Biological, Serological, and Molecular Characterization of 
*Pepper mild mottle virus* (PMMoV) in Tunisia

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**ABSTRACT**


In Tunisia, the main virus disease infecting protected pepper (*Capsicum annuum* L.) has been *Tobacco mosaic virus* (TMV) common strain. The F1 pepper hybrid J-27 carrying the *L1* resistance gene to TMV was obtained in a local breeding program and cultivated for several years. Severe necrosis on fruits and mottling on leaves have been observed. Two isolates called Sah.11-00 and Teb.24-00 were determined as *Pepper mild mottle virus* (PMMoV) on the basis of DAS-ELISA. Biological classification based on pathotype-genotype interaction demonstrated that local isolates, Sah.11-00 and Teb.24-00, reacted as PMMoV(1-2) specially by their ability to infect *Capsicum* species containing *L1* and *L2* resistance genes and their inability to infect tomato species and *Capsicum* genotypes containing *L3* and *L4* genes. Antibodies produced against local isolate Sah.11-00 strongly reacted with its homologous antigens as well as with the isolate Teb.24-00 and reference strains of PMMoV. Cross-reactions with TMV common strains were minimized after absorption with heterologous antigens. A modified procedure involving immunocapture (IC) followed by reverse transcription polymerase chain reaction (RT-PCR) in a single step reaction was developed for detection of PMMoV and differentiation of pathotypes using *Eco* I restriction enzyme analysis (RFLP). This procedure was proven to be useful for diagnosis and will be helpful for epidemiological investigations. This new occurrence of such breaking-resistance isolates of PMMoV in Tunisia may create a new challenge for the national breeding program for multivirus resistance in pepper.

**Keywords:** Pepper, PMMoV, *Tobamoviruses*, pathotype, resistance, antibodies, IC-RT-PCR, RFLP

In Tunisia, pepper (*Capsicum annuum* L.) is grown in several areas starting from the North regions to the South in the oasis. The plains of the centre (Kairouan and Sidi Bouzid) and the east coastal area are the main production areas. Pepper is cultivated under cold plastic tunnels as an early crop from September to July, and as a full-season vegetable as an open field crop from March to November. Full season pepper crop is strongly affected by *Potato virus Y* and *Cucumber mosaic virus* (PVY and CMV) which are estimated to cause up to 50% losses of potential production in pepper varieties cultivated in Tunisia (2, 14, 22). However these two viruses are less frequent under cold plastic tunnels (14). The main virus infecting protected culture is *Tobacco mosaic virus* (TMV) common strain (16). It affects strongly the yield and fruit quality of local cultivars Baker and Baklouti (14, 22). The F1 pepper hybrid (J 27) carrying the *L1* resistance gene against the common strain of TMV and adapted to cold protected crop was obtained in a local breeding program (14) and cultivated for several years. *Tobamoviruses* were mentioned to cause significant economic losses on infected pepper in the world (1, 24) and especially
in Mediterranean area (12, 30). These Tobamoviruses causing Pepper mild mottle virus (PMoV) disease have been ordered on the basis of their interaction with the L gene for resistance in pepper genotype. Based upon their ability to overcome the resistance conferred by allelic series of Capsicum spp. genes known as $L^1$, $L^2$ and $L^3$ they have been designed as pathotypes $P_1$, $P_{1,2}$, respectively (11). In the Capsicum genus, four alleles of the Tobamovirus resistance gene $L$ have been discovered. The allele $L^4$ confers resistance to the more aggressive pathotype PMoV$_{1,2,3}$ which was originally found in some lines of Capsicum chacoense and was introduced in the Hungary pepper type “Himes F1” (24).

In the last five years, severe necrosis on fruits and mottling on leaves similar to those caused by Tobamoviruses have been observed on the J27 F1 resistant hybrid cultivated in the central coastal area. Paying attention to these observations, we attempt in our study to identify and characterize this virus using biological, serological, and molecular tools. Host ranges, serological and molecular reactions of these isolates were compared to those of reference isolates.

**MATERIALS AND METHODS**

**Virus isolates and strains.** Two virus isolates Sah.11-00 and Teb.24-00 were taken in this study with reference isolates of four strains belonging to the Tobamovirus group, kindly supplied by Gebre-Selassie from INRA Avignon-France (1998). They are described in Table 1. The studied isolates were collected from J27 pepper hybrid plants grown under cold plastic tunnels during early season crop 2000/2001 in two regions Teboulba and Sahline situated in central coastal area of Tunisia. Severe necrotic symptoms on fruits and mottling have been observed on leaves of J27 infected plants. All reference and local isolates were long-term stored by desiccation of small amounts of leaf materials of pepper over calcium chloride (CaCl$_2$) at 4°C according to the method described by Bos (3).

**Biological tests.** Fresh collected or calcium chloride conserved dried leaves were ground in 0.03 M phosphate buffer (pH 7.2) and containing 0.2% Sodium diethyldithiocarbamate (Na-DIECA) at the rate 1:4. Activated charcoal (75 mg/ml buffer) and celite were added to the extract. Inoculation was made by rubbing the extract on 5-leaf stage plants for each isolate tested. The isolates were cloned after two successive passages on Nicotiana glutinosa. A single local lesion was used to propagate the virus in each time. Virus cultures were maintained in

<table>
<thead>
<tr>
<th>Table 1. Strains and isolates used in this study</th>
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<tr>
<td>Isolates</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>ToMV: SM2</td>
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<tr>
<td>TMV: Vi 76</td>
</tr>
<tr>
<td>PMoV (1-2): Adam</td>
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<tr>
<td>PMoV (1-2-3): Eve</td>
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<tr>
<td>Sah.11-00</td>
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<td>Teb.24-00</td>
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pepper J27 F1 hybrid and propagated on *Nicotiana benthamiana*. Inoculated pepper plants and other indicator plants were subsequently transferred into an insect proof glasshouse with temperature ranging from 25 to 28°C during day and from 12 to 15°C at night. Host range tests were performed with two to four plants per species (Table 3). Symptoms were scored four to five weeks after inoculation. Back inoculation to at least two plants of *Datura stramonium* or *N. glutinosa* from non-inoculated leaves was made.

**Serological tests.** Serological assays were carried out using the standard sandwich DAS ELISA according to Clark and Adams (1977) on pepper samples showing virus like symptoms to check for common viruses TMV, PVY, CMV and for new virus infection with PMMoV. Indirect ELISA test was performed according to Lommel and collaborators (19) to determine the obtained antibody titer. The antibodies used to detect PVY were locally produced in the laboratory of plant protection at INRAT; those used to detect CMV were kindly supplied by Drs Marchoux and Gebre-Selassie from INRA Avignon. PMMoV sera were purchased from BIO-RAD Phyto-diagnostics (France) and used according to the manufacturer’s conditions.

**Virus purification and antiserum production.** The isolate Sah.11-00 was purified according to the method described by Gebre-Selassie and Marchoux (11). *N. benthamiana* was used for propagating the virus. Systemically infected leaves were homogenized in 0.5 M phosphate buffer pH 7.2 (Na$_2$HPO$_4$-KH$_2$PO$_4$) containing 1% (v/v) of 2-mercaptoethanol with the ratio of 100 g leaf material per 200 ml of buffer. The homogenate was clarified by adding 8% (v/v) n-butanol using centrifugation at a low speed centrifugation at 10,000 x g for 30 min. PEG 6,000 and NaCl were added to the supernatant in order to give a final concentration of 3% and 1% (w/v), respectively. A second precipitation by PEG was performed with Triton X-100 being added (5% v/v) to the supernatant. Virus particles were further purified by two cycles of differential centrifugation: 90,000 x g for 90 min and 10,000 x g for 15 min. The obtained pellet was resuspended in a 1 mM EDTA buffer, pH 7.2. Purified virus was stored at –18°C until use.

Virus concentration and purification yield were estimated using a specific extinction coefficient (A$_{0.1% \text{~cm} \text{~at} \text{~260} \text{~nm}}$) = 3.18 (30).

A rabbit was immunized by injecting 0.5 mg of purified virus emulsified with an equal volume of Freund’s complete adjuvant. Four intramuscularly injections were performed at one-week interval and three more injections two week spaced. Bleedings started one month after the first immunization.

**Absorption of serum.** IgGs of the obtained antiserum were purified by chromatography column sepharose CL-4B protein A (from Pharmacia) and absorbed according to Dijkstra and Jager (10) against virus-free plant material and SM2 TMV strain. Sap extracts from virus-free plants (*N. benthamiana*) and SM2 infected plants were squeezed through cheesecloth and diluted 20 times in PBS-T. Antibodies were diluted in the obtained plant sap. The mixture was incubated for one hour at 37°C and centrifuged at 8,000 x g for 20 min. The supernatant (absorbed antiserum) was used in indirect ELISA.

**Molecular tests.**

**Oligonucleotide primers.** In this investigation, the set of PMMoV specific primers P12/3 (5’-ACAgCgTTTgTCTTAgTAT-3’) and P12/3A (5’-gTgCggTCTTTAATAACCTCA-3’) were used (28).

**IC-RT-PCR.** Immunocapture (IC) was performed according to Wetzel and collaborators (31). Each PCR tube (0.2 ml Biozym) was incubated 3 hours at 37°C with 100 µl of PMMoV or TMV antiserum diluted in carbonate buffer pH 9.6 as used in ELISA test, then washed.
three times with 150 µl of PBST at pH 7.4 (137 mM NaCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl) containing 0.05 % (v/v) Tween 20. The plant samples infected by PMMoV and TMV were ground in ELISA extraction buffer (PBST containing 2% (w/v) of Polyvinylpyrrolidone and 0.2 % (w/v) of bovine serum albumin). The homogenate was spun at 2,000 x g for 5 min at 4°C. Hundred microliters of the supernatant were added to coated tubes and submitted to an immunocapture phase for 2 hours at 30°C. Then, they were washed 3 times with PBST buffer and one more with sterile bidistilled water. The detection method was based on one-tube RT-PCR assays using Titan kit from Roche diagnostics (Penzberg, Germany) according to manufacturer’s conditions. A mix of 50 µl was prepared as following: 0.2 mM of each dATP, dCTP, dGTP, dTTP, 0.4 µM of each primer; 5mM DTT; 10 µl of (5 x RT-PCR) buffer containing 1.5 mM MgCl₂ and 1 µl of AMV and Expand High fidelity PCR system. The volume of mix was adjusted with sterile bidistilled water and added to each coated tube.

The amplification was carried out in a Biometra Uno II programmed for one cycle of 50°C for 30 min and a second cycle of 94°C for 2 min followed by 35 cycles (denaturing at 94°C for 30 s, annealing at 50°C for 1 min and extension at 68°C for 1 min) and terminated with a final extension cycle at 68 °C for 10 min.

IC-RT-PCR-RFLP. RFLP analysis was performed for Sah.11-00 and Teb.24-00 isolates and reference strains PMMoV (1-2) and PMMoV (1-2-3) by digesting the amplified product obtained from IC-RT-PCR with primers P12/3 and P12/3A with EcoR I enzyme according to manufacturers conditions. PCR products and digested fragments were run in a 1% (w/v) agarose gel contained ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) under a 100 mA and were visualized with a UV transilluminator at 312 nm.

RESULTS

Purification and serology. Concentration of purified virus of isolate Sah.11-00 was estimated to 0.4 g/kg plant tissues. DAS-ELISA tests demonstrated that extracts from Sah.11-00 and Teb.24-00 infected plants strongly reacted with antiserum produced against PMMoV from Bio-Rad but neither against PVY, CMV nor TMV antiserum. The titre of our anti-PMMoV antiserum when tested by indirect ELISA was found to rise to 10⁻⁵ against homologous antigens and other PMMoV strains, 10⁻⁴ against TMV strains Vi76 and SM2 whereas it was around 10⁻³ against healthy plant sap (Fig. 1).

Absorbed antibodies with healthy plant still produce a good homologous reaction with Sah.11-00 and against PMMoV (1,2) but they still cross-reacted with the common strain of TMV (Vi 76 and SM2) (Fig. 2). These absorbed IgGs were secondly absorbed with SM2. They still produce a good reaction with Sah11-00 but did not react positively with healthy plant or with SM2 infected plant (data not shown).
Reactions on various indicator plants. Sah.11-00 and Teb.24-00 isolates were inoculated as described previously on a range of indicator plants and at the same time to a pepper host range with known pathotype-genotype interaction of Tobamoviruses (Table 2).

Reactions of tested plants are summarized in Table 3. Three weeks after inoculation, our two isolates reacted as following:
- *D. stramonium*, *N. tabacum* Xanthi–nc and *N. glutinosa* displayed small necrotic local lesions,
- *N. tabacum* cv Samsun did not show neither local nor systemic symptoms and the virus could not be detected from newly developed leaves,
- *N. occidentalis* displayed a systemic mosaic and leaf deformation,
- *N. benthamiana* reacted with systemic chlorosis, leaf deformation and growth reduction,
- *Physalis floridana* showed systemic mottling and some leaf deformation. Symptoms were more severe in the case of rise of temperature,
- *Lycopersicon esculentum* F1 hybrid Razan was immune to the virus as proven by back-inoculation,
- *Solanum melongena* on which only faint chlorotic local lesions appeared but the virus could not be detected neither from inoculated nor from non-inoculated leaves.

Typing on various Capsicum genotypes. Reactions on *Capsicum* species were carefully observed in order to classify the isolates studied on the basis of virus-host genotype interactions.
- On local pepper varieties Baker and Bakkouri inoculated leaves were fallen down one week after inoculation, plants developed systemic chlorosis, mottling and leaf rugosity, some necrosis may appear on the stems.
- Hybrid J27 showed no local symptoms, systemic infection started by vein clearing followed by systemic necrotic spots and mottling.
- Hybrids Roda/Drago (*L*¹) and Greygo (*L*²) displayed a systemic mottling two weeks after inoculation.
- Hybrids Novi (*L*³), Rapires (*L*³), Cuzco (*L*⁴), Himes (*L*⁴) and *C. chacoense* PI260429 (*L*⁴) displayed local necrotic lesions five days after inoculation which then started to enlarge and coalesce. The ultimate stage is the falling down of the inoculated leaves. No virus activity was detected on top leaves, i.e. no symptoms and no infection as verified by back inoculation.

IC-RT-PCR for detection of PMMoV. IC-RT-PCR test was applied to common strain of TMV, to our isolates of PMMoV Sah.11-00 and Teb.24-00, to reference strains PMMoV (1-2), PMMoV (1-2-3), and to healthy sample. The size of the amplified product was as expected around 836 bp with local isolates and reference strains of PMMoV but neither with TMV (Vi 76) (lane 2) nor with healthy sample (lane 7) (Fig. 3).

Differentiation of PMMoV strains with IC-RT-PCR-RFLP. RFLP analysis was performed to Sah.11-00, to Teb.24-
00 isolates and PMMoV (1-2) and PMMoV (1-2-3) by digesting the amplified fragment obtained from IC-RT-PCR with primer pair P12/3 and P12/3A. Restriction digestion with EcoRI enzyme yielded one single fragment with PMMoV (1-2), Sah.11-00 and Teb.24-00, whereas it produces two fragments of 260 bp and 570 bp with PMMoV (1-2-3) reference strain (Fig 4).

Table 2. Pepper host range used in biological tests, genotypes and origins

<table>
<thead>
<tr>
<th>Host</th>
<th>Genotype</th>
<th>Origins</th>
</tr>
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<tbody>
<tr>
<td>C. annuum: cv Baker</td>
<td>L1&lt;sup&gt;0&lt;/sup&gt;: Hamza, INRAT unpublished data</td>
<td></td>
</tr>
<tr>
<td>C. annuum: cv Baklouti</td>
<td>L1&lt;sup&gt;0&lt;/sup&gt;: Hamza, INRAT unpublished data</td>
<td>Hamza, N., INRAT, Tunisia.</td>
</tr>
<tr>
<td>C. annuum: J27 F1 Hyb</td>
<td>L1&lt;sup&gt;1&lt;/sup&gt;: Hamza and collaborators (14)</td>
<td></td>
</tr>
<tr>
<td>C. annuum: Greygo</td>
<td>L2&lt;sup&gt;1&lt;/sup&gt;: Kalman and collaborators (17)</td>
<td>Gáborjányi, Vezprém university, Georgikon Faculty of Agricultural Science, Hungary.</td>
</tr>
<tr>
<td>C. annuum: Rapiroes F1 Hyb</td>
<td>L1&lt;sup&gt;2&lt;/sup&gt;: Kalman and collaborators (17)</td>
<td></td>
</tr>
<tr>
<td>C. annuum: Himes F1 Hyb</td>
<td>L4&lt;sup&gt;1&lt;/sup&gt;: Sagi and Salamon (24)</td>
<td></td>
</tr>
<tr>
<td>C. chacoense: PI 260429</td>
<td>L4&lt;sup&gt;4&lt;/sup&gt;: Boukema (4) and Gebre-Selassie and collaborators (12)</td>
<td></td>
</tr>
<tr>
<td>C. annuum: Roda/Drago (P824) F1 Hyb</td>
<td>L4&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Syngenta</td>
</tr>
<tr>
<td>C. annuum: Cuzco (BK162) F1 Hyb</td>
<td>L4&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
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</table>

Fig. 3. One percent agarose gel electrophoresis analysis of IC-RT-PCR product obtained after amplification with primers pair P12/3 and P12/3A. IC-RT-PCR product from Vi76 (Lane 2); PMMoV (1-2) (Lane 3); PMMoV (1-2-3) (Lane 4); Sah.11-00 (Lane 5); Teb.24-00 (Lane 6); healthy sample (Lane 7). Molecular weight marker Gene Ruler 100 base pair ladder plus from Roche (Lanes 1 and 8).
**DISCUSSION**

DAS-ELISA tests showed that Sah.11-00 and Teb.24-00 isolates were determined as PMMoV. The antibodies produced against purified isolate Sah.11-00 were used in indirect ELISA tests. They reacted strongly with PMMoV strains, less with TMV, and only slightly with healthy plant. Our produced immunogen was probably not totally pure. Indeed, we did not perform any gradient step at the end of purification; this can explain the reaction against healthy plant sap.

The obtained cross reaction against the common strain of TMV can be explained by the close serological properties of PMMoV to some viruses belonging to *Tobamovirus* group, mainly TMV and ToMV as reported by Wetter and Conti (29) and Gebre-Selassie and Marchoux (11). Serological identification of species, which are related, might lead to ambiguous results especially within the *Tobamovirus* genus. Differentiation problems resulted from heterologous reactions of antisera detecting conserved epitopes (18). Our results demonstrated that after absorption with virus-free plant, the obtained IgGs still produce a good reaction with their homologous antigens but reacted with TMV strains (Vi 76 and SM2) (Fig. 2). This absorption allowed to discard the antibodies produced against plant proteins. The double absorption with sap of healthy plant and with SM2 infected plant gave better reaction with Sah. 11-00 and minimize there activities with TMV common strains (data not shown).

Subgrouping of PMMoV isolates is important for elucidation of PMMoV epidemiology and for practical detection of PMMoV pathotypes. Classification system based on virus-host genotype interactions in which pathogenicity or virulence of strain is expressed in Arabic numerals relating to L-genes resistance in *Capsicum* sp. was used (Table 2). According to the obtained results (Table 3) and to the descriptions of Gebre-Selassie and collaborators (12) and Gebre-Selassie and Marchoux (11) Sah.11-00 and Teb.24-00 isolates behaved like the PMMoV pathotype 1-2, by their ability to infect *Capsicum* species J27 and Greygo hybrids F1 carrying respectively L¹ and L² resistance gene and their inability to infect *L. esculentum* genotype and especially by their capacity to induce hypersensitive reaction on *Capsicum* genotypes carrying L⁴ genes (PI260429, Himes and Cuzco) and on *Capsicum* genotypes carrying L³ genes (Rapires and Novi).

RT-PCR methods have been reported for detecting plant viruses within many genera such as *Potyvirus*, *Tobamovirus*, *Luteovirus* and *Cucumovirus* (6, 7, 9, 13, 15, 18, 23, 26, 28). RT-PCR-RFLP has been also used to differentiate pathotype of PMMoV.
Table 3. Comparative host range of local strains to reference strain

<table>
<thead>
<tr>
<th>Host range</th>
<th>Genotype</th>
<th>Sah.11-00</th>
<th>Teh.24-00</th>
<th>PMMoV (1-2): Adam</th>
</tr>
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<tbody>
<tr>
<td>Capsicum annuum cv Baker</td>
<td>L°</td>
<td>- / S</td>
<td>- / S</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum cv Baklouti</td>
<td>L°</td>
<td>- / S</td>
<td>- / S</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum cv J27</td>
<td>L¹</td>
<td>- / S</td>
<td>- S</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Drago/Roda</td>
<td>L¹</td>
<td>- S</td>
<td>- S</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Greygo</td>
<td>L²</td>
<td>- S</td>
<td>- S</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Novi</td>
<td>L³</td>
<td>L / -</td>
<td>L / - (-/s)</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Rapires</td>
<td>L³</td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Himes</td>
<td>L³</td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Capsicum annuum Hyb F1 Cuzco</td>
<td>L³</td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Capsicum chacoense PI 260429</td>
<td>L⁴</td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Lycopersicon esculentum Hyb F1 Razan</td>
<td></td>
<td>.* / -</td>
<td>.* / -</td>
<td></td>
</tr>
<tr>
<td>Nicotiana tabacum cv Xanthi- nc.</td>
<td></td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Nicotiana tabacum cv Samsun</td>
<td>1 / -</td>
<td>1 / (s/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana benthamiana</td>
<td>.* / S</td>
<td>-* / S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana glutinosa</td>
<td>L / -</td>
<td>L / - *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana occidentalis</td>
<td>.* / S</td>
<td>-* / S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physalis floridana</td>
<td>.* / S</td>
<td>-* / S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solanum melongena F1 cv Black enorma</td>
<td></td>
<td>L / -</td>
<td>L / -</td>
<td></td>
</tr>
<tr>
<td>Datura stramonium</td>
<td>L / -</td>
<td>L / -</td>
<td></td>
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**Explanation of symbols:** local reaction / systemic reaction; L: necrotic local lesions; l: latent local infection; S: systemic symptoms; s: latent systemic infection; -: no infection as verified by back inoculation. -*: infection not tested; ( ) symbols between brackets refer to inconsistent or erratic symptoms depending on growing conditions.

These methods based on RT-PCR encountered several problems in epidemiological research of the viral diseases. RNA extraction is troublesome and molecules are also readily degraded due to the ubiquitous presence of RNase. In contrast, the IC-RT-PCR method avoided the extraction of viral or plant total RNA, and was easily carried out in a single tube. Such procedures have been developed for detecting many plant viruses including Grapevine leafroll-associated virus 1, Pepino mosaic virus, and Plum pox virus (5, 20, 25, 31). Here, we applied IC-RT-PCR and IC-RT-PCR-RFLP procedure respectively for detecting PMMoV and for pathotypes identification. In IC-RT-PCR, we found that a single strong band of about 830 nucleotides could easily be amplified from plant saps containing PMMoV Tunisian isolates and reference isolates (Fig. 3). This result confirms those obtained by Velasco and collaborators (28) using total RNA in RT-PCR test.

Results also showed that a single strong band of about 830 nucleotides was amplified from plant saps containing PMMoV(1-2) and local isolates, whereas two bands of about 570 and 260 bp were obtained from plant saps containing PMMoV(1-2-3) when digested with EcoRI (Fig. 4). From sequence alignment of fragments obtained using the primer pair (P12/3 and P12/3A); Velasco and collaborators (28) demonstrated that the pathotypes (1-2) and (1-2-3) are differentiated by the absence of EcoRI restriction site in the first one. Restriction patterns of EcoRI enzyme were then sufficient to differentiate pathotypes of tested species. Pattern shown in Fig. 4 fits well with the assignment of the local isolates and the reference strain.
PMMoV(1-2) into the pathotype (1-2). Whereas the pattern obtained with reference strain of PMMoV(1-2-3) demonstrated the presence of the EcoRI restriction site which confirms its assignment to pathotype (1-2-3).

Moreover IC-RT-PCR-RFLP confirms that PMMoV reference isolates Adam and Eve belonged respectively to the pathotypes (1-2) and (1-2-3) as reported by Gebre-Selassie and Marchoux (11) using biological classification. Characterization of the two local isolates and the reference strains using both pathotyping and IC-RT-PCR-RFLP showed a good correlation between both methods. Both appeared to be reliable. However pathotyping was time-consuming, whereas IC-RT-PCR-RFLP method was efficient, time saving and could be widely used for identification on a larger scale.

Based upon biological, serological, and IC-RT-PCR-RFLP reactions, we can conclude that Sah.11-00 and Teb.24-00 can be assigned as isolates belonging to the strain (1-2) of PMMoV. This finding demonstrates the occurrence of this breaking-resistance pathotype in pepper crops in Tunisia. To our knowledge this virus has not been described in Tunisia and our investigation reports for the first time the natural occurrence of PMMoV in this country. This new occurrence may create a new challenge for the Tunisian breeding program for multivirus resistance in pepper (14).

To get more information concerning the prevalence of PMMoV pathotypes, continuous surveys from year to year in different crops and locations will be required. The possible presence of pathotype (1-2-3) must be checked on a larger scale in Tunisian farms in order to pertinently introduce efficient resistant \textit{L}^3 gene against PMMoV (1-2). The introduction of suitable resistance gene in plants will offer a powerful and accurate tool to avoid PMMoV (1-2) infection especially in early-protected pepper crops of central coastal regions and possibly in regions where this virus could be identified.

ACKNOWLEDGEMENTS
Authors gratefully acknowledge Drs. Gebre-Selassie and Georges Marchoux (INRA Montfavet) for kindly providing reference strains and CMV antibodies, Drs. Alain Palloix (INRA Montfavet), R. Gaborjanyi, and G. Kazinczi (University of Veszpraem, Hungary) for kindly providing \textit{Capsicum} species carrying resistance gene and Dr. Naceur Hamza (INRA Tunisia) for kindly providing local pepper varieties and hybrids.

RESUME

En Tunisie, la souche commune du virus de la mosaïque du tabac (\textit{Tobacco mosaic virus} TMV) sur piment (\textit{Capsicum annuum} L.) est la cause de la maladie virale la plus répandue en culture protégée. L’hybride F1 J-27 porteur du gène de résistance \textit{L}^1 vis-à-vis des souches communes de TMV a été obtenu dans le cadre d’un programme d’amélioration du piment en Tunisie et a été cultivé pendant plusieurs années. Des symptômes de nécroses sévères sur les fruits et des mosaïques sur les feuilles ont été observés. Deux isolats appelés Sah.11-00 et Teb.24-00 ont été identifiés en ELISA en utilisant un kit d’anticorps commercial spécifique vis-à-vis du virus de la marbrure légère du piment (\textit{Pepper mild mottle virus} PMMoV). La caractérisation biologique basée sur l’interaction pathotype-génotype démontre l’appartenance des isolats tunisiens au PMMoV pathotype (1,2). En effet, les isolats Sah.11-00 et Teb. 24-00 infectent les génotypes du piment porteurs des gènes de résistance \textit{L}^1 et \textit{L}^2 et n’infecte pas ceux porteurs des gènes \textit{L}^3 et \textit{L}^4 ainsi que l’espèce de tomate. Un antisérum a été préparé à partir de l’isolat Sah.11-00. En ELISA, les anticorps obtenus déetectent l’antigène homologue, l’isolat Teb.24-00 et les antigènes des souches de référence du PMMoV. L’élimination par absorption des anticorps
réagissant avec les extraits de plantes saines a permis d’obtenir un réactif très fiable de détection du PMM0V. Une procédure modifiée basée sur l’immunocapture (IC) suivie d’une RT-PCR en une seule étape a été développée pour l’identification du PMM0V et la différenciation des pathotypes en analysant les fragments obtenus après RFLP. Cette procédure, fiable, rapide et facile à mettre en pratique pour le diagnostic, pourrait être d’une grande utilité pour les études épidémologiques futures. La nouvelle présence en Tunisie du pathotype 1-2 du PMM0V devra obligatoirement être prise en compte par les sélectionneurs et se traduire par une réorientation de leur programme d’amélioration du piment pour la résistance aux maladies virales.

Mots clefs: Piment, PMM0V, Tobamovirus, pathotype, résistance, anticorps, IC-RT-PCR, RFLP

ملخص

مناري حطاب، منيرة وكريم الزار. 2006. التشخيص البيولوجي والسريع لعدوى البيبيجيا الجزيرة للفيروس PMM0V . Tunisian Journal of Plant Protection 1: 12-21

لا تعتبر السلالات العادلة لفيروس ثيراس الشدو (TMV) (Capsicum annuum L.) الأكثر توافدا في البيوت المحمية في تونس. أنتج النهج J27 الحامل لجين تعدد الفيروسية لـ PMM0V للسلالات العادلة لفيروس TMV خلال برنامج وطني لتحسين الفيروس بتونس ووقع إشارات بطيئة من سنوات عدة، كما لوحظت أعراض موت موضعية حاد على ثمار الفيروس ونفرط على الأوراق. وعند الوصول إلى عزل وتشخيص سلالة تين تين من فيروس ثيراس الشدو بسهل تبة 11-2004 وSah11-2004-00-التمثيل الأصلية PMM0V، اكتشف أن ELISA المعبط على تفاعل النسيم المرجي، أن السلالات التوساوية تتم إلى صف PMM0V، حيث تصبح الفيروس بيضاء مقصورة مقاومة L1 و L2، بينما لا تعصف الفيروس البيضاء لبيضاء L3 و L4، مما كتب أن PMM0V لا تعصف الفيروس البيضاء لبيضاء L3 و L4، مما كتب أن PMM0V لا تعصف الفيروس البيضاء لبيضاء L3 و L4، مما كتب أن PMM0V لا تعصف الفيروس البيضاء L3 و L4، مما كتب أن PMM0V لا تعصف لـ PMM0V. وعند تعريض الفيروس البيضاء لـ PMM0V، تسبب الفيروس بيضاء مقصورة مقاومة L1 و L2، بينما لا تعصف الفيروس البيضاء لبيضاء L3 و L4، مما كتب أن PMM0V لا تعصف الفيروس البيضاء L3 و L4، مما كتب أن PMM0V لا تعصف لـ PMM0V. وعند تعريض الفيروس البيضاء L3 و L4، مما كتب أن PMM0V لا تعصف لـ PMM0V. وعند تعريض الفيروس البيضاء L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما كتب أن PMM0V لا تعصف L3 و L4، مما K:\abraham\Desktop\PMM0V.pdf

LITTERATURE CITÉ

