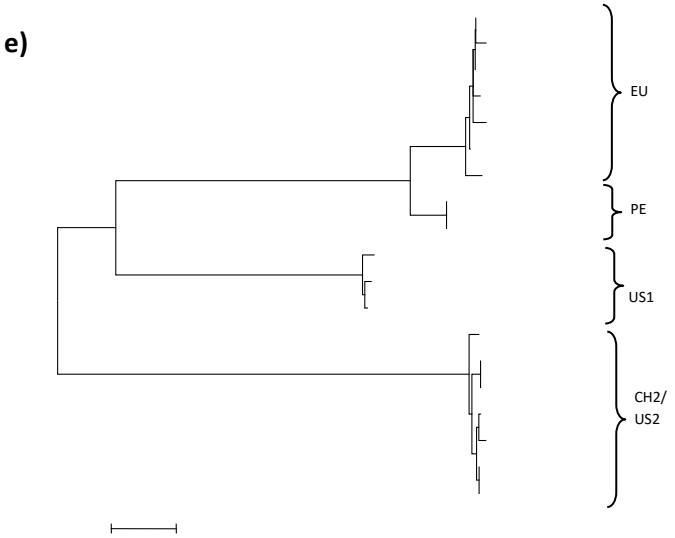

**TGBp1**




**TGBp2**




**TGBp3**




**CP**