SHORT COMMUNICATION

HOST RANGE, SEED TRANSMISSION AND DETECTION BY ELISA AND LATERAL FLOW OF AN ITALIAN ISOLATE OF *PEPINO MOSAIC VIRUS*

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SUMMARY

The potexvirus *Pepino mosaic virus* (PepMV) has recently been found infecting tomato in different European countries but little is known on the biodiversity of the different isolates or their relationships with the type strain from Peru. The experimental host range of an Italian isolate from tomato (PepMV-To) differed markedly from that of the isolate from pepino and to a less extent from that of a Spanish isolate from tomato. Among cultivated Solanaceae, eggplant but not pepper or potato were found to be susceptible to PepMV-To. The virus was detected by serology in tomato seeds but it was not seed transmissible to 50 seedlings; it was easily purified by a new procedure, and polyclonal antisera were produced. In tomato and other hosts the virus was efficiently detected by different ELISA procedures and by a quick field immunochromatographic system based on lateral flow, with 100% correlation between DAS ELISA and this new technique.

Key words: tomato, Italy, potexvirus, diagnosis, immunochromatography.

The potexvirus *Pepino mosaic virus* (PepMV) was originally found in pepino (*Solanum muricatum*) in Peru in 1974 and characterized by Jones *et al.*, 1980. The virus is a typical highly infectious potexvirus, with a narrow host range mainly limited to Solanaceae, no known arthropod or fungal vectors and not reported to be seed transmissible. PepMV was found only in pepino and for a long time there were no other reports of its occurrence (Review of Plant Pathology, from 1973 to 2000). Interest in the virus revived at the end of the 90s when it was found in tomato in the UK, the Netherlands, Germany, Spain, France and Italy (see Roggero *et al.,* 2001). The virus was also found on tomato in North America in 2000 (French *et al.,* 2001). In Spain PepMV was also detected in some asymptomatic weed plants collected around affected tomato glasshouses (Jordá *et al.*, 2001). The symptoms in tomato include chlorosis and scattered necrotic spots on older leaves, mosaic on stems and young leaves and alteration of the fruit colour resulting in uneven ripening. Although symptoms seem related to environmental conditions and possibly the cultivar, damage is always high, with unmarketable fruits. How the virus is spread within Europe is unknown but spread within glasshouse occurs easily by mechanical transmission, as the virus is highly contagious. PepMV is now included on the EPPO Alert List.

Little is known on the biodiversity of the European isolates of PepMV from tomato (PepMV-To) or their differences from the original isolate from pepino (PepMV-Pe). van der Vlugt *et al.* (2000) and Roggero *et al.* (2001) reported a serological difference between PepMV-To and the original PepMV-Pe. High homology was found in a stretch of less than 600 nucleotides of PepMV-Pe and PeMV-To from the Netherlands (van der Vlugt *et al.,* 2000).

In this paper we report the experimental host range of an Italian isolate of PepMV-To, an assay to determine a possible seed transmission in tomato, a new simplified procedure for purification of the virus, production of polyclonal antisera and developments of classical serological tests based on ELISA, as well as new detection procedures based on immunochromatography.

The isolate of PepMV-To was obtained in March 2001 from tomato cv. 'Camone' in Sardinia (Roggero *et al.,* 2001). No other viruses were detected in the sample, using electron microscopy after negative staining and mechanical inoculation to experimental hosts. It was maintained by mechanical inoculation in *Nicotiana benthamiana* and *Datura stramonium.*

Plants were grown in an insect-proof glasshouse in sterilized soil. For safety purposes, inoculated plants were kept in a growth chamber (photoperiod of 14 h provided by Sylvania Gro-Lux and Philips TL 95 fluorescent lamps, and 28-20°C day/night temperatures). At the end of the experiments, infected material was sterilized. At least 10 plants of each species were inocu-

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lated. Symptoms were scored weekly and presence of the virus was checked by ELISA one month after inoculation in inoculated and uninoculated leaves. Results are shown in Table 1.

Systemic infection was found only among Solanaceae, with severe symptoms in *N. megalosiphon* and *D. stramonium*, and mild mosaic in *N. benthamiana*, eggplant and tomato. Infection was latent in an unknown cultivar of pepino. The host range differed from that reported by Jones *at al.*, 1980 for PepMV-Pe since they did not find infection of *Chenopodium quinoa* and *C. amaranticolor* but their virus locally infected *Cucumis sativus* and systemically all *Nicotiana* species tested and also different potato cultivars.

Our isolate also seems different from the Spanish isolate (Jordá *et al.,* 2000), which was not able to infect the two *Chenopodium* spp. or tobacco, but infected *Gomphrena globosa* and potato, whereas for other test plants the reaction was similar.

Seeds were collected from naturally infected fruits testing positive by ELISA for PepMV, washed with water and dried at room temperature. The virus was detected by lateral flow in individual homogenates of 3 dried seeds. One month after emergence of the seedlings at the 2-3 true leaf stage they showed any symptoms, and leaves from 52 plants tested negative by ELISA. We conclude that the virus is not seed transmissible in tomato. This suggests that the virus is not seed transmissible, at least with a high transmission rate.

The virus was purified from naturally infected tomato or experimentally infected *N. benthamiana*. Leaves were homogenized with 5 vol. of ELISA extraction buffer (PBS pH 7.2 containing 0.05% Tween-20, 2% PVP Mr about 30,000 and 10 mM Na-DIECA). After filtration through nylon stocking, an equal volume of chloroform and 1:10 volume of n-butanol were added and the mixture shaken for 10 min. After low speed centrifugation, the aqueous phase was mixed with 5%

Table 1. Host range of the Italian isolate of *Pepino mosaic virus*.

Family	Species[a]	Inoculated leaves[b]	Uninoculated leaves[b]
Amaranthaceae	*Amaranthus retroflexus*	+, latent	–
	Gomphrena globosa	–	–
Chenopodiaceae	*Chenopodium amaranticolor*	+, cll	–
	C. quinoa	+, cll	–
Cucurbitaceae	*Cucumis sativus*	–	–
	Cucurbita pepo	–	–
Labiatae	*Ocimum basilicum*	–	–
Solanaceae	*Capsicum annuum,* cv. Quadrato d'Asti	–	–
	Datura stramonium	+, cs	+, mosaic
	Lycopersicon esculentum, cv. Marmande	+, cs	+, mosaic
	Nicotiana benthamiana	+, cs	+, mosaic
	N. clevelandii	+, cll	–
	N. glutinosa	–	–
	N. megalosiphon	+, cs, ns	+, mosaic, necrosis
	N. rustica	+, cll	–
	N. tabacum, White Burley type	+, latent	–
	Petunia x hybrida	–	–
	Solanum melongena	+, cs	+, mosaic
	S. muricatum	+, latent	+, latent
	S. nigrum	+, cs	+, mosaic
	S. tuberosum, cv. Spunta	–	–

[a] A minimum of 10 plants was inoculated for each species.
[b] +, virus detected by DAS ELISA one month after inoculation. –, virus not detected by DAS ELISA. Abbreviations: cll: chlorotic local lesions; cs: chlorotic spots; ns: necrotic spots.

PEG (Mr 6000), 1% NaCl and 0.2% Triton-X100 and stirred for 1 hour. Virus was recovered by low speed centrifugation, resuspended in extraction buffer and finally centrifuged in Beckman SW28 rotor for 150 min over a 20% sucrose cushion dissolved in PBS. The pellet was resuspended in PBS. It contained large amount of non-aggregated potexvirus-like particles observed by electron microscopy. Yield was 40 mg from 100 g of *N. benthamiana*, assuming A_{260}, 1 mg ml^{-1} = 3. This procedure is simpler than that reported by Jones *et al.,* 1980.

One rabbit and one hen were injected intramuscularly twice with 2 mg of the purified preparations after emulsion with Freund's adjuvant. Bleedings and eggs were taken three months after the first injection.

ELISA plates were coated overnight at 4°C with *N. benthamiana* sap diluted 1:100 in coating buffer pH 9.6 from healthy plants or infected with PepMV-To or PepMV-Pe (DSMZ PV-0554). Plates were blocked for 1 h at room temperature with PBS-Tween containing 2% PVP and 2% defatted milk. Rabbit antisera against PepMV-Pe (DSMZ-AS-0554) and PepMV-To and hen yolk were diluted with PBS-Tween containing 2% PVP, 2% defatted milk and 1% healthy *N. benthamiana* sap and tested in a twofold dilution series. Incubation was 2 h at 37°C. Bound antibodies were detected with anti-rabbit or anti-chicken antibodies conjugated with alkaline phosphatase from SIGMA. The titre was considered to be the last dilution having an absorbance 3 times or higher than the healthy control. The rabbit antiserum and hen yolk against PepMV-To had the same titre of 1:2,048,000 and 1:64,000 respectively with both virus isolates. DSMZ-As-0554 against PepMV-Pe had a homologous titre of 1:512,000 and a titre of 1:32,000 against PepMV-To. The difference in homologous and heterologous reactivity of the antiserum against PepMV-Pe was similar to that obtained by a decoration test in electron microscopy (Roggero *et al.,* 2001).

Rabbit serum and hen yolk were cross-absorbed with healthy tomato and *N. benthamiana* leaves before purification. Rabbit antibodies were purified by affinity chromatography with Protein G (Amersham Pharmacia Biotech). Yolk antibodies were precipitated with isopropanol and acetone and finally purified by affinity chromatography with HiTrap IgY column (Amersham Pharmacia Biotech). The antibodies were conjugated with alkaline phosphatase by using glutaraldehyde and a two-step procedure. DAS and TAS ELISA were compared in parallel with lateral flow-immunochromatography. ELISA plates (Greiner high binding type) were coated with 2 µg ml^{-1} of rabbit or hen antibodies and the same concentration was used when the antibodies were used as second antibodies. For DAS ELISA, conjugated antibodies were used diluted 1:500. With TAS ELISA bound antibodies were detected with anti-rabbit or anti-chicken antibodies conjugated with alkaline phosphatase from SIGMA. For immunochromatography, a lateral flow system (Danks and Barker, 2000) was developed with purified rabbit antibodies against PepMV-To. Predator Chromatographic membrane, absorbent pad, conjugate pad and sample pad were from Pall Corporation. Colloidal gold with a diameter of about 40 nm was from British Biocell and anti-rabbit antibodies were from SIGMA. As capture line, anti PepMV-To antibodies in PBS were used at 1 mg ml^{-1} and for the control line anti rabbit antibodies were used at 0.25 mg ml^{-1}. Colloidal gold was activated with anti PepMV-To antibodies according to the Pall manual with minor modifications. The final buffer was PBS containing 1% BSA, 1% sucrose and 0.25% Tween-20. After deposition of the reagents, membranes and conjugate pad were dried at 37°C for 6 h. Membranes were cut into strips about 5 mm wide and mounted in a plastic device, and the conjugate pad contained 12 µl of gold conjugate having an absorbance of about 3 at 530 nm. The devices were stored desiccated at room temperature. The samples were diluted 1:50 with ELISA extraction buffer containing also 0.5% Triton-X100 and about 150 µl was applied to each strip.

Tomato samples were collected in Sardinia at the end of May in infected glasshouses. A total of 20 leaf and 6 fruit samples was tested in parallel with all the procedures.

DAS ELISA with rabbit antibodies provided the best results, with high readings from infected samples and low background, whereas hen antibodies were ineffective after conjugation. With TAS ELISA, coating the plates with rabbit antibodies followed by hen antibodies also provided similar results, whereas coating with hen antibodies followed by rabbit antibodies resulted in a high background. Lateral flow detected the virus in all samples, with a clear band easily visible in 3-5 min. ELISA and lateral flow results from some samples are shown in Fig. 1.

The end point of detection of the virus by DAS ELISA and lateral flow was determined using two-fold dilution of a leaf tomato sample down to 16,384. The virus was detected by DAS ELISA up to 1:8192 whereas with lateral flow a band was still visible at dilution of 1:2048.

DAS ELISA and lateral flow were further compared for PepMV detection using 51 further samples. Of these, 31 were tomato either fruit or leaves; PepMV was detected in 4 samples with both methods. The other 20 samples were different experimentally infected plants and again the correlation between the 2 techniques was 100%.

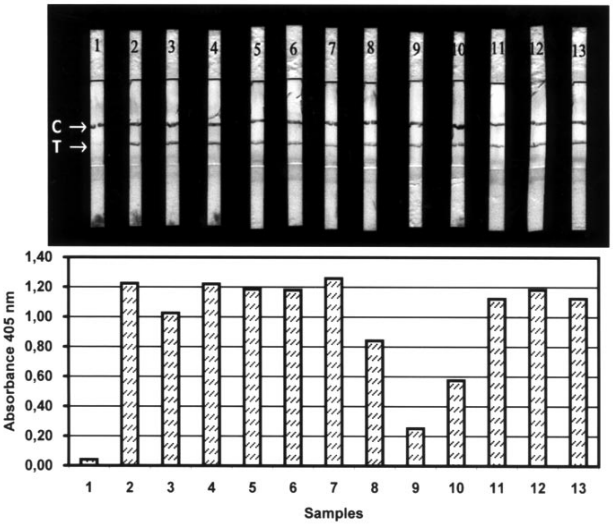

Fig. 1. Detection by lateral flow and DAS ELISA of *Pepino mosaic virus* in tomato. Sample 1 is healthy control, samples 2 to 8 are from leaves and samples 9 to 13 are from fruits. T indicates the test line and C indicates the control line.

The different serological procedures used with the two antisera against PepMV-To provide effective detection of the virus. For a large number of samples and with laboratory facilities available, the best method is ELISA, whereas lateral flow represents a reliable field detection system, providing results within few minutes, without the use of any equipment. The exact correlation between the two techniques provides assurance of the reliability of the newer technology of lateral flow. Among the ELISA procedures, the TAS protocol with non-conjugated antibodies from different animal species is generally able to detect more distantly related viruses, but DAS ELISA is preferred since the number of steps is lower.

In conclusion based on the test plant results, the Italian isolate of PepMV from tomato seems different from the original isolate from pepino and also different from the isolate from tomato found in Spain. Further studies are needed to clarify the picture of the biodiversity of PepMV isolates from tomato in Europe. The virus was not found to be seed transmissible in the tomato cv. 'Camone'. Aspects of the long-distance dissemination of the virus should be considered, like the trade in living plants or contamination of pots, or the possibility of seed transmission among other tomato cultivars and other *Lycopersicon* species used for grafting *L. esculentum*. Of interest is the possibility to detect the virus by lateral flow in seeds, to avoid any use of infected material with the possible risk of contamination of the environment.

ACKNOWLEDGEMENTS

Research at the Istituto di Fitovirologia Applicata supported by National Research Council of Italy. Special Project "Diagnosi precoce di malattie nelle piante di interesse agrario e forestale", paper no. 13. We thank R. Lenzi and C. Perrone for skillful technical assistance, S. Ena and F. Coghe, ERSAT, Cagliari for collecting infected samples, S. Winter of DSMZ for providing viruses and antisera, M. Conti and R.G. Milne for revision of the manuscript.

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Received 13 September 2001
Accepted 7 December 2001