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# Distribution of *Peach latent mosaic viroid* in Commercial Orchards of Peach in the North of Tunisia

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## Abstract

The presence of *Peach latent mosaic viroid* (PLMVd) was monitored during 2 years in peach orchards located in the North of Tunisia. PLMVd population was surveyed using a specific RT-PCR test adapted to crude sap extract. During the first year (2004), 228 samples were randomly selected in 22 peach orchards (eight cultivars) and tested for the presence of PLMVd. Results showed that PLMVd is highly and equally present in four regions of the North of Tunisia. Analyses of some key factors in relation to PLMVd incidence revealed that the tree age did not influence the infection rate. The eight studied cultivars were clustered in three groups according to their PLMVd incidence. Furthermore, the early and season cultivars were statistically more infected than the late cultivars. Prospections in May and October 2005 were performed in four selected orchards. Each two orchards contained Early May Crest (early cultivar) and Carnival (late cultivar) cultivars, respectively. The difference in PLMVd incidence observed in 2004 was confirmed. Furthermore, no correlation between the tree physiological state and the viroid detection was observed. Further investigations on the origin of the differences in PLMVd incidence between Early May Crest and Carnival cultivars would be of interest.

## Introduction

The first report of peach latent mosaic (PLM) disease was made in France in the course of graft indexing of peach germplasm imported from USA and Japan on peach GF305 indicator (Desvignes, 1976). PLM disease is induced by *Peach latent mosaic viroid* (PLMVd) which is a covalently-linked circular single-stranded RNA molecule ranging in size from 335 to 351 nucleotides and presenting a high degree of base pairing (Malfitano et al., 2003). In peach (*Prunus persicae*), PLMVd can induce a broad variety of symptoms: mosaic, large white patches covering most of the leaf blade, irregularly shaped colourless fruits with cracked sutures and enlarged pits, bud necrosis and delay in foliation, flowering and maturity and a general wilting (Desvignes, 1986). The incidence of PLMVd reached up to 60% in North American germplasm (Shamloul et al., 1995; Hadidi et al., 1997), 40% in Syrian germplasm (Ismaeil et al., 2001), 50% in Italian germplasm (Loreti et al., 1998), 64% in Chinese germplasm (Zhang et al., 2000), 68% in Australian germplasm (Di Serio et al., 1999), 80–85% in Spanish germplasm (Flores et al., 1992) and 90% in Japanese germplasm (Osaki et al., 1999). In orchards, the infection rate of commercial varieties of peach and nectarine in five USA states reached 50% (Skrzeczkowski et al., 1996). In Spain, while the native cultivars were not infected, the percentage of imported and infected varieties reached 85% (Badenes and Llacer, 1998). PLMVd has been detected in other species of fruit trees, such as apricot (*Prunus armeniaca*), plum (*Prunus domestica*), sweet cherry (*Prunus avium*), cultivated pear (*Pyrus communis*) and wild pear (*Pyrus amygdaliformis*) from European countries (Faggioli et al., 1997; Hadidi et al., 1997; Kyriakopoulou et al., 2001) and recently, pear and almond (*Prunus amygdalus*) from Tunisia (Fekih Hassen et al., 2004, 2005). The economic importance of PLMVd derives from the fruit alterations, reduced tree longevity and increased susceptibility to other biotic or abiotic stress (Flores et al., 2006).

Previous study showed that PLMVd is readily transmissible by grafting and budding but not through seed (Desvignes, 1986). During greenhouse experiments, the viroid was transmitted to healthy plants by *Myzus persicae* but not by *Aphis gossypii* and *Aphis spiraecola* (Desvignes et al., 1992; Flores et al., 1992). PLMVd can be transmitted through blades contaminated with PLMVd (Flores et al., 1990; Hadidi et al., 1997). This latter result indicates that contaminated pruning tools may play a role in viroid spread within commercial

orchards. So far, few studies have been carried out to study the viroid incidence on various cultivars within a growing area. For example, Canizares et al. (1998) studied the *Hop stunt viroid* (HSVd) incidence in five commercially important apricot cultivars in south-eastern Spain. This study revealed an infection rate of 81% and no significant differences in infection level between cultivars. To our knowledge and despite the worldwide spread and economical importance of PLMVd, the influence of the cultivar and the age of the tree on its incidence have never been studied so far.

Peach culture has an economic importance in Tunisia as it occupied more than 22 000 hectares producing approximately 108 000 tons in 2005. A preliminary survey revealed that the PLMVd was present in Tunisian orchards and it represents one of the most important threats for the peach production (Fekih Hassen et al., 2004). Several questions rose from this preliminary study: what is the incidence of PLMVd in different Tunisian regions? What is the relationship between the cultivar or the tree age and the PLMVd incidence? Does the sampling period influence the PLMVd detection?

So, using a previously published RT-PCR protocol (Fekih Hassen et al., 2006), a large survey of PLMVd incidence was undertaken in eight peach cultivars in the main production areas of Tunisia. During this survey, data such as the geographical origin, cultivar and rootstock identity and origin and age of the trees were collected. The influence of some of these parameters was statistically evaluated. Together with this first survey, the spatio-temporal distribution of PLMVd in four selected orchards was studied during 2 years.

## Materials and Methods

### Peach sampling for PLMVd incidence study

Twenty-two orchards of peach trees were prospected in May 2004 in the main growing regions of the North of Tunisia (Bizerte, Ben Arous, Manouba and Nabeul). For each orchard, the localization, age of the trees, identity and origin of the rootstock and cultivar were collected. For 18 orchards, the rootstock was GF305 while two orchards had GF677 and two other had almond trees. The rootstock origin was Spain or Italy for peach trees and Tunisia for almond trees. The majority of the sampled cultivars were imported from USA and multiplied in Tunisia. The tree age ranged from 5 to 18 years. Orchards were further classified according to the region (Table 1), the age (Table 2) and the cultivar (Table 4). Two hundred twenty-eight trees were randomly sampled. Three 1-year-old branches were collected in the canopy of each tree.

### Peach sampling for PLMVd spatio-temporal distribution study

After the first sampling in 2004, four orchards from Ben Arous and Nabeul were prospected in May 2005 and October 2005. The characteristics of each orchard (location, identity and origin of the rootstock and the cultivar and age of the trees) are summarized in Table 5. Fifty trees were randomly sampled in each orchard. Three 1-year-old branches were collected in the canopy of each tree.

Table 1
*Peach latent mosaic viroid* (PLMVd) prevalence and incidence in each studied region in the north of Tunisia in May 2004

| Region | Number of orchards (samples) | PLMVd prevalence (%) | PLMVd incidence (%) |
|---|---|---|---|
| Ben Arous | 8 (111) | 88 | 68 |
| Nabeul | 5 (38) | 100 | 68.42 |
| Manouba | 7 (60) | 100 | 80 |
| Bizerte | 2 (19) | 100 | 63.15 |
| Total number | 22 (228) | – | – |

Table 2
*Peach latent mosaic viroid* (PLMVd) incidence for each age in May 2004

| Orchard's age (years) | Number of orchards (samples) | PLMVd incidence (%) |
|---|---|---|
| 5 | 2 (17) | 52.94 |
| 6 | 6 (45) | 95.55 |
| 7 | 4 (25) | 84 |
| 8 | 3 (46) | 47.82 |
| 10 | 2 (55) | 96.09 |
| 12 | 3 (21) | 80.95 |
| 18 | 2 (19) | 63.15 |
| Total number | 22 (228) | – |

### Source of positive controls

Twigs of PLMVd-infected GF305 peach seedlings were kindly provided by Dr Pradier (Station de Quarantaine des Ligneux, Lempdes, France). These materials were viroid-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).

### Preparation of crude sap extract

Leaf tissues (0.2 g) were grounded in Sample Extraction Pouches (Agdia, Germany) with 1 ml of 2x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7) containing 1% sodium sulfite as anti-oxidant. The mix was transferred to 1.5 ml eppendorf tube. After centrifugation at 16 000 × ***g*** during 30 min the supernatant was collected, diluted 100x and used directly for RT-PCR test.

### RT-PCR assay

RT-PCR was performed using the one tube RT-PCR Titan kit (Roche diagnostics, Penzberg, Germany). The reverse transcription and the amplification steps were performed sequentially in the same tube. Primer pairs previously reported for RT-PCR amplification

for PLMVd (Loreti et al., 1999) were used in this study. Homologous PLMVd primer (5′-CCCGATAGAAAGGCTAAGCACCTCG-3′) and complementary PLMVd primer (5′-AACTGCAGTGCTCCGAATAGGGCAC-3′) are identical and complementary to positions 117–141 and 92–116, respectively, of the PLMVd reference sequence (Hernandez and Flores, 1992). Synthesis and purification of the primers were performed by Eurogentec (Eurogentec, Seraing, Belgium). Two microlitres of the diluted (1/100) crude sap extract were mixed with 0.5 $\mu$l of the complementary primer (400 mM). This mixture was heated for 5 min at 100°C and immediately chilled on ice. The RT-PCR reaction mixture (total volume 25 $\mu$l) contained 1 × RT-PCR buffer, 5 mM dithiothreitol, 200 mM of dATP, dCTP, dGTP and dTTP each, 400 mM of the homologous primer, 0.5 U of enzyme mix (AMV reverse transcriptase and High Fidelity Taq-Polymerase). Diethylpyrocarbonate treated water was added to a volume of 22.5 $\mu$l. After addition of the denatured extract/complementary primer mixture, the RT-PCR reaction was submitted to the following cycling parameters: 50°C for 1 h (cDNA synthesis), followed by 30 cycles at 95°C for 30 s (3 min for the first cycle), 60°C for 45 s and 72°C for 45 s with a final extension at 72°C for 7 min.

Each RT-PCR run included a positive control, a water control and a healthy plant control. The latest corresponded to crude sap extract from a PLMVd-free peach.

### Statistical analysis

The influence of the tree age and the cultivar on PLMVd incidence was analysed by the ANOVA procedure of the MINITAB 13.20 software (Minitab Inc., State College, PA, USA). Statistical significance was judged at the $P < 0.05$ (significant differences) or $P < 0.01$ (highly significant differences) levels. Whenever analysis revealed statistically significant differences, the Tukey test for separation of means was performed. Using the same software, the influence of the physiological state of the tree on PLMVd incidence was analysed by applying the general linearized model procedure.

## Results

### Incidence and prevalence of PLMVd in four Tunisian regions

In order to study the PLMVd prevalence (the number of infected orchards divided by the total number of tested orchards in one region) and incidence (the number of infected trees divided by the total number of tested trees) in the main producing area of Tunisia, prospections were performed in 22 peach orchards during May 2004. Between Tunisian regions, the prevalence of the pathogen ranged from 87.5% to 100% and its incidence ranged from 63% to 80% (Table 1). No significant differences of PLMVd incidence were observed between the different regions ($F$ was not significant at $P = 0.694$). Fig. 1 shows an example of results obtained with RT-PCR tests on crude sap extracts from PLMVd-infected peachs.

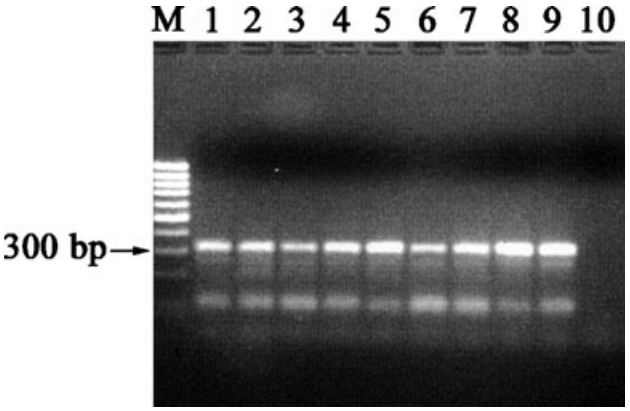


Fig. 1 Agarose gel electrophoretic analysis of RT-PCR products amplified from crude sap extracts of *Peach latent mosaic viroid* (PLMVd)-infected peachs (lanes 1–9). Negative control included crude sap extract from healthy peach (lane 10). M: 100 bp DNA ladder plus (GIBCO BRL)

### Influence of the tree age on PLMVd incidence

The results were clustered according to the age of the sampled trees. An ANOVA analysis was applied to evaluate the effect of the tree age on the PLMVd incidence. No significant relationship was observed ($F$ was not significant at $P = 0.497$) (Table 2).

### Influence of the cultivar on PLMVd incidence

Among our results, some examples of orchards (Table 3) showed interesting features as they contained each two or three cultivars presenting the same characteristics (location, agricultural practices, tree age, origin and identity of the rootstock and origin of the cultivar). The first example corresponded to two adjacent orchards containing Early May Crest and Carnival cultivars, respectively. A clear difference in PLMVd incidence was observed: 100% for Early May Crest and 23% for Carnival (Table 3). The second example corresponded to three adjacent orchards containing respectively, Early May Crest, Royal Glory and Carnival cultivars. The PLMVd incidence was 100, 83 and 40%, respectively (Table 3). In the third example, two neighbouring orchards containing the cultivars Plate de Chine and Mygold had infection rates of 73 and 50%, respectively (Table 3). These three examples highlighted the difference in PLMVd incidence between the studied cultivars. A larger statistical analysis, including eight cultivars, was undertaken. The ANOVA showed significant differences in the incidence of the pathogen ($F$ was significant at $P = 0.013$) between the studied cultivars. The Tukey test allowed their classification into three groups (Table 4). Group I (highly infected) contained cultivars Early May Crest and Suwanee which showed an incidence of 96 and 100%, respectively. Group II (fairly infected) contained cultivars Plate de Chine, Seville, Henry, Royal Glory and Mygold with infection rates ranging from 50% to 74%. Group III (less infected) corresponded to the cultivar Carnival with an infection rate of 16%. Interestingly, group I contained one early

Table 3
*Peach latent mosaic viroid* (PLMVd) incidence according to the cultivar in May 2004

| Example | Orchard | Region | Cultivar | Age (years) | Rootstock origin/identity | Cultivar origin | PLMVd incidence (%) |
|---|---|---|---|---|---|---|---|
| 1 | A | Ben Arous | Early May Crest | 10 | Spain/peach GF677 | USA | 100 |
| | B | Ben Arous | Carnival | 10 | Spain/peach GF677 | USA | 23 |
| 2 | A | Nabeul | Early May Crest | 12 | Spain/peach GF305 | USA | 100 |
| | B | Nabeul | Carnival | 12 | Spain/peach GF305 | USA | 40 |
| | C | Nabeul | Royal Glory | 12 | Spain/peach GF305 | USA | 83 |
| 3 | A | Bizerte | Plate de Chine | 18 | Tunisia/Almond | USA | 73 |
| | B | Bizerte | Mygold | 18 | Tunisia/Almond | USA | 50 |

A, B and C are neighbouring orchards.

Table 4
*Peach latent mosaic viroid* (PLMVd) incidence according to the cultivars and the maturity period of the cultivar in May 2004. Cultivars and cultivar maturity groups with statistically significant difference in PLMVd infection rates were designated with different letters

| Maturity | Cultivar | Number of orchards (samples) | PLMVd incidence according to cultivar (%) | PLMVd incidence according to the maturity period of the cultivar (%) |
|---|---|---|---|---|
| Early | Early May Crest | 8 (98) | 95.91 a | 93 a |
| | Seville | 1 (12) | 66.66 ab | |
| Saison | Suwanee | 2 (20) | 100 a | 73 a |
| | Plate de Chine | 3 (19) | 73.98 ab | |
| | Royal Glory | 3 (19) | 52.63 ab | |
| | Mygold | 1 (8) | 50 ab | |
| Late | Henri | 1 (9) | 55.55 ab | 23 b |
| | Carnival | 3 (43) | 16.27 b | |
| | Total number | 22 (228) | – | – |

a: highly infected cultivars; ab: fairly infected cultivars; b: less infected cultivars.

Table 5
*Peach latent mosaic viroid* (PLMVd) incidence in the prospected orchards of Ben Arous and Nabeul regions in May 2004, May and October 2005

| Orchard | Region | Cultivar | Age (years) | Rootstock origin/identity | Cultivar origin | PLMVd incidence | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | May 2004 (%) | May 2005 (%) | October 2005 (%) |
| 1A | Ben Arous | Early May Crest | 10 | Spain/peach GF677 | California | 100 | 100 | 100 |
| 2A | Nabeul | Early May Crest | 12 | Spain/peach GF305 | California | | | |
| 1B | Ben Arous | Carnival | 10 | Spain/peach GF677 | California | 26 | 46 | 49 |
| 2B | Nabeul | Carnival | 12 | Spain/peach GF305 | California | | | |

A and B are adjacent orchards.

cultivar and one season cultivar, group II contained one early cultivar, three season cultivars and one late cultivar and group III contained one late cultivar. So, in order to search for a correlation between the cultivar maturity group and the PLMVd incidence, the eight studied cultivars were also clustered into three groups according to the maturity period (early, season and late) (Table 4). The ANOVA showed a highly significant differences of PLMVd incidence between the cultivar's maturity groups ($F$ was highly significant at $P = 0.002$). The Tukey test allowed the clustering of the early and season cultivars into one group with high incidence of the pathogen (73–93%) and the late cultivars into another group with low infection rate (23%) (Table 4).

**Influence of the physiological state of the tree on PLMVd incidence**

To refine our knowledge on the spatio-temporal evolution of PLMVd infection, two additional surveys were carried out in May and October 2005 through four selected orchards located in Ben Arous and Nabeul. Each two orchards were adjacent and differed only by the cultivar: Early May Crest (highly infected) or Carnival (less infected) cultivars (Table 5). These two cultivars are widely cultivated in Tunisia. Early May Crest and Carnival being mature in May and October, respectively. Another goal of these experiments was to evaluate the influence of the physiological state of the tree on the PLMVd incidence.

Results showed that the differences in PLMVd incidence observed in May and October 2005 between the two cultivars were similar to those observed the previous year in May 2004 (Table 5). The influence of the physiological state of the tree on PLMVd incidence was evaluated by the general linearized model. No significant differences of PLMVd incidence in Carnival cultivar were observed between the three periods of prospections in May 2004, May and October 2005 ($F$ was not significant at $P = 0.615$). For Early May Crest cultivar, the infection rate was 100% whatever its physiological state and the year of sampling.

## Discussion

These 2-year experiments studied for the first time the prevalence and the incidence of PLMVd in various regions of Northern Tunisia. The results were analysed to evaluate the influence of the cultivar, the tree age and the sampling period on PLMVd incidence. This analysis underlined for the first time high PLMVd distribution and significant differences in PLMVd incidence between eight cultivars. Additionally, the tree age did not have a significant effect on PLMVd incidence. The diagnostic method used was able to detect PLMVd during two sampling period corresponding to different physiological states of the tree.

The eight cultivars were classified into three groups of different infection rates. This clustering seemed to be related with the maturity of the cultivar. Further analysis revealed that early and season cultivars belonged to a group, with high infection rate, significantly different from the late cultivars, corresponding to another group with low infection rate. These results could be due to a relation between the level of viroid replication and the physiological state of the tree. Indeed, all the samples were taken in May 2004, which corresponds to the maturity period for only the early cultivars. Therefore, the PLMVd incidence for an early cultivar (Early May Crest, group I) and a late cultivar (Carnival, group III) was further studied in May (maturity of Early May Crest) and October (maturity of Carnival) 2005. While these two cultivars were grown under the same conditions (region, agricultural practices, tree age, origin and identity of the rootstock and origin of the cultivar), they exhibited significant differences in PLMVd infection rates whatever the physiological state of the tree. Nevertheless, these preliminary results should further be investigated to confirm this observation.

Different hypothesis may explain the wide distribution of PLMVd throughout Tunisian orchards and the observed differences between cultivars. First of all, as the PLMVd can be latent in the plant nurseries for a long period of time (Flores et al., 2006), the disease can be propagated by the use of non-certified material as rootstock or as grafted scion. The risk is real as 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; Hadidi et al., 1997; Loreti et al., 1998; Kyriakopoulou et al., 2001). So, the exchange of infected materials between Tunisia and these countries could be in part responsible for the presence of this viroid in Tunisia. Indeed, while PLMVd is of certification importance in Europe (Smith et al., 1992), PLMVd is not on the list of the diseases for quarantine or certification and control in USA (e.g. State of California) (California Department of Food and Agriculture (CDFA), 1998) from which the Early May Crest and Carnival cultivars were imported. It can be suggested that the differences of infection rates between Carnival and Early May Crest cultivars might come from different infection levels in the scion mother trees used in the nursery for propagation. So, the use of virus free and virus tested material should be recommended and legislation should be strengthened to avoid plantation of non-virus-free material.

After planting, the infection can be further extended by agricultural practices (Hadidi et al., 1997) or by a potential insect vector (*M. persicae*) (Desvignes et al., 1992). So, adoption of good agricultural practices with disinfection of the tools should be recommended to moderate viroid propagation. Additionally, the role of a potential insect vectors could not be discarded. Experimental proof of *M. persicae* transmission has been provided (Desvignes et al., 1992) but other vectors could not be excluded. In Tunisia, the emergence peaks of *M. persicae* correspond to April and September, e.g. months before our sampling period in May and October, respectively. So, as the *M. persicae* population density is nearly identical in both periods and if the transmission with this vector is real in field conditions, its affinity to Early May Crest and to Carnival cultivars might be different. These differences could also explain the observed differences between both cultivars. Finally, the observed differences in infection rates between Early May Crest and Carnival cultivars might be due to intrinsic resistance of Carnival cultivar to PLMVd infection. Nevertheless, this assertion remains to be proved.

In conclusion, the obtained results showed a high PLMVd incidence in Tunisian orchards. Furthermore, significant differences in PLMVd incidence were observed between cultivars, which were clustered in three different groups of different incidences. The Carnival cultivar was the less infected one while the Early May Crest cultivar was highly infected. The most important factor explaining the high PLMVd incidence and the variability between cultivars could be the propagation of infected scion mother in nurseries. Nevertheless, other factors such as the differential affinity of a vector to peach cultivars and the partial resistance of some cultivars against PLMVd can not be ruled out. Further experiments will address some of those hypotheses through bioassays.

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