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

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Abstract *Pepino mosaic virus* (PepMV, 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 discolouration, similar to those induced by PepMV. Consequently, an extensive survey was conducted in all tomato producing areas of the country to identify the incidence and prevalence of PepMV in protected and open field tomato crops. Analysis of 3500 leaf samples from tomato plants and weeds with DAS-ELISA and real-time RT-PCR showed that PepMV was present in all tomato growing areas of the island. The virus was detected in both protected and open field tomato plants, as well as in 20 weed species in the families of *Amaranthaceae*, *Chenopodiaceae*, *Compositae*, *Convolvulaceae*, *Malvaceae*, *Plantaginaceae* and *Solanaceae*. All Cypriot isolates assayed belonged to the CH2 genotype. Biological assays with two Cypriot isolates showed that they could infect cultivated and weed species including *Vigna unguiculata*, *Solanum melongena*, *Nicotiana tabacum*, *Malva parviflora*, *Sonchus oleraceus*, *Solanum nigrum*, *Convolvulus arvensis*, *Chrysanthemum segetum* and *Calendula arvensis*. To our knowledge, this is the first study to report *Chrysanthemum segetum* and *Calendula arvensis* as hosts of PepMV.

Keywords Genotype · PepMV · Potexvirus · Real-Time RT-PCR · Weed hosts

Abbreviations

CH2	chile 2 genotype
PepMV	*Pepino mosaic virus*
RT-PCR	reverse transcription- polymerase chain reaction

During the last ten years, *Pepino mosaic virus* (PepMV) (Genus *Potexvirus*, Family *Flexiviridae*) has emerged as an important viral disease of greenhouse tomato crops worldwide. PepMV was originally described in Peru during the 1970's infecting *Solanum muricatum* Ait. (Jones et al. 1980) and since then the virus has been reported to cause

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significant losses to tomato crops (*Solanum lycopersicum* L.) in many countries around the globe. The virus was first detected on the European continent in 1999, in glasshouse tomatoes in the UK (Mumford and Metcalfe 2001) and the Netherlands (van der Vlugt et al. 2000). The disease spread rapidly to many European countries, leading EU plant health authorities to create and enforce legislation against the introduction of the virus from third countries and the further spread of the virus within the EU (EU Decision 2004/200/EC).

PepMV is highly contagious and can be transmitted easily and efficiently by mechanical means (Wright and Mumford 1999) and through the seed to young tomato seedlings (Córdoba-Sellés et al. 2007; Hanssen et al. 2009). Infected tomato plants show a high variability in symptoms including mosaic, nettled heads, bubbling and yellow spots on the leaves, as well as yellow mottling, mosaic, discolouration, marbling and uneven ripening of fruits. As a result, PepMV infection causes a significant reduction in fruit quality (Spence et al. 2006).

The virus has a high level of genetic diversity. Four different PepMV genotypes have been recognized (EU, PE, CH2, US1) to date, with an inter-genotype RNA sequence identity of at least 80%. However, no correlation has been found between symptoms and genotypes (van der Vlugt 2009; Hanssen and Thomma 2010). Epidemiological and host range studies have shown that, in addition to tomato, PepMV can infect eggplant, potato, tobacco and other plants (Jones et al. 1980; van der Vlugt et al. 2002; Verhoeven et al. 2003; Pospieszny et al. 2008), as well as several weed species (Jordá et al. 2001a; Córdoba et al. 2004).

In Cyprus, the first PepMV-like symptoms were observed in January 2009 on greenhouse tomatoes showing leaf mosaic, mottling, yellow discolouration of fruits and uneven ripening. The virus was identified as PepMV using the PepMV AgriStrip kit (Bioreba, AG, Switzerland) and a previously described conventional reverse transcription (RT) polymerase chain reaction (PCR) assay (Ling et al. 2007). An extensive survey was performed during 2009 and 2010 in protected and open field tomato crops to determine the disease incidence on the island. More than 2500 samples were collected from 23 different locations in the main tomato growing areas of the country (Fig. 1, Table 1). Approximately 20–30 fully developed leaf samples were randomly collected from each greenhouse or field surveyed. At the same time, the presence of PepMV-like symptoms such as leaf mosaic and fruit discolouration was estimated and recorded for each surveyed crop. In addition, the presence of PepMV was assessed on 964 weed samples from 12 different families. Weed samples were randomly collected from a PepMV-infected greenhouse in Parekklisia (Lemesos) and a PepMV-infected open field tomato field in the village of Odou (Larnaka) (Fig. 1, Table 2).

Tomato and weed samples were tested for virus infection using serological and molecular methods.

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

Table 1 Tomato crop surveys for PepMV presence 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. The areas with PepMV infection are indicated in bold

Map Code	Area	District	Greenhouses			Fields			TOTAL PepMV positive samples
			No. surveyed greenhouses	No. samples collected	PepMV positive samples[a]	No. surveyed fields	No. samples collected	PepMV positive samples[a]	
1	**Parekklisia**	Lemesos	12	415	182 (8)	5	115	58 (3)	240 (45.3%)
2	**Pyrgos**		9	326	139 (7)	1	25	12 (1)	151 (43.1%)
3	Kellaki		4	82	0	0	0	0	0
4	**Zakaki**		4	54	15 (1)	0	0	0	15 (28.8%)
5	**Odou**	Larnaka	0	0	0	7	218	112 (5)	112 (51.4%)
6	**Melini**		0	0	0	4	64	16 (1)	16 (25%)
7	Kiti		4	52	0	0	0	0	0
8	**Zygi**		6	127	21 (1)	0	0	0	21 (16.5%)
9	Maroni		0	0	0	1	26	0	0
10	Kalavasos		4	82	0	0	0	0	0
11	Kokkinotrimithia	Lefkosia	0	0	0	2	41	0	0
12	Akaki		0	0	0	1	18	0	0
13	Farmakas		0	0	0	5	134	0	0
14	Deftera		0	0	0	2	41	0	0
15	Lempa	Pafos	4	72	0	0	0	0	0
16	Mandria		4	79	0	0	0	0	0
17	Kissonerga		3	55	0	0	0	0	0
18	Chlorakas		4	61	0	0	0	0	0
19	Argaka		4	64	0	0	0	0	0
20	Gialia		4	68	0	0	0	0	0
21	Agia Marina		5	81	0	0	0	0	0
22	**Sotira**	Ammochostos	5	92	21 (2)	2	55	0	21 (14.3%)
23	**Paralimni**		3	46	6 (1)	2	51	0	6 (16.6%)
24	Derynia		1	26	0	0	0	0	6 (16.6%)
TOTAL			81	1782	384 (20)	32	788	198 (10)	582

[a] The number of greenhouses or fields from which the positive samples were collected are shown in parentheses.

Serological tests were carried out using the standard double-antibody sandwich enzyme linked immunesorbent assay (DAS-ELISA) using commercial polyclonal antibodies (Bioreba, AG) according to the supplier's instructions. Samples were considered infected when absorbance values measured at 405 nm were greater than three times the average of the corresponding healthy plant extract. In addition, all samples were analyzed by real-time RT-PCR with primers KL05-48, KL05-49, KL05-51, KL05-52 and probe KL05-50, designed to amplify a 200 bp product from all PepMV genotypes (Ling et al. 2007). 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's instructions. Three PepMV isolates belonging to the EU, CH2 and US1 genotype (kindly provided by Plant Research International, The Netherlands and National Institute of Biology, Slovenia), were included as positive controls in all tests. A negative control obtained from a healthy tomato plant was also used in each test.

For genotype discrimination and typing of EU, PE, CH2 and US1 PepMV genotypes, a real-time qRT-PCR method (Gutiérrez-Aguirre et al. 2009) was used for the rapid screening of all PepMV positive samples originating from tomato and weed samples. Based on the results obtained from genotyping Cypriot PepMV isolates and due to the homogeneity of genotype distribution detected in the country, two isolates were

Table 2 List of weed species sampled during the surveys conducted in 2009 and 2010. All samples were tested using DAS-ELISA and real-time RT-PCR

Family	Species	No of plants infected/ tested	Collection sites of PepMV infected weeds Greenhouse/ Open field
Amaranthaceae	*Amaranthus retroflexus* L.	2/41	0/2
	Amaranthus viridis L.	3/18	0/3
	Amaranthus graecizans L.	5/51	1/4
Chenopodiaceae	*Chenopodium album* L.	0/21	0/0
	Chenopodium murale L.	2/30	0/2
Compositae	*Calendula arvensis*	6/58	0/6
	Chrysanthemum coronarium	0/12	0/0
	Chrysanthemum segetum	1/19	0/1
	Matricaria recutita	0/12	0/0
	Onopordum cyprium	2/21	0/2
	Sonchus asper	6/28	2/4
	Sonchus oleraceus	2/31	0/2
	Sonchus tenerrimus	1/19	0/1
Convolvulaceae	*Convolvulus arvensis*	4/27	3/1
	Convolvulus humilis	1/11	1/0
Cruciferae	*Hirschfeldia incana*	0/5	0/0
	Raphanus raphanistrum	0/9	0/0
	Sinapis alba	0/15	0/0
	Sinapis arvensis	0/22	0/0
Euphorbiaceae	*Chrozophora tinctoria*	0/8	0/0
	Euphorbia helioscopia	0/14	0/0
	Mercurialis annua	0/12	0/0
Geraniaceae	*Erodium ciconium*	0/11	0/0
	Erodium cicutarium	0/6	0/0
Malvaceae	*Malva cretica*	0/16	0/0
	Malva neglecta	1/29	0/1
	Malva nicaeensis	5/92	0/5
	Malva parviflora	7/71	2/5
	Malva sylvestris	2/54	0/2
Plantaginaceae	*Plantago lagopus*	1/18	1/0
	Plantago major	1/16	1/0
Solanaceae	*Datura innoxia*	2/25	0/2
	Datura stramonium	0/31	0/0
	Solanum nigrum	9/55	3/6
	Solanum villosum	0/12	0/0
Umbelliferae	*Scandix pecten-veneris*	0/5	0/0
Urticaceae	*Urtica urens*	0/39	0/0
		63/964	14/49

selected for molecular and biological characterization: Isolate CY-Parekklisia was collected from a greenhouse tomato plant showing severe fruit discolouration and leaf mosaic, and isolate CY-Odou was collected from an open field, tomato plant with mild leaf mosaic symptoms. The two isolates tested were negative to other plant viruses (*Cucumber mosaic virus*, *Tomato mosaic virus*, *Potato virus* Y, *Tomato yellow leaf curl virus* and *Tomato chlorosis virus*), which were previously reported to infect tomatoes on

the island (Papayiannis et al. 2008). Both isolates were then analysed using a multiplex RT-PCR assay for PepMV, as described by Alfaro-Fernández et al. (2009) and RT-PCR amplification products were directly sequenced to confirm the genotype identification. Different fragments of the virus genome were amplified by RT-PCR using SuperScript III one step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen Life Technologies, Barcelona, Spain) with three different pairs of primers: Pep3/Pep4, CP-D/CP-R (Pagán et al. 2006), and PepTGB-D/PepTGB-R (Alfaro-Fernández et al. 2008) and sequenced directly. 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 analysis based on nucleotide and amino acid sequences was 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.

Biological characterization was carried out by inoculating 12 species of cultivated plants (Solanaceae: *S. lycopersicum* hybrid F179, *Capsicum annuum*, *S. tuberosum*, *S. melongena* and *Nicotiana tabacum*; Cucurbitaceae: *Cucumis sativum*, *C. melo*, *Citrullus lanatus*; Leguminosae: *Phaseolus vulgaris* and *Vigna unguiculata*) and six species of weeds (*Malva parvoflora, Solanum nigrum, Sonchus oleraceus, Convolvulus arvensis, Chrysanthemum segetum* and *Calendula arvensis*) with the two Cypriot PepMV isolates using five plants per species. The inoculated plants were maintained in a greenhouse at 25°C with 14–16 h of natural lighting for 6 weeks. Symptom development was monitored weekly, and plant leaf tissues were analyzed for PepMV presence by DAS-ELISA and real-time RT-PCR as described previously in this work.

The results of the study showed that PepMV was present in three districts of Cyprus (Table 1). The incidence of PepMV was higher in Lemesos and Larnaka (406 and 149 positive samples out of 1017 and 569, respectively), whereas only 27 positive samples out of 244 were detected in Ammochostos (Table 1). In Larnaka, most of the positive samples were found in open field cultures (128 positive samples out of 149) in the area of Odou and sometimes in mixed infections with *Tomato yellow leaf curl virus* (TYLCV) and *Tomato spotted wilt virus* (TSWV) (data not shown). As estimated, the occurrence of PepMV-like symptoms, ranged between 50–80% and 10–30% in all infected greenhouses and open field tomato crops, respectively. Symptoms were not observed in the virus-free tomato crops.

PepMV was not detected in Cyprus in large scale surveys conducted during 2005–2008, in response to EU directives (Papayiannis et al. 2008). The results of the current study show that the virus has rapidly spread and established in several tomato production areas of the island. The sudden appearance of PepMV in Cyprus is still under investigation and possible virus pathways include contaminated seed, as well as imported infected tomato fruits (Hanssen and Thomma 2010). The spread of the virus on the island could have been facilitated through the sharing of plastic package boxes which is common between farmers, its easy mechanical transmission, the proximity of tomato cultures, and farmers' unawareness for PepMV hygiene protocols. Furthermore, 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). PepMV symptoms were visible on plants during the winter and early spring of 2009 and 2010, when temperatures were below 20°C.

A total of 63 weeds belonging to 20 species in the families of *Amaranthaceae*, *Chenopodiaceae*, *Compositae*, *Convolvulaceae*, *Malvaceae*, *Plantaginaceae* and *Solanaceae* were also found to be infected with PepMV (Table 2). Most of the infected weeds (49 samples) were collected outdoors in the area of Odou, where weeds were abundant. The remaining 14 virus-positive weeds were collected from a greenhouse in Parekklisia. So far, PepMV has been mainly reported as a glasshouse/greenhouse disease (Jordá et al. 2001a; Soler et al. 2002; Córdoba et al. 2004). In this study, the virus was also detected in open field tomato crops and several weed species. Weeds 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).

A partial sequence of 2406 nt was obtained for both CY-Parekklisia (GenBank Accession number GU119903) and CY-Odou (GenBank Accession num-

ber GU119904) isolates. The obtained sequence included part of the polymerase gene (RdRp) and the full length of the TGB and CP genes. BLAST analyses revealed 98% to 99% nt identity with sequences from the CH2 genotype of PepMV published in GenBank (GenBank accession nos FJ612601 and DQ000985, respectively). The two Cypriot 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 nucleotide identities between the Cypriot isolates and the CH2 isolate (Accession no. DQ000985) were 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 genes, respectively. Phylogenetic studies grouped both Cypriot PepMV isolates within the CH2 clade in all genome fragments studied. In addition, all Cypriot isolates tested, including 300 positive tomato and 60 weed samples, were typed as CH2 genotype. The CH2 type, which is the predominant genotype in several European countries, has been gradually replacing the EU type in Europe in recent years (Gómez et al. 2009; Hanssen and Thomma 2010).

Biological assays revealed that both CY-Parekklisia and CY-Odou isolates infected tomato, black-eyed pea (*V. unguiculata*), eggplant (*S. melongena*) and tobacco (*N. tabacum*) plants. Symptoms of mild mosaic were recorded on tomato and tobacco plants, whereas eggplant and pea plants showed no symptoms. The virus did not infect cucumber, melon, watermelon, zucchini, pepper, bean and potato plants which can be used in a crop rotation to control the virus. In addition, seven weed species that were found to be naturally infected with PepMV (Table 2) were artificially infected without symptom development. The host range analyses of the two Cypriot PepMV isolates were similar to those performed previously with isolates belonging to EU and CH2 genotypes (Jorda et al. 2001b; van der Vlugt et al. 2002; Verhoeven et al. 2003; Pospieszny et al. 2008), To our knowledge, the current study is the first to report *Calendula arvensis* and *Chrysantemum segetum* as natural hosts of PepMV

Implementation of strict hygiene protocols are needed to avoid further introductions and spread of the virus on the island. Control measures must include the use of 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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