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DETECTION AND EPIDEMIOLOGICAL CHARACTERISTICS OF PEACH LATENT MOSAIC VIROID IN TUNISIA

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ABSTRACT

A rapid and sensitive assay was developed for the detection and identification of Peach latent mosaic viroid (PLMVd) by reverse transcription-polymerase chain reaction (RT-PCR) in infected tissues from Tunisian orchards. The test was initially performed by using total RNA preparations from selected isolates and then applied on total RNA preparations from leaf or bark tissues of fruit trees collected in 2003 in 20 orchards in the North of Tunisia and the Sahel. PLMVd occurred in peach and pear trees. The identity of the detected viroid was confirmed by comparison of its sequence with other isolates previously characterized. The test was then simplified by direct use of diluted crude plant extracts. The results obtained from crude sap extracts of leaves or bark tissues are identical to those obtained from total RNA preparations.
Epidemiological characteristics of PLMVd on peach trees have been investigated. A survey of peach trees was carried out in 32 orchards in May 2004. The obtained results showed that (1) PLMVd is highly and equally present in several regions of the north of Tunisia rather than the central, the Sahel and the southern regions, (2) infection percentage increases with the age of the tree and (3) the studied cultivars are classified into three groups of sensitivity.

Keywords: PLMVd, fruit trees, RT-PCR, viroids, epidemiology, Tunisia.

INTRODUCTION

Tunisian fruit trees have an important economic place and represent an important source of export of the agricultural products (Jabari *et al.*, 1991). However, fruit quality can be damaged by viroid infections leading to severe yield losses. Peach latent mosaic viroid (PLMVd) mainly affects peach (*Prunus persica*) (Flores *et al.*, 1992; Osaki *et al.*, 1999; Loreti *et al.*, 1998). It induces an early aging of fruit trees and irregularly shaped, flattened and colorless fruit which causes a decrease in yield and a reduction of fruit quality and vigour (Desvignes, 1986; Flores *et al.*, 1990; Llacer, 1998). During the last few years, it has been shown that other species of stone and pome fruit trees are natural hosts of PLMVd, such as apricot (*Prunus armeniaca*), plum (*Prunus domestica*), sweet cherry (*Prunus avium*) (Hadidi *et al.*, 1997) and cultivated pear (*Pyrus communis*) and wild pear (*Pyrus amygdaliformis*) (Kyriakopoulou *et al.*, 2001). This fruit tree viroid is now present in different countries of the European, American and Asian continents (Flores *et al.*, 1990; Hadidi *et al.*, 1997; Kyriakopoulou *et al.*, 2001) where its control is considered compulsory in all certification programs aimed at obtaining vi-

roids free propagative material. The development of a diagnostic method that is rapid, sensitive and specific is a pre-requisite for the successful management of the certification programs. As viroids do not code for any peptide, serological detection is not possible. Biological detection for this viroid is time-consuming and labor intensive. Such assays are not appropriate for screening a large number of samples, mostly due to the requirements of greenhouse facilities and plant care. Molecular techniques, such as specific double gel electrophoretic techniques (Schumacher *et al.*, 1983; Flores *et al.*, 1985) and nucleic acid hybridization (Ambros *et al.*, 1995; Kyrikopoulou *et al.*, 2001) have been used for a reliable detection of PLMVd in fruit trees. However, such tests are not highly sensitive and low concentrations of viroids in woody material may be undetected, giving false negative results. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) tests have been developed for more sensitive detection of this viroid (Loreti *et al.*, 1999, Shamloul *et al.*, 2002). Although these RT-PCR protocols are highly sensitive, they rely on labour intensive preparation of the nucleic acid extracts from plant tissues submitted to the tests and need to be improved for a routine detection of viroids in fruit trees.

Concerning the epidemiological characteristics of PLMVd, it's known that it can be transmitted by grafting (Desvignes, 1986) or through agronomic practices like pruning (Hadidi *et al.*, 1997). Furthermore, it has been demonstrated only experimentally that it can be transmitted by aphids (*Myzus persicae*) (Desvignes *et al.*, 1992).

The main objectives of this study were to screen for the presence of PLMVd on peach and pear in naturally infected field trees in Tunisia. For this purpose a rapid RT-PCR test appropriate for analyses of a large number of samples was developed and then simplified by direct use of crude sap for easy detection during routine diagnosis. Furthermore, this test was used for studying (1) the distribution of PLMVd, (2) and the influence of two factors on PLMVd incidence which are the age of the trees and the cultivar.

MATERIAL AND METHODS

Source of orchard fruit trees

Twenty orchards, 11 located in the North of Tunisia (6 of pears trees, 4 of peach trees and 1 of peach and pear trees) and 9 in the Sahel (5 of peach and pear, 1 of peach and 3 of pear) were prospected. Peach and pear trees, mainly showing different symptoms on leaves and barks potentially caused by PLMVd were tested. Three one-year-old branches per tree were collected in May 2003. This material was stored at 4°C. Bark and leaf tissues of these branches were analysed within 8 days. To minimize the effects of potential uneven viroid distribution, the samples were taken from different parts of the three branches collected from each tree. Total RNA and crude sap were extracted from these bark and leaf samples.

For PLMVd epidemiological investigation, thirty-two orchards of peach trees have been prospected in May 2004. Twenty-two were located in the North of Tunisia (Bizerte, Ben Arous, Manouba, Nabeul and Zaghouan), one in the center (Kairouan), two in the Sahel (Mahdia) and one in the South (Sfax). Table 1 represents the number of orchards and samples for each studied

regions. The prevalence of the pathogen was determined by the number of infected orchards on the total number of tested orchards in one region. The incidence of the pathogen corresponded to the number of infected samples on the total number of tested samples.

Table 1. Number of orchards and samples in each studied regions

Regions	Number of orchards	Number of samples
Ben Arous	7	131
Nabeul	3	42
Manouba	12	90
Bizerte	3	25
Zaghouan	3	26
Kairouan	1	20
Mahdia	2	24
Sfax	1	12
Total number	32	370

Data such as identity and origin of the rootstock and the cultivar and the age of the tree were taken for each orchard. For the majority of the prospected orchards, rootstock corresponded to GF305 peach trees, except for seven orchards where we found GF677 peach trees (4 orchards) or almond trees (3 orchards). The origin of these rootstocks was Spain or Italy for peach trees and autochthones for almond trees. The majority of the studied cultivars were imported from USA and multiplied in Tunisia. They represent the principal varieties cultivated in this country. Table 2 represents the maturity and the number of orchards and samples for each cultivar. The age of the tree varied from 1 to 18 years (Table 3). Three one-year-old branches per trees were collected and processed as described in the first paragraph.

Table 2. Number of orchards and samples for each cultivar

Cultivars (maturity)	Number of orchards	Number of samples
Swanier (of saison)	2	20
Early may crest (early)	11	130
Plate de chine (late)	5	32
Cvi (early)	1	12
Mygold (of saison)	1	8
Henry (late)	1	9
Royal glory (of saison)	5	40
Carnival (late)	5	57
Alberta (late)	1	10
Cristal (late)	1	6

Table 3. Number of orchards and samples for each age

Orchard's age (years)	Number of orchards	Number of samples
1	2	8
4	4	45
5	2	17
6	4	45
7	7	59
8	4	56
10	2	55
12	1	25
18	3	25

Statistical tools

Analysis of the variance for one-way ($P<0,05$) was employed to highlight possible differences between the studied factors. In the case of significant differences, the test of comparison of the averages (Tukey with the threshold of

5%) was carried out to classify them in different groups by using statistical software MINITAB 13 UK.

Source of positive controls

Twigs of PLMVd-infected GF 305 peach seedlings were kindly provided by B. Pradier (Station de Quarantaine des Ligneux, Lempdes, France). These materials were positive as revealed by chip budding of infected material on peach seedling indicator plants grown under greenhouse conditions.
Leaves of a PLMVd-infected peach tree were generously provided by Dr Kyriakopoulou (Agricultural University of Athens, Votanikos, Athens, Greece). Two PLMVd-infected peachs were also generously supplied by Dr. R. Flores (Instituto de Biologia Molecular y cellular de Plantas, Valencia, Spain).

Total RNA extraction from plant tissue

Total RNA extracts were prepared according to the method described by Grasseau *et al.* (1998), with some modifications. Leaf and bark tissues (0.2 g) were powdered in liquid nitrogen and homogenized with 1 ml of 2X SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7) containing 1 % sodium sulphite. After centrifugation at 16000× *g* for 30 min, the pellet was discarded and the supernatant was mixed with 500 µl of 10 % non-ionic CF-11 cellulose (Whatman, England). After shaking for two hours, the cellulose was collected by centrifugation and washed twice with 1 ml of STE buffer (10 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA pH 8) containing 35 % ethanol. The total RNA was released from the cellulose by washing twice with 300 µl of STE buffer and precipitated with 700 µl of isopropanol and 64 µl of sodium acetate (5 M, pH 5.2). Finally, the pellet was dried in speed-vac (Savant, Farmingdale, USA) and suspended in 30 µl of diethylpyrocarbonate (DEPC) treated water.
Concentration and extraction purity were estimated after determining the absorbance at 260 nm and 280 nm with a spectrophotometer (Ultrospec II, LKB Biochrom, UK).

Preparation of crude sap extract

Crude sap extract was prepared from the same plant material as used for the preparation of the total RNA extracts. Leaves and bark tissues (0.2 g) were ground in a mortar with liquid nitrogen. Frozen pulverized tissue was transferred to 1.5 ml eppendorf tube and mixed with 1 ml of the extraction buffer. Different extraction buffers were tested: (1) 136 mM NaCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, 2 mM KCl, 15 mM NaN_3, 1 % PVP, 0.05 % Tween (ELISA buffer); (2) 136 mM NaCl, 16 mM $Na_2HPO_4.2H_2O$, 1 mM K_2HPO_4, 2 mM KCl, 3 mM NaN_3, 80 mM $NaSO_3$, 0.05 % Tween 20, pH 7.2-7.4 (B. Lockhart, University of Minnesota, personal communication); (3) 200 mM Tris-HCl, pH 8.5, containing 1.5 % sodium dodecylsulfate, 300 mM lithium chloride, 10 mM EDTA, 1 % sodium deoxycholate, 1 % NP-40 (Spiegel *et al.*, 1996); (4) 2X SSC buffer buffer (3 M NaCl, 0.3 M sodium citrate, pH 7) complemented with 1 % Triton; (5) 2X SSC buffer complemented with 1 % Tween; (6) 2X SSC containing 1 % sodium sulfite as anti-oxydant.

After centrifugation at 16000× *g* for 30 min the supernatant was collected. Different dilutions (10×, 50×, 100×, 500×, 1000×) were prepared and stored at –20°C.

Primer design

Primer pairs previously reported for RT-PCR amplification for PLMVd (Loreti *et al.*, 1999) have been used in this study. hPLMVd (5'CCCGATAGAAAGGC-TAAGCACCTCG3') is complementary to residues 116-140 and cPLMVd (5'AACTGCAGTGCTCCGAATAGGGCAC3') is complementary to PLMVd residues 91-115. Synthesis and purification of the primers were performed by Eurogentec (Seraing, Belgium).

RT-PCR assay

RT-PCR was performed on the total RNA preparations and crude sap extracts by using the One tube RT-PCR Titan kit from Roche diagnostics (Penzberg, Germany). This kit allows reverse transcription and amplification to be performed sequentially in one same tube. Two µl aliquot containing 200 ng of nucleic acid extract or 2 µl of diluted crude sap extract were mixed with 0.4 µM of the complementary primer. This mixture was heated for 5 min at 100°C and immediately chilled on ice. RT-PCR reaction mixes (total volume 25 µl) contained 5 µl of 5 X RT-PCR buffer, 1.25 µl of 100 mM dithiothreitol (DTT), 0.5 µl of 10 µM dATP, dCTP, dGTP and dTTP each, 0.4 µM of the homologous primer, 0.5 µl of enzyme mix (AMV reverse transcriptase and High fidelity Taq-polymerase) and Diethylpyrocarbonate (DEPC) treated water to a volume of 22.5 µl. After addition of the denatured extract/complementary primer mixture, the RT-PCR reaction was submitted to the following cycling parameters: 1 h at 50°C for cDNA synthesis, denaturation at 95°C for 3 min at first cycle and for 30 s at following ones, primer annealing at 60°C for 45 s, and extension at 72°C for 45 s, for 30 amplification cycles with a final extension at 72°C for 7 min.
Each RT-PCR run included a water control. Total RNA preparations from a PLMVd-free peach, a PBCVd and HSVd-free pear and a ASSVd-free apple tree were included as healthy controls.

Analysis of amplified RT-PCR products

Aliquots (10 µl) of each amplification product were electrophoresed in ethidium bromide stained agarose gel (2%) in 1× TAE buffer (40 mM/l, Tris-acetate 1 mM/l pH 8.0).

Cloning and sequencing of viroid cDNA

RT-PCR products obtained from PLMVd-infected orchard fruit trees were directly cloned into the PCR® 2.1 cloning vector following the instructions of the supplier, TA cloning Kit (Invitrogen, Groningen, the Netherlands). The nucleotide sequences of cloned amplification products obtained for PLMVd isolates were determined at GATC Biotech AG (Konstanz, Germany). Two

clones per positive fruit tree were sequenced. Sequence analysis was performed using DNAMAN software (BioSoft, Lynnon, Canada).

RESULTS

Development of an RT-PCR test for pImvd detection in Tunisian orchards

RT-PCR test was initially performed by using total RNA preparations from selected isolates. It showed specific amplification products with expected size of about 337 bp for PLMVd. No bands were observed for negative controls (water and preparations from healthy trees). This test was then applied on total RNA preparations from leaf or bark tissues of fruit trees collected in 2003 in 20 orchards of peach and pear in the North of Tunisia and the Sahel. The results are summarised in Table 4.

Table 4. Number of orchard's trees infected with PLMVd in 2003

Nature of tree	Total number of tested trees	Region	Number of tested tree in each region	Number of trees infected with PLMVd
Peach trees	37	North	22	10
		Sahel	15	2
Pear trees	73	North	31	0
		Sahel	42	2

Twelve of the 37 peach trees tested were found positive for PLMVd. Ten PLMVd-positive peach trees were located in the North and 2 were located in the Sahel. Twenty five were negative for PLMVd. Among 73 pear trees tested, two were positive for PLMVd and located in the Sahel. Whatever the fruit tree species, the observed symptoms were not related with the detection of viroids.

To control the specificity of the RT-PCR test developed, the amplification products of PLMVd, obtained from different fruit trees were cloned and sequenced. PLMVd shared 85-98% homology with the PC-C32 Italian isolate of PLMVd from peach (GenBank Accession No. AJ550905).

Simplification of the RT-PCR tests by using crude plant extract

Six extraction buffers with several dilutions have been tested. The higher intensity of the signal was observed when samples were ground with SSC buffer supplemented with sulfite sodium, diluted 50× or 100× and added directly into the RT-PCR mix. Figure 1 shows an example of PLMVd detection. With that selected buffer the test using crude plant extract gave similar results as using total RNA preparations (Figure 2).

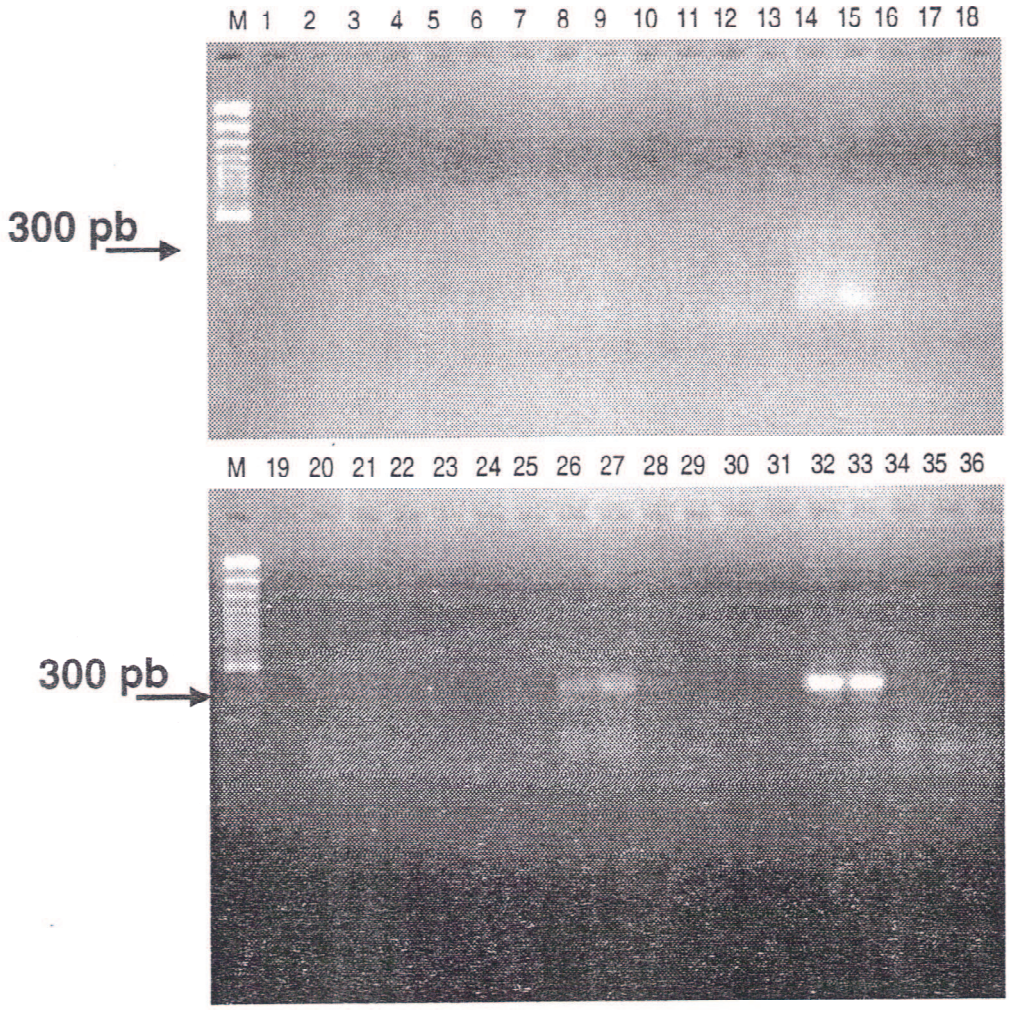


Figure 1. Comparative analysis of RT-PCR amplification products obtained from diluted crude sap extracts of PLMV-infected peach prepared with six different extraction buffers. **Lanes 1-5**: ELISA buffer; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lanes 7-11**: Lokhart buffer; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lanes 13-17**: Spiegel buffer; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lanes 19-23**: SSC buffer complemented with Triton; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lanes 25-29**: SSC buffer complemented with Tween; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lanes 31-35**: SSC buffer complemented with Sodium sulfite; dilutions 1/10, 1/50, 1/100, 1/500 and 1/1000, respectively. **Lane 6, 12, 18, 24, 30** and **36**: RT-PCR product amplified from crude sap extract of healthy peach prepared respectively with the ELISA, Lokhart, Spiegel, SSC complemented with Triton, Tween or sodium sulfite buffers (dilutions 1/100). M: 100 bp DNA ladder plus (GIBCO BRL).

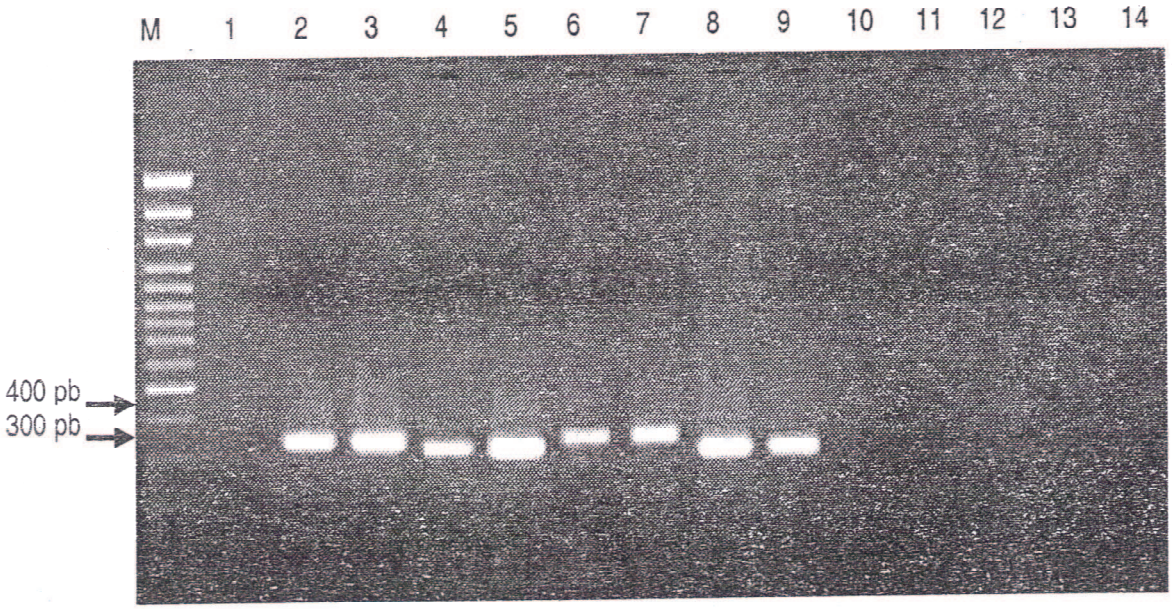


Figure 2. Comparative analysis of RT-PCR amplification products obtained from diluted crude sap extracts prepared with SSC buffer complemented with Sodium sulfite and total RNA extracts from viroid-infected tissues. **Lane 1**: crude sap extract of ASSVd-infected apple; dilution 1/10. **Lane 2**: crude sap extract of ASSVd-infected apple; dilution 1/100. **Lane 3**: total RNA extract of ASSVd-infected apple. **Lane 4**: crude sap extract of PBCVd-infected pear; dilution 1/100. **Lane 5**: total RNA extract of PBCVd-infected pear. **Lane 6**: crude sap extract of PLMVd-infected peach; dilution 1/100. **Lane 7**: total RNA extract of PLMVd-infected peach. **Lane 8**: crude sap extract of HSVd-infected almond; dilution 1/100. **Lane 9**: total RNA extract of HSVd-infected almond. Negative controls included water and crude extracts from healthy apple, pear, peach and almond (**Lanes 10, 11, 12, 13** and **14** respectively). M: 100 bp DNA ladder plus (GIBCO BRL).

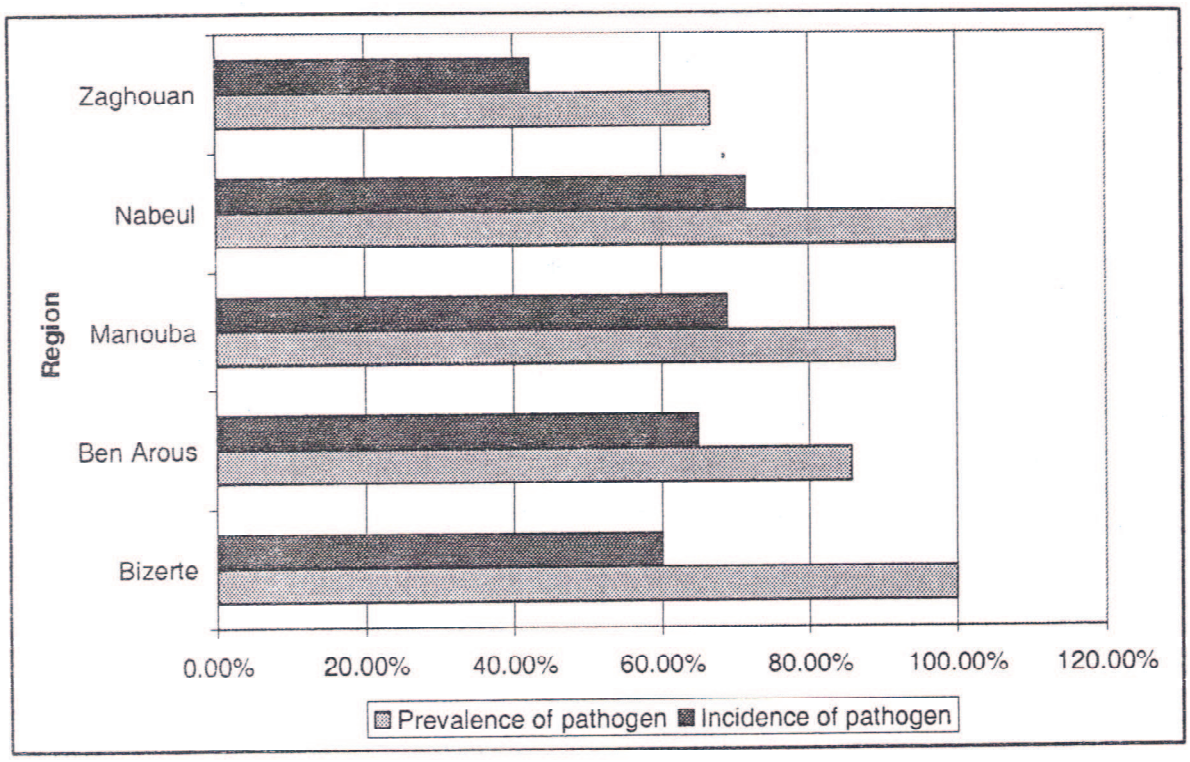
Epidemiological characteristics of PLMVD in the North of Tunisia

Incidence and prevalence of PLMVd

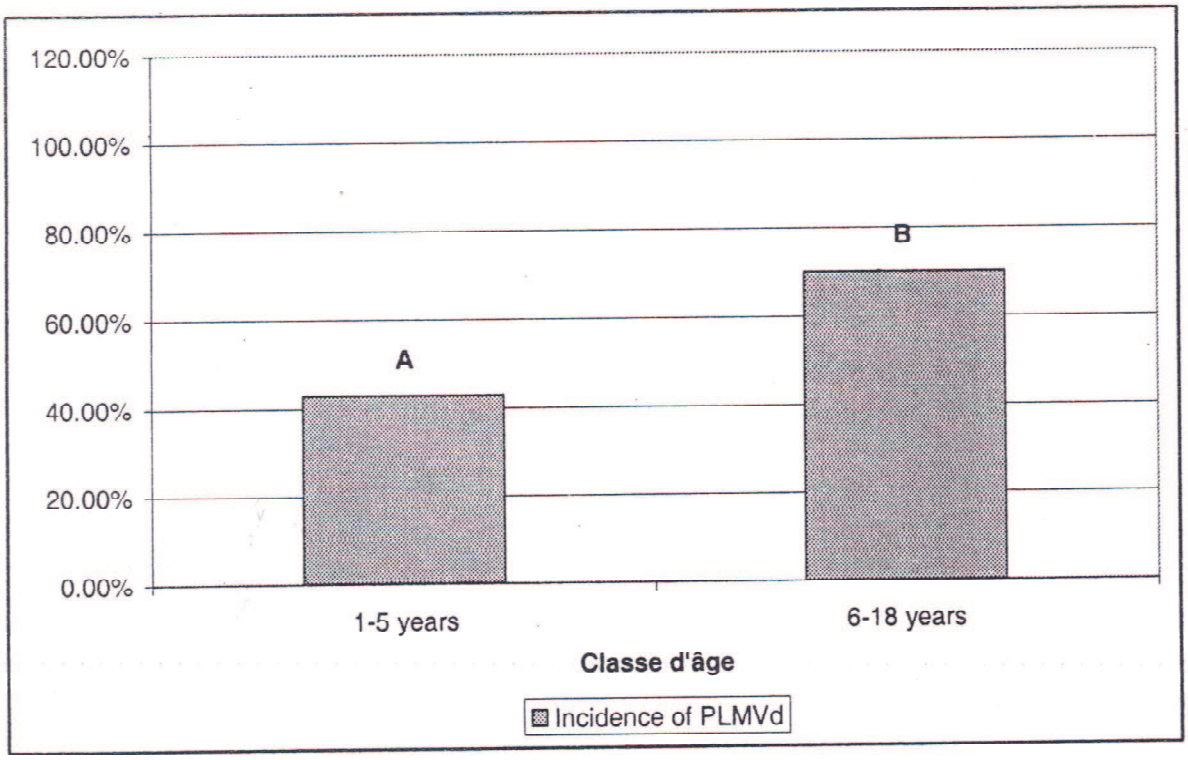
The prevalence and the incidence of the pathogen were evaluated in different regions of the north: Bizerte, Ben Arous, Manouba, Nabeul and Zaghouan in 2004. This analysis revealed an important prevalence of the pathogen from 66-100% with an incidence ranged between 41% and 72% (Graph 1). No significant differences of PLMVd incidence were observed between the different regions ($P>0.05$).

Influence of the age of the tree on PLMVd incidence

The PLMVd incidence is 43% for the age class 1-5 years and 70% for the age class 6-18 years. A significant difference of PLMVd incidence was detected between these two age classes (Graph 2).



Graph 1. Prevalence and incidence of the pathogen in the northern regions



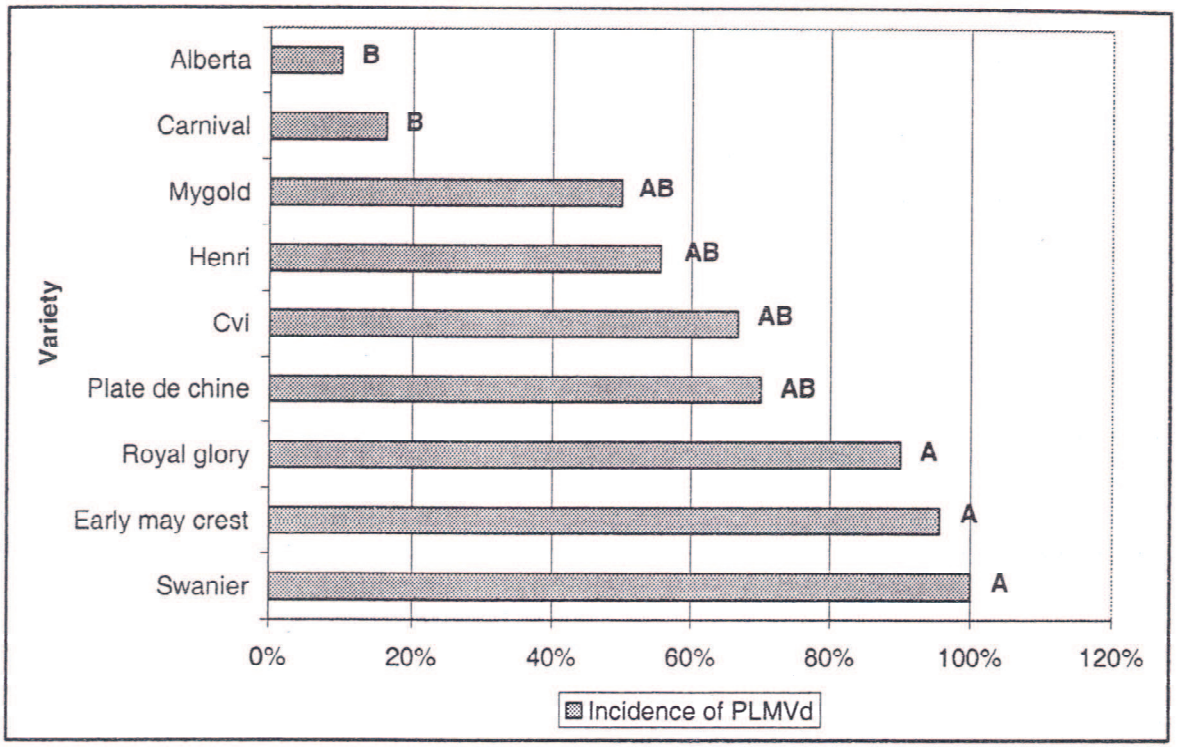
Graph 2. PLMVd incidence according to the age of North of Tunisia (The letters A and B correspond to two different groups)

Influence of the cultivar on PLMVd incidence

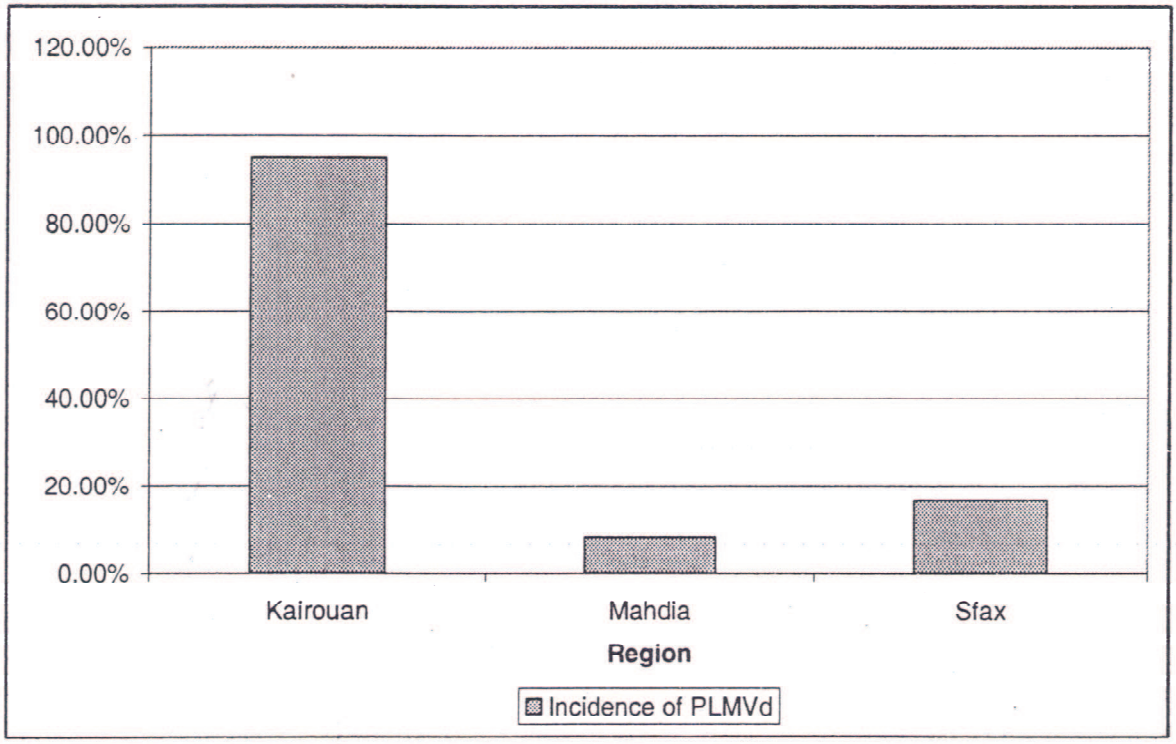
In order to study the sensitivity of the cultivar to PLMVd infection, four examples of orchards located in the north were selected because cultivars were from the same region, with similar age and origin (including rootstock).
The first example correspond to two orchards separated by a Cyprus line and differed only by the cultivar, which in Early may crest for one and Carnival for the other. A clear difference in PLMVd incidence was recorded: 100% for Early may crest and 23% for Carnival. The cultivar Carnival appears less sensitive than Early may crest.
The second example correspond to an orchard containing three cultivars: 4 trees rows for each of the cultivars Early may crest and Royal glory and 3 tree rows for the cultivar Carnival. PLMVd incidence is respectively 100, 83 and 40%. In this example the cultivar Carnival seems to be less sensitive than the two others.
The third example correspond to two neighbouring orchards containing the cultivar Alberta for one and Henry for the other. According to this example the cultivar Alberta appears less sensitive than the cultivar Henry as PLMVd incidence is 10 and 56% respectively.
Finally in a fourth example, two neighbouring orchards containing the cultivars Plate of china and Mygold had an infection rate of 73 and 50% respectively.
These examples suggest that there is a difference in sensitivity between the studied cultivars. So a large number of samples collected in different regions of the North of Tunisia for 9 cultivars of different fruit maturity and belonging to the age class of 6 to 18 years (having high incidence of the pathogen) were considered to confirm the hypothesis. The cultivar Cristal was excluded from this statistical analysis because it was found only in the Sahel.
The analysis of the variance showed very highly significant differences between the studied cultivars for the incidence of the pathogen (P= 0.001). The Tukey test allowed to classify them into three groups (Graph 3).

Incidence of PLMVD in three regions of the centre, the Sahel and the South of Tunisia

The study was extended to other regions of Tunisia in the Centre (Kairouan), the Sahel (Mahdia) and the South (Sfax) in order to detect the presence of PLMVd. The incidence of the pathogen is important in one orchard visited in Kairouan (95%), while two orchards in Mahdia and one orchard in Sfax seem to be less touched (8 and 17%, respectively) (Graph 4).



Graph 3. PLMVd incidence according to the cultivars in the North of Tunisia.
a : Cultivars very sensitive
ab : Cultivars fairly sensitive
b : Cultivars less sensitive



Graph 4. PLMVd incidence in three regions of the Centre, the Sahel and the South.

DISCUSSION

An RT-PCR test has been developed for the detection of PLMVd. This test was first developed by using total RNA preparations from materials known to be infected with selected isolates. With such assay, all the tested isolates were detected. These tests were then applied on total RNA preparations from plant material collected in several orchards in Tunisia, showing that cultivated fruit trees were infected with PLMVd. The analyses of the nucleotide sequences of cloned PLMVd amplification products revealed a high nucleic acid identity with previously characterized sequence variants from other isolates of this same viroid (Hernandez and Flores, 1992), demonstrating the specificity of the test developed here.

The test was then simplified for direct use of diluted crude plant extracts. Samples were ground in an extraction buffer, diluted and added directly into the RT-PCR mix. The inhibitory effects of plant polysaccharides or phenolic compounds of crude plant extracts on PCR amplification were avoided by their dilutions. This rapid and simple extraction method avoids the use of organic and harmful solvents. It gave as good response as the classical viroid extraction protocol making the test adapted for routine diagnostic of large number of samples for viroid detection and identification. The test is performed in a single closed tube which reduces the risk of contaminations between the reverse transcription and the amplification steps. The processing of thirty samples relys on 7 hours.

The epidemiology of PLMVd was undertaken by studying its incidence and its prevalence according to the locality, the age of the tree and the cultivar in different regions of the North of Tunisia. The results confirmed the importance of the disease in these regions.

There was a relationship between the age and PLMVd incidence. This can be due to transmission by the tools and the potential existence of transmission by the insect vectors *Myzus persicae* (Desvignes *et al.*, 1992).

There was a difference in sensitivity between the studied cultivars to PLMVd infection and they were classified into three groups of different sensitivities. Group I contains cultivars Swanier, Early may crest and Royal glory showing high incidence of the pathogen from 90 to 100%. They are considered as very sensitive. Group II contains cultivars Plate of china, Cvi, Henry and Mygold with infection rate from 50 to 70%. They are fairly sensitive. Group III considered as less sensitive, includes cultivars Carnival and Alberta with infection rate from 10%-16%.

These differences in sensitivity were related with the maturity of the cultivar. Indeed, the early cultivars and the cultivars of season belong to group I and II, the late cultivars belong to group II and III, and the third group contains only late cultivars. This would be due to a relation between level of replication and physiology of the tree since all the samples were taken at the same period (May). On the other hand, an interaction between the physiology of the tree and the cycle of a potential insect vector could exist. Indeed, this vector-plant interaction could depend on the precocity state of the tree.

Analysis of PLMVd incidence in three other areas of Tunisia showed a high infection rate in the prospected orchard in Kairouan. This could be explained by the culture of a sensitive cultivar, in particular Early may crest, which confirms the results obtained for the northern regions. The areas of Mahdia

and Sfax seem to be less infected. The culture of late cultivars potentially less sensitive as suggested by our study, in particular Plate of China and Cristal, could explain this low incidence in the prospected orchards in these areas.

PLMVd is very widespread in the world and particularly in the Mediterranean countries (France, Spain, Italy, Greece, Yugoslavia, Algeria and Morocco) and USA (Flores *et al*, 1992; Loreti *et al*, 1998; Hadidi *et al*, 1997). Furthermore, the origins of the rootstock and the cultivar in Tunisia are Spain, Italy and USA. The exchange of infected materials between Tunisia and these countries could be in part responsible for the presence of this viroid in Tunisia. So, it is interesting to develop a phytosanitary certification program for this viroid to limit its propagation.

In conclusion, this work shows the presence of PLMVd in tunisian orchards and offer a rapid technique adapted to crude plant extract for viroid detection. Furthermore, we report the influence of two factors on PLMVd incidence which are the age of the trees and the cultivars. All these results will be confirmed by new prospections. This work will carry on (1) by the study of molecular variability of PLMVd according to the cultivars and (2) could lead to the selection of resistant cultivars that will be recommended for an efficient control of the peach latent mosaic disease.

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