

OUTBREAK OF PLUM POX VIRUS IN TUNISIA

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SUMMARY

The appearance of virus-like symptoms in leaves and fruits of Japanese plums in a stone-fruit tree germplasm collection in Tunisia led us to screen them for the presence of known stone-fruit viruses. Chip-budding of dormant bark from suspected plums onto seedlings of the biological indicator GF 305, and subsequent testing of these plants by serological and molecular tests, confirmed the presence of *Plum pox virus* (PPV) and demonstrated for the first time the occurrence of this virus in Tunisia. The use of monoclonal antibodies and restriction fragment length polymorphism of amplified cDNA identified the virus as belonging to strain D. Two Japanese plums cultivars '606' and 'Fortune' were severely affected. Seven other plum cultivars were also infected with PPV.

Key words: Tunisia, Sharka virus, serology, molecular assays.

INTRODUCTION

Stone-fruit trees can flourish in both temperate and Mediterranean areas, which has led to extensive exchange of germplasm between European and North African countries; peach, plum and apricot represent the major crops exchanged through formal and informal partnerships. Although many adapted species were introduced long ago, progress in developing germplasm collections has been hampered by the unintentional introduction of virus diseases. In Tunisia, virus diseases cause significant yield reduction and affect the fruit quality of stone fruit crops. *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) have been reported and characterized biologically, serologically and by molecular tools (Boulila and Marrakchi, 2001a,b; Boulila, 2002a,b).

At the beginning of 2000, Tunisian mother blocks,

particularly those of the Groupement Obligatoire des Viticulteurs et Producteurs de Fruits (GOVPF) and Groupement Interprofessionnel des Agrumes et Fruits (GIAF) were tested for viruses. Random collection of suspicious samples did not at this time result in the detection of PPV. It is important to note that damage caused by PPV depends on the species, the cultivar and the virus strain (Kegler *et al.*, 1998). Therefore, optimization of the timing of the observations of symptoms, and sampling of leaves and fruit that show symptoms has proved to be critical for the detection of PPV. Sharka, known in Europe as a virus disease affecting peach, plum, apricot, almond and cherry (Crescenzi *et al.*, 1997), was revealed for the first time in Tunisia in Japanese plum stocks.

PPV is very variable but two major serotypes can be distinguished: Dideron (PPV-D) and Marcus (PPV-M) (Candresse *et al.*, 1995). In addition, two atypical PPV isolates including El Amar (PPV-E) found in Egypt (Wetzel *et al.*, 1991a) and Cherry (PPV-C) in central Europe (Nemchinov *et al.*, 1996) represent another two recognized strains. PPV-M is found mostly in the Mediterranean and Balkan areas. The recent outbreak in North America (USA and Canada) (Levy *et al.*, 2000) has been identified as PPV strain D.

In the absence of information on PPV epidemiology in Tunisia, the purpose of the present study was to identify the causal agent of virus-like symptoms in Japanese plums. Our analyses confirmed that PPV strain D is present in Tunisian orchards.

MATERIALS AND METHODS

Plant material. Plum material was collected from two regions in Tunisia. From the Cap Bon region (Grombalia) we collected nine plants cv 606, ten plants cv Fortune, eight plants cv Delbarazur, four plants cv Black Amber 1 and three plants cv Black Gold. From the Kairouan region we collected eleven plants cv Angelino, five plants cv Black Diamond, thirteen plants cv Black Amber 2, seven plants cv Santa Rosa, ten plants cv Kelsey, twenty one plants cv Methley and fifteen plants cv Golden Japan from Sbikha.

Mechanical transmission. All samples were mechanically inoculated by crushing tissues in 0.1 M phosphate buffer, pH 7.4, containing 0.1% 2-mercaptoethanol, and rubbing the extract onto celite-dusted leaves of *Nicotiana benthamiana* and *N. clevelandii*. Infected *Nicotiana* plants were kept in a greenhouse (22-24°C).

Woody indexing. The material was chip-budded to four-month-old GF 305 peach seedlings. After two weeks, these were pruned to encourage the development of axillary buds and the expression of symptoms.

Serology and PCR. Triple-antibody sandwich ELISA (TAS-ELISA) was done using a kit supplied by Bio-Rad (Marnes-La-Coquette, France) for field samples, using the REAL PPV kit supplied by Durviz (Valencia, Spain) for greenhouse sampling to detect PPV strain D (Cambrà *et al.*, 1994) or using a kit provided by Agritest (Valenzano, Italy) that contains PPV strain M-specific monoclonal antibodies (Boscia *et al.*, 1997). The same samples were also assayed by indirect double-antibody sandwich (DAS-ELISA) using polyclonal antibodies raised to *Apple chlorotic leaf spot virus* (ACLSV) (Loewe Biochemica GmbH, Sauerlach, Germany).

Immuno-trapping virus particles from plant sap and negative staining with uranyl acetate for immunosorbent-electron microscopy (IEM) were done as described by Abou-Ghanem *et al.* (1997).

To verify the hypothetical size of CPs, we collected symptomatic leaves of infected plants and extracted soluble total proteins (Berger *et al.*, 1989) for analysis by Western blotting with a polyclonal antiserum (INRA-Bordeaux) according to Ravelonandro *et al.* (1992).

Anti-PPV specific immunoglobulins (INRA-Bordeaux) were used for immunocapture of virus particles that were then used for cDNA synthesis, followed by PCR detection of the 243 bp cDNA fragment (Wetzel *et al.*, 1991b, 1992).

To confirm the identity of the PPV isolate, heminested-PCR (Olmos *et al.*, 1997) and restriction fragment length polymorphism analysis (RFLP) was used following the digestion of amplified fragments by *Alu* I and *Rsa* I. Amplicons and restriction products were analyzed by electrophoresis in 1.5% agarose gels. Bands were visualized by ethidium bromide staining (Wetzel *et al.*, 1992).

RESULTS

Plum pox virus detection in trees and transfer to herbaceous plants. Collections of Japanese plums grown in two different areas (Cap Bon and Kairouan regions) that carried fruit with suspicious symptoms were inspected visually. Two cultivars showed clear symptoms. Cv 606 showed the most severe damage, with chlorotic and necrotic ring patterns on leaves (Fig. 1A and 1B). Fruits were deformed and dropped prematurely (Fig. 1C). Cv Fortune showed bark splitting and deformed leaves with conspicuous necrotic blotches. All other cultivars showed only a slight yellowing of leaves.

Initial testing of leaf or bark samples from these trees did not detect PPV. This may have been because the detection of PPV in Japanese plums can be masked for unknown reasons (high temperature in the environment, low virus concentration or the possible presence of another virus). For this reason, dormant buds were grafted onto 'GF 305' peach and forced. Shoots from cultivars produced the typical leaf symptoms on 'GF 305' (vein clearing or corkscrew twisting of the leaves) developed within 1 to 2 months (Fig. 1B). The samples from 'Delbarazur', 'Black Gold' and 'Golden Japan' did not induce this response.

Mechanical transmission from the field plums and 'GF 305' test plants to herbaceous hosts induced systemic chlorotic mottling in *N. benthamiana* and slight

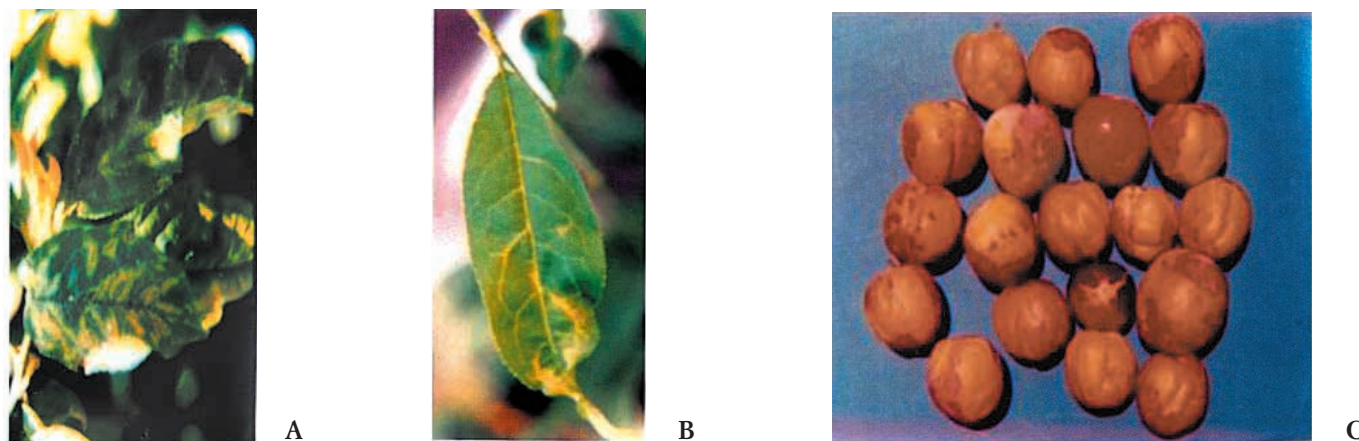


Fig. 1. Symptoms displayed by PPV: **A)** on Japanese plum cv 606 in the field; **B)** on graft-inoculated 'GF305' peach; **C)** PPV-like symptoms on fruit of cv 606.

Table 1. Rate of PPV infection in different cultivars of *Prunus salicina* located in the two inspected regions.

Location	Cultivars	Symptoms ^a	TAS-ELISA ^b	Infection ^c (%)
Grombalia	606	9/52	9/52	17.3
Grombalia	Fortune	10/74	8/74	10.8
Grombalia	Black Amber 1	4/23	3/23	13.0
Grombalia	Delbarazur	8/99	0/99	0.0
Grombalia	Black Gold	3/37	0/37	0.0
	Total	34/285	20/285	7.0
Sbikha	Angelino	11/88	10/88	11.4
Sbikha	Santa Rosa	7/61	5/61	8.2
Sbikha	Black Diamond	5/74	3/74	4.1
Sbikha	Black Amber 2	13/102	5/102	4.9
Sbikha	Kelsey	10/114	6/114	5.3
Sbikha	Methley	21/157	8/157	5.1
Sbikha	Golden japan	15/31	0/31	0.0
	Total	82/627	37/627	5.9

^a Number of trees with suspicious symptoms/number of total trees of the same cultivar.

^b TAS-ELISA using monoclonal antibody to PPV-D.

^c Percentage of trees infected with PPV.

local chlorotic lesions followed by systemic mottling in *N. clevelandii*. These symptoms were evident in 90% of plants inoculated with crude extracts of infected cv 606 and Fortune (not shown).

Immunodetection of PPV and co-existence of PPV associated with ACLSV. Characterization by TAS-ELISA of the PPV isolates found in field collections or those from greenhouse-indexed plants showed a positive reaction with the monoclonal raised to PPV strain D (Table 1) but none with monoclonal antibody raised to PPV-M (not shown). More than 45% (37/82) of the

suspected plants in Sbikha and 58% (20/34) in Grombalia tested positive for PPV-D. The remaining samples were negative by TAS-ELISA, indicating that PPV was not present. In addition, leaves from a few apricot and peach trees growing in the vicinity of these Japanese plums were assayed by TAS-ELISA but none gave a positive result (not shown).

To verify the possible occurrence of a mixed infection, the suspicious leaves collected in the two Tunisian regions were assayed by DAS-ELISA using polyclonal antibodies raised to PNRSV, PDV or ACLSV. No sample tested positive for PNRSV or PDV (not shown). However, a few Japanese plums (1.1 %) in Sbikha, but not in Grombalia, contained both PPV and ACLSV (Table 2).

To better characterize the occurrence of PPV in these woody plant tissues, ISEM was used to visualize the virus particles. Fig. 2A shows flexuous virus particles that were observed in infected but not healthy sap. Also, total protein extracts from these plants were analyzed by Western blotting (Fig. 2B). The capsid protein (CP) mobilities of strain M and strain D differed and the CP detected in both isolates PPV-606 and PPV-Fortune co-migrated with that of D type, further confirming that these isolates resemble PPV strain D.

Molecular detection assay and RFLP analysis. When plants were analyzed by IC-RT-PCR, eight positive PPV isolates ('606', 'Fortune', 'Angelino', 'Santa Rosa', 'Kelsey', 'Black Diamond', 'Black Amber 2' and 'Methley') yielded the expected 243 bp fragment (Fig. 3A). Using the nested primer PD, amplification in hemi-nested PCR (H-PCR) gave a fragment of 198 bp, as shown in Fig. 3B for the positive control, 606, Fortune and Black Amber 1 isolates. The nested PCR resulted in

Table 2. Rate of PPV and ACLSV infection in cultivars of *Prunus salicina* in Sbikha.

Cultivars	PPV infection ^a (%)	ACLSV ^b	PPV+ACLSV ^c (%)
Angelino	10/88 (11.4)	2/88	2.3
Santa Rosa	5/61 (8.2)	1/61	1.6
Black Diamone	3/74 (4.1)	1/74	1.4
Black Amber 2	5/102 (4.9)	0/102	0.0
Kelsey	6/114 (5.3)	1/114	0.8
Methley	8/157 (5.1)	2/157	1.3
Golden Japan	0/31 (0.0)	0/31	0.0
Total	37/627 (5.9)	7/627	1.1

^a Number of PPV infected plants / number of total trees of the same cultivar and (percentage of infected plants).

^b Biassed assays of PPV infected samples for DAS-ELISA using polyclonal antibodies to ACLSV.

^c Percentage of trees infected with PPV+ACLSV.

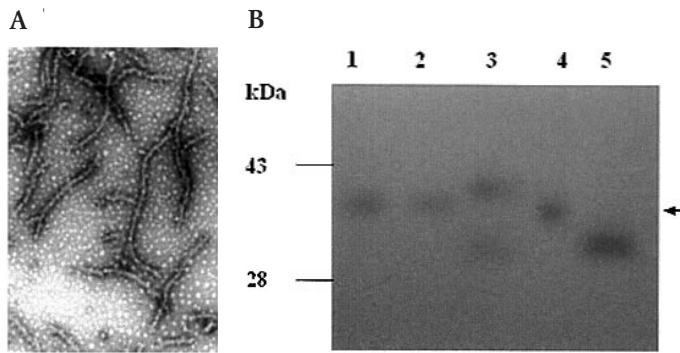


Fig. 2. A) Immunosorbent electron microscopy of plum pox virus particles, magnification 40,000x, from ‘GF 305’ peach inoculated with PPV ‘Fortune’ stained with 2% uranyl acetate. Bar = 200 nm. B) Western blot analysis of total protein extracts using polyclonal antibody anti-PPV. Extracts from ‘GF 305’ peach infected with PPV were isolated from cv 606 (lane 1), cv Fortune (lane 2), PPV-M (lane 3) and PPV-D (lane 4). Lane 5 represents the total protein from *N. benthamiana* infected with PPV-NAT. Positions of marker proteins are shown on the left.

dramatically improved sensitivity. In its current version, this assay can detect about 2,000 virus particles (10 femtograms of virus) diluted in 100 ml of crude plant sap. This is equivalent to a sensitivity of about 2,000 times better than that of a standard ELISA assay (Candresse *et al.*, 1995).

RFLP analysis with the amplified fragments showed that they produced two bands of 168 bp and 75 bp with *Alu* I and of 182 bp and 61 bp with *Rsa* I. This further indicated that PPV isolates from ‘606’, ‘Fortune’ and ‘Black Amber 1’ were D type (Fig. 3B).

The results obtained by molecular detection (IC-RT-PCR, H-PCR, RFLP analysis) indicated that PPV-D was present in nine Japanese plum cultivars (‘606’, ‘Fortune’, ‘Angelino’, ‘Santa Rosa’, ‘Kelsey’, ‘Black Diamond’, ‘Black Amber 1’, ‘Black Amber 2’ and ‘Methley’) (Fig. 3). These were in agreement with the results of TAS-ELISA (Table 1).

DISCUSSION

Our results show that PPV strain D is present in Tunisia. Since these trees did not show symptoms in the first years, there is no doubt that propagative material was introduced into the country from abroad without phytosanitary evaluation at the borders. The fact that the nine PPV isolates identified belong to the same serotype and the exclusive detection of Sharka in Japanese plums at the two localized areas, strongly suggests the unintentional introduction of PPV in Tunisia *via* infected materials. The low numbers (6 to 7%) of infected trees found in Grombalia or in Sbikha (Table 1) suggest that PPV infection is spreading slowly. It is conceivable that the propagation of materials by grafting reflects the uneven distribution of PPV in the plant materials that were introduced. As a consequence of such grafting experiments, we suggest that a few trees were initially produced from the introduced budsticks that contained high virus concentrations. This may explain why cv 606, Fortune and Black Amber 1 in Grombalia with PPV infection rate of 17.3, 10.8 and 13% and cv Angelino or Santa Rosa with 11.4 and 8.2% of infected trees in Sbikha (Table 1) were the first trees to show symptoms. These cultivars of Japanese plums may therefore be regarded as sensitive to PPV, but little is known about the behaviour of the three following cultivars, ‘Delbarazur’, ‘Black Gold’ and ‘Golden Japan’ that were uninfected.

Based on symptomatology, the PPV isolates differ from those commonly observed in Spain (Llacer and Cambra, 1986) because the expected symptoms were not seen. Limited tests found no evidence for co-infection of PPV with ilarviruses (PDV or PNRSV) but PPV was found with ACLSV in a few trees.

Once PPV had been detected, field inspection of potential aphid vectors was conducted. A few possible

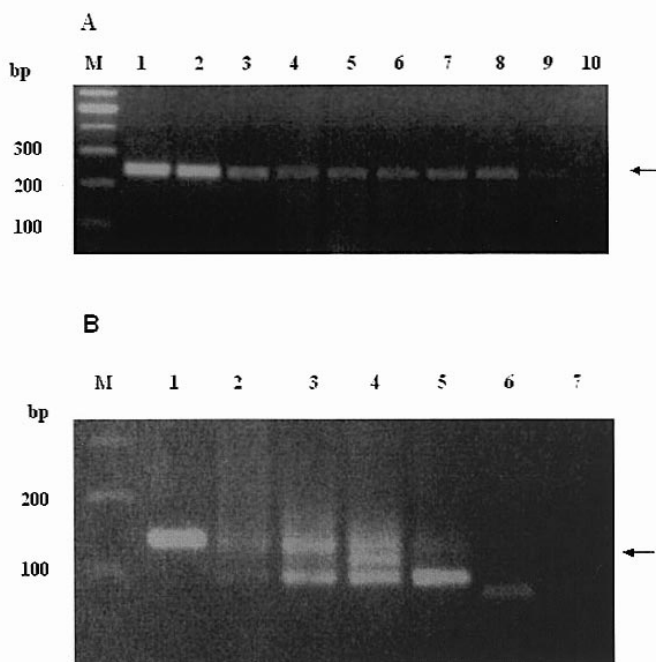


Fig. 3. A) Agarose gel electrophoresis of the amplified fragments (243 bp, indicated by the arrow) from the suspected Japanese plum cultivars. The amplified DNA from: positive control (lane 1), ‘606’ (lane 2), ‘Fortune’ (lane 3), ‘Angelino’ (lane 4), ‘Santa Rosa’ (lane 5), ‘Kelsey’ (lane 6), ‘Black Diamond’ (lane 7), ‘Black Amber 2’ (lane 8), ‘Methley’ (lane 9) and the healthy control (lane 10). B) Agarose gel analysis of the nested-PCR products (indicated by the arrow) of the 3’ coding region of PPV CP hemi-nested PCR products (198 bp). Positive control non-reamplified (lane 1), positive control (lane 2), ‘606’ (lane 3), ‘Fortune’ (lane 4), ‘Black Amber 1’ (lane 5), ‘606’ amplicon digested with *Rsa* I (lane 6) ‘Fortune’ amplicon digested with *Rsa* I (lane 7). Size (bp) of molecular weight markers is indicated on the left.

aphid vector species found in Mediterranean area (*Aphis spiraeicola*, *Brachycaudus helichrysi*, *Myzus persicae*) were observed but the involvement of these vectors in PPV spread could not be tested because control measures have eliminated the infected trees.

Intensified effort is needed to set up strict measures to control PPV in Tunisia. The results reported here suggest that the present quarantine system used to control imported material does not work effectively enough. Facing a real threat, it becomes extremely urgent to prevent the spread of Sharka in Tunisia by launching an eradication campaign in mother block stands and orchards, strengthening regulations regarding the importation and movement of propagative materials, using certified virus-free materials, better and accurate checking by quarantine services and regular inspections and surveys in orchards and nurseries.

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