

## **Pepino mosaic virus and Tomato chlorosis virus causing mixed infection in protected tomato crops in Sicily**

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**Summary.** An unusual virus-like yellow leaf disorder associated with fruit marbling was observed during the winter of 2005 in some greenhouse tomato crops in the province of Ragusa Sicily (Southern Italy). Leaf samples from 250 symptomatic tomato plants were serologically tested by DAS-ELISA technique for 5 viruses: *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV) and *Pepino mosaic virus* (PepMV). PepMV was detected in 215 of the samples. The virus was mechanically transmitted to cucumber, wild melon, wild tobacco and 'Rio Grande' tomato. The experimental host range of PepMV-Ragusa differed from that of the PepMV found in Sardinia in 2001, which infected 'Camone' tomato. By applying RT-PCR to 25 PepMV-infected tomato plants, the expected 844 bp DNA fragment for PepMV and the expected 439 bp DNA fragment for *Tomato chlorosis virus* (ToCV) were obtained from all the samples tested. Sequences of the obtained amplicons were used to study the phylogenetic relationships of the viruses with isolates from other countries. Nucleotide sequence alignments showed that the sequence CP-PepMV-Ragusa (Genbank acc. No. DQ 517884) were 99% homologous with both US2 and Spain-Murcia isolates, while those of ToCV-Ragusa (Genbank acc. No. DQ517885) isolate HSP70, were 99% homologous with the Florida isolate, and 98% with the Lebanon isolate. The results proved that the unusual disorder found in greenhouse tomatoes in Sicily can be associated with infections by PepMV and ToCV, reported for the first time in a mixed infection.

**Key words:** Italy, host range, RT-PCR, phylogenetic analysis.

### **Introduction**

A number of viruses infect tomato (*Lycopersicon esculentum* Mill.) crops, reducing fruit quality and yield. These viruses include *Pepino mosaic virus* (PepMV) and *Tomato chlorosis virus* (ToCV).

*Pepino mosaic virus* (genus *Potexvirus*, family *Flexiviridae*) was first found in pepino or pear melon (*Solanum muricatum* Aiton) growing in the coastal region of Peru in 1974 and was characterized in

1980 (Jones *et al.*, 1980). At the beginning of 1999 this virus was detected in protected tomato crops in the Netherlands (Van der Vlugt *et al.*, 2000) and thereafter in other European and non-European countries, such as Germany (Lesemann *et al.*, 2000), Italy (Roggero *et al.*, 2001), Belgium (Mumford and Metcalfe, 2001), Spain (Jordà *et al.*, 2001), the United Kingdom (Mumford and Metcalfe, 2001), Canada (French *et al.*, 2001), France (Cotillon *et al.*, 2002), the United States (French *et al.*, 2001; Maroon-Lango *et al.*, 2005) and Austria (Verhoeven *et al.*, 2003). In addition to cultivated tomato and pepino species, PepMV was also found to infect some asymptomatic weeds collected around affected

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tomato glasshouses in Spain (Jordà *et al.*, 2001), and wild tomato and other *Lycopersicon* species in Peru (Soler *et al.*, 2002). PepMV virions consist of elongated particles of 470×13 nm, with a monopartite genome of positive single-stranded RNA (6450 nt). The genome organization of PepMV is typical of the genus *Potexvirus* with the following reading frame order: ORF 1, encoding a putative replicase; ORFs 2-4, triple gene block proteins (TGBp) 1-3; and ORF 5, coat protein (CP). The symptoms of PepMV infection in tomato include chlorosis, necrotic spots in older leaves, mosaic on the younger leaves; marbling on the stems and fruits and non-ripening of fruits. As the virus is highly contagious, it spreads easily within greenhouses by manual handling, contamination of pots, grafting, etc., by pollinating insects and, in the case of some tomato cultivars, also by seed (Córdoba *et al.*, 2007), (i.e., PepMV was not found to be seed transmissible in the tomato cv. Camone) (Salomone and Roggero, 2002).

ToCV is the causal agent of a tomato disease called 'yellow leaf disorder', reported for the first time in Florida (USA) (Wisler *et al.*, 1998a), and currently also occurring in the Mediterranean area (Portugal, Italy, Greece, Spain, Cyprus, Lebanon and Israel), in Réunion Island and Taiwan (Lozano *et al.*, 2006). ToCV is a typical member of the *Closteroviridae*, with long flexuous virions varying from 800 to 850 nm in length. All members of the *Closteroviridae* have large genomes of positive ssRNA but those belonging to the genus *Crinivirus* are divided into two RNAs: RNA 1 is organized into four ORFs and encodes proteins involved in replication; RNA 2 is composed of nine ORFs and encodes among others an HSP70 homolog and two proteins, involved in the encapsidation of viral RNA and referred to as the major coat protein (CP) and the minor coat protein (CPm). The CPm of ToCV is probably involved in determining the transmissibility of the virus by its vectors. (Wintermantel *et al.*, 2005). Vectors include the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood), the bandedwinged whitefly (*T. abutilonea* Haldeman) and sweetpotato whitefly (*Bemisia tabaci* Gennadius) biotypes A and B (Wisler *et al.*, 1998a). ToCV-infected tomato plants show interveinal leaf yellowing and become thicker and crispy on older leaves, flowers abort and the number and size of fruits decrease (Wintermantel *et al.*, 2005; Lozano *et al.*, 2006).

PepMV and ToCV (Roggero *et al.*, 2001; Accotto

*et al.*, 2001) have both been found in Italy infecting tomato crops but they have not been molecularly characterized.

During the winter of 2005, an unusual yellow leaf disorder associated with interveinal leaf yellowing and fruit marbling, was observed in some greenhouse tomato crops in Sicily (Vittoria - Ragusa province, Southern Italy). This paper describes the results of the research to identify and molecularly characterize PepMV and ToCV, the two viruses found for the first time to be involved together in a tomato disease.

## Materials and methods

### Field observations

The first leaf symptoms were observed in only one greenhouse on tomato plantlets grown from an imported non-commercial seed lot of unknown origin. The crop was periodically visually inspected in order to determine: symptom development on both leaves and fruit, percent incidence of symptomatic plants, and virus spread to other greenhouses located in the Vittoria area.

### Mechanical transmission

Symptomatic tomato leaf samples, were used for mechanical transmission to herbaceous plants belonging to the *Chenopodiaceae*, *Cucurbitaceae*, *Lamiaceae* (*Labiatae*), *Fabaceae* (*Leguminosae*) and *Solanaceae* families. At least four plants of each species were inoculated. The inoculated plants were inspected visually and symptoms scored weekly for four weeks. PepMV was detected in both symptomatic and symptomless inoculated plants by the double antibody sandwich, enzyme-linked-immunosorbent-assay (DAS-ELISA) (see below) and was confirmed by electron microscopy (leaf-dip preparation).

### Serology

Samples (two leaves each) from 250 plants of tomato showing the symptoms described above, were collected and tested by DAS-ELISA. Sera of the following viruses were employed: *Tomato spotted wilt virus* (TSWV, BR01), *Impatiens necrotic spot virus* (INSV), *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV DTL/ToRS) and PepMV; polyclonal antibodies from the Loewe-Phytopathologica Biochemica (Sauerlach, Germany) were used. All plantlets inoculated by sap were tested by DAS-ELISA.

### Molecular characterization

Twenty-five leaf samples, collected from 25 symptomatic tomato plants previously tested by DAS-ELISA, were used to extract total RNAs with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) (according to manufacturer's instructions), and to extract total DNA using a procedure previously described (Noris *et al.*, 1994). Total RNA extracts were used as templates for reverse transcription and polymerase chain reactions (RT-PCR) to amplify genes from PepMV, ToCV and *Tomato infectious chlorosis virus* (TICV), a *Crinivirus* serologically unrelated to ToCV, but responsible in tomato for similar symptoms (Duffus *et al.*, 1996; Wisler *et al.*, 1998b); total DNA extracts was used to amplify *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl sardinia virus* (TYLCSV), two viruses endemic in Ragusa province.

To amplify RNA-viruses the following primer pairs were used: PepMV-TGB and PepMV-UTR that amplify a fragment of 844 bp containing the CP gene, part of the TGB gene and UTR3 of PepMV (Mumford and Metcalfe, 2001) for PepMV; ToCV-172(+) and ToCV-610(-) which amplified a fragment of 439 bp containing part of the HSP70 gene of ToCV (Louro *et al.*, 2000) for ToCV; TICV-32(+) and TICV-532(-) which amplified a fragment of 500 bp containing part of the HSP70 gene (Vaira *et al.*, 2002) of TICV. To amplify DNA-viruses, primers MA272(+) and MA273(-) were used to amplify a fragment of 670 bp containing part of

the REP gene, intergenic region and part of the CP gene for TYLCSV, and primers MA272(+) and MA274(-) which amplified a fragment of 675 bp containing part of the REP gene, intergenic region and part of the CP gene for TYLCV (Garcia-Andres *et al.*, 2007). All the primer sequences are listed in Table 1.

RT-PCR for RNA-viruses was performed in one-step in a 25  $\mu$ l reaction final volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, 1  $\mu$ M of each primer, 4U of RNaseOut, 20 U of SuperScript II reverse transcriptase-RNaseH and 2U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out in a Peltier Thermal Cycler PTC 100 (M.J. Research INC., Waltham, MA, USA), under the following conditions and parameters: 42°C for 30 min, 94°C for 2 min, 35 cycles of 30s at 94°C, 30s at 56°C, and 30s at 72°C with a final elongation of 4 min at 72°C.

PCR for DNA-viruses was performed in a 25  $\mu$ l volume reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, 1  $\mu$ M of each primer and 2U of Taq DNA polymerase (Invitrogen). PCR was carried out in the same thermal cycler PTC 100, under the following conditions and parameters: 94°C for 2 min, 35 cycles of 15s at 94°C, 20s at 60°C, and 20s at 72°C with a final elongation of 4 min at 72°C. Total RNA and DNA extracts of tomato plants grown in a greenhouse of the Dipartimento di Scienze e Tecnologie Fitosani-

Table 1. Sequence of primers utilized in PCR and RT-PCR analysis for virus detection.

Primer	Sequence	Specificity	Amplicon size	Reference
PepMV-TGB	5'-CACACCAGAAGTGCTTAAAGCA-3'	PepMV	844 bp	Mumford and Metcalfe, 2001
PepMV-UTR	5'-CTCTGATTAAGTTTCGAGTG-3'	PepMV		
ToCV-172(+)	5'-GCTTCCGAAACTCCGTCTTG-3'	ToCV	438 bp	Louro <i>et al.</i> , 2000
ToCV-610(-)	5'-TGTCGAAAGTACCGCCACC-3'	ToCV		
TICV-32(+)	5'-TCAGTGCGTACGTTAATGGG-3'	TICV	500 bp	Vaira <i>et al.</i> , 2002
TICV-532(-)	5'-CACAGTATACAGCAGCGGCA-3'	TICV		
MA273(-)	5'-GGTTCGTAGGTTTCTTCAACTAG-3'	TYLCSV	670/675 bp	Garcia-Andres <i>et al.</i> , 2007
MA274(-)	5'-GCTCGTAAGTTTCTCAACGGAC-3'	TYLCV		
MA272(+)	5'-CTGAATGTTYGGATGGAAATGTGC-3'	TYLCSV, TYLCV		

tarie (DISTEF), University of Catania, Italy, were utilized as negative controls.

PCR fragments were cloned in an apCR2.1–TOPO vector Kit (TOPO-TA Cloning, Invitrogen) and transformed to *Escherichia coli* Math1 cell (Invitrogen) according to manufacturer's instructions. Purified recombinant plasmids were sequenced on an ABI PRISM DNA 377 sequencer (Perkin-Elmer, Boston, MA, USA) using standard M13 forward and reverse primers. The sequences obtained from PepMV (named in this work PepMV-*Ragusa*) were trimmed to remove the contaminating TGB and UTR sequences, leaving only the 714 nt long putative CP gene. The sequences of CP PepMV-*Ragusa* and partial HSP of ToCV obtained (named in this work ToCV-*Ragusa*) were aligned with those of related viruses using the CLUSTAL W program (Thompson *et al.*, 1994).

#### Phylogenetic relationship

The sequences obtained were employed to further study the phylogenetic relationship of the two viruses identified. The MEGA program (Kumar *et al.*, 2001) was applied to estimate the nucleotide distances between pairs of sequences (number of nucleotide differences per site) using the Kimura 2-parameter correction (Kimura, 1980). To compare the PepMV-*Ragusa* isolate with isolates from other countries, 14 nucleotide sequences were retrieved from GenBank entries: France (AJ438768), Estonia (AF510038), Spain (AJ606359; AJ606360; AM113811; AM113807; NC004067; AM113840), United States (AY509926; AY509927; AY508411), Peru (AJ606361; AM109896), England (AF340024). To compare the ToCV-*Ragusa* isolate with isolates from other countries, 12 sequences were retrieved from GenBank entries: Lebanon (DQ234079), Cyprus (AM158958), Florida (AY903848), St. Pierre Réunion (AJ968494; AJ968495; AJ968496), Portugal (AF234029), Spain (AF215817; AF215818; AF233435; DQ136146), Sicily (AY048854). Phylogenetic relationships were inferred using the MEGA based neighbour-joining method with a 1,000 replicate bootstrap value.

## Results

#### Field observations

During the first inspection in the greenhouse where the tomato plantlets were growing, inter-

veinal leaf yellowing symptoms were observed on almost 50% of the plants. In the other greenhouse crops of the same tomato grower, disease incidence was variable, with a maximum of 5% of symptomatic plants inside the nearest greenhouse; no diseased plants were found in greenhouses of other tomato growers in the Vittoria area. A second inspection after a month, revealed a severe increase in virus infection in all the tomato crops (from 50% and 5% to almost 100% and 30% respectively); in addition, a few symptomatic plants were found at other growers.

#### Mechanical transmission

Mechanical inoculation produced local infection only on 'Cubit' cucumber (*Cucumis sativus* L.) which showed chlorotic lesions, wild metel (*Datura metel* L.), wild tobacco (*Nicotiana benthamiana* L.) and 'Rio Grande' tomato which showed chlorotic lesions followed by systemic mosaic symptoms.

No infections occurred on nettle-leaves (*Chenopodium murale* L.), quinoa (*C. quinoa* Willd.), beetroot (*Beta vulgaris* L.), basil (*Ocimum basilicum* L.), bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), broadbean (*Vicia faba* L.), cowpea (*Vigna unguiculata* Endl.), 'Lungo della Cina' cucumber, potato (*Solanum tuberosum* L.) of an unknown cultivar, pepper (*Capsicum annuum* L.) 'California Wonder' and 'Lombardo', and also on different tomato cultivars ('Roma', 'Napoli', 'Principe Borghese').

#### Serology

All 250 samples tested were negative for TSWV, INSV, TMV and CMV, while 215 were positive to PepMV. Among the inoculated plantlets, symptomatic cucumber, metel, wild tobacco and 'Rio Grande' tomato were positive to PepMV.

#### Molecular characterization

RT-PCR technique to 25 tomato leaf samples (selected from the 215 PepMV-infected ones), all samples yielded the expected 844 bp DNA fragment for PepMV and the expected 439 bp DNA fragment for ToCV. No amplification was obtained with specific primers for the other viruses tested (TICV, TYLCV, TYLCSV) and no amplification was obtained from tomatoes grown in the DISTEF greenhouse and used as negative controls. Nucleotide sequence alignments of CP-PepMV-*Ragusa* between the sequences obtained and the sequences from GenBank,

were 99% homologous with isolates US2 and Spain-Murcia (Fig. 1). Nucleotide sequence alignments of HSP70 of ToCV between the sequences obtained and the sequences retrieved in GenBank were 99% homologous with the Florida isolate and 98% with Lebanon isolate.

## Discussion

Greenhouse tomato is an economically important crop in Sicily, with more than 5,000 hectares in Ragusa province. Tomato crops are commonly infected by several viruses, including TYLCV, TYLCSV and ToCV (Accotto *et al.*, 2001; Accotto *et al.*, 2003; Davino *et al.*, 2006). For this reason, since 1988 DISTEF of Catania University has been working hard to avoid the introduction and spread of new pathogens on the EPPO Alert Lists, such as PepMV. Our results provide evidence that this *potexvirus*, hitherto only discovered in diseased Sardinian tomatoes (Roggero *et al.*, 2001), is spreading to other Italian regions, causing severe symptoms also in combination with other viruses. The unusual

disorder found in the Sicilian greenhouse tomatoes was associated to PepMV and ToCV, found for the first time in a mixed infection.

The symptomatology described is 'unusual', if it is considered that in tomato plants the most prominent effects of PepMV are the colour alterations on the fruits (marbling), while those of ToCV consist of interveinal yellowing on the leaves. The disease found in mixed virus-infected tomatoes in Sicily, seems to combine both the effects of PepMV and ToCV alone, making a reliable differential diagnosis based on field symptoms difficult.

From an epidemiological point of view, periodic inspections of tomato grower crops, clearly indicate imported non-commercial seed as the origin of PepMV spread. In fact, a high percentage (50%) of leaf symptoms was first observed only in the greenhouses where tomato plantlets from these seeds were introduced. A month later, PepMV infected tomato plants were found in almost all the greenhouses in the same area. The virus spread was probably due to mechanical operations, soil movement and/or mechanical agricultural operations carried out by the tomato growers.

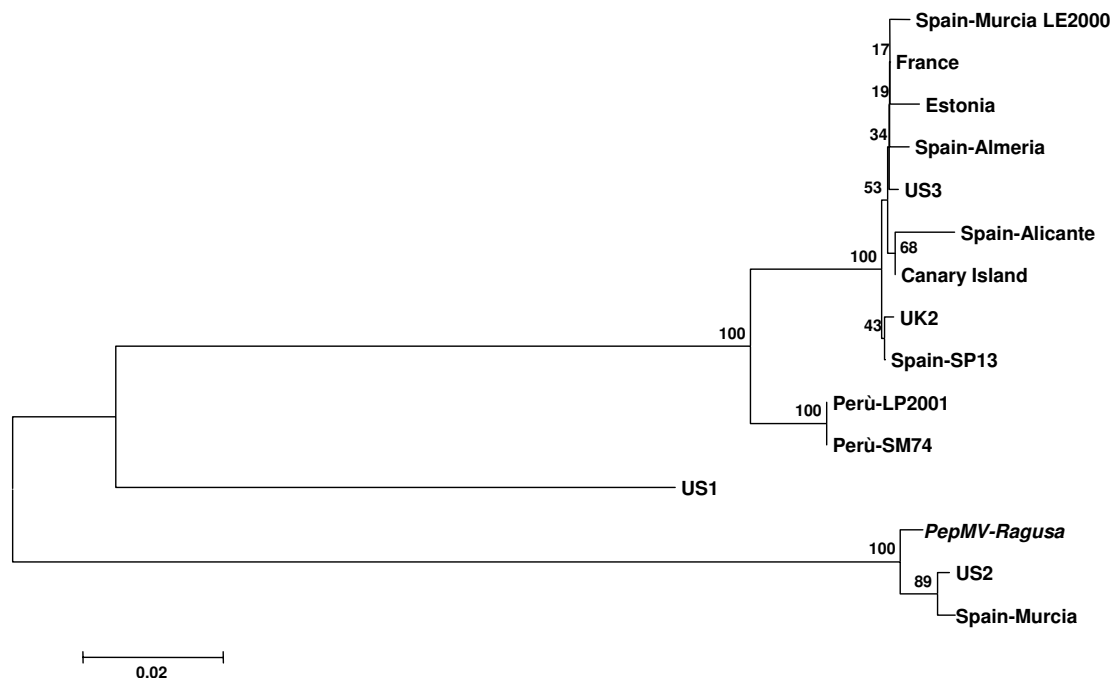


Fig. 1. Bootstrap consensus trees of CP of PepMV-Ragusa complete sequence, constructed with the MEGA program according to the neighbour-joining method. Bootstrap values (percentages) are indicated in the nodes; strain acronyms are as in the text.

PepMV-Ragusa was mechanically transmitted to 'Cubit' cucumber and some *Solanaceae* species, including melon and wild tobacco. This host range differs from that reported by Salomone and Roggero (2002) for PepMV in Sardinia which did not infect cucumber, but this virus locally infected *Chenopodiaceae* species: *Chenopodium amaranticolor* and *C. quinoa*. PepMV-Ragusa seemed more similar to the Spanish isolate which did not infect *Chenopodiaceae* species, but infected melon inducing systemic mosaic (Jordà *et al.*, 2001).

As regards wild tobacco, like almost all *Nicotiana* species, this plant is susceptible to PepMV infection, but also to that of ToCV (Wisler *et al.*, 1998b). However, Wintermantel *et al.*, (2005) to determine the complete nucleotide sequence of this *closterovirus*, isolated dsRNA from ToCV-infected wild tobacco plants previously infected by viruliferous sweetpotato whitefly or bandedwinged whitefly. After mechanical inoculations of the crude leaf sap of diseased tomato from Sicily, wild tobacco infections by PepMV alone were confirmed by both DAS-ELISA tests and electron microscopy.

PepMV virus was detected with both serological and RT-PCR techniques, while those of ToCV by TR-PCR only. No other viruses were serologically and/or molecularly detected, in particular CMV, TMV, TSWV, INSV, TICV, TYLCV, TYLCSV, indicating that the unusual disease found in greenhouse tomato crops can only be associated to the presence of *Potexvirus* and *Crinivirus* described above. Phylogenetic analysis using the MEGA programme generated a phylogenetic tree showing that the PepMV isolate in Sicily is different from those found in middle and northern Europe, yet very similar to the US2 and Spain-Murcia isolates, both present only in a few regions of the world and exactly in subtropical ones. These results show that probably all isolates of PepMV discovered in Europe do not have a common origin.

From an epidemiological point of view, aspects of the long-distance dissemination of these two viruses should be considered, like the trade in living plantlets, seeds, contaminated pots (for PepMV) or viruliferous whiteflies, such as sweetpotato whitefly and greenhouse whitefly (for ToCV) which, in Southern Italy, occur simultaneously, whether out in the open or in greenhouse crops. More restrictive controls are required to avoid PepMV spreading to other Italian regions as Apulia and Campania where tomato crops are widely spread.

The results showed that all the isolates of PepMV discovered in Europe probably do not have a common origin. Since PepMV-Ragusa the isolate was homologous to the US2 isolate discovered in USA (Maron-Lango *et al.*, 2005) and to the isolate found in Spain (GenBank Acc. No. AM113840)

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