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SHORT NOTE

Occurrence and distribution of pome fruit viruses in Tunisia

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Summary. The phytosanitary status of pome fruit trees was examined in Tunisia, in surveys conducted in spring 2009 and 2010, in the main Tunisian mother blocks. A total of 248 samples were collected (111 from apple, 106 from pear and 31 from quince), and tested for the presence of *Apple chlorotic leaf spot virus* (ACLSV) and *Apple mosaic virus* (ApMV) using ELISA and RT-PCR, and for *Apple stem pitting virus* (ASPV) using RT-PCR. 37% of the samples were infected by at least one virus. ACLSV was the most widespread virus (34% of samples), followed by ApMV (4%). Furthermore, molecular analysis showed that 69% of the sampled trees were infected and apple was the most infected species (80%), followed by pear (75%) and quince (10%). ASPV was the most prevalent virus (46%), followed by ACLSV (39%) and ApMV (10). Mixed infections occurred in several trees, and the most common combination was ASPV+ACLSV (23%). This is the first report on the presence of viruses infecting pome fruits in Tunisia.

Key words: apple, pear, quince, ELISA, RT-PCR.

Introduction

Pome fruit trees are traditionally grown in Tunisia, with a total cultivated area of 28,000 ha. Apple is the predominant crop (19,000 ha), followed by pear (8,500 ha) and quince (600 ha), and the pome fruit production areas are mostly concentrated in the northern part of the country (Anonymous, 2010).

Many viruses and virus-like diseases known under different names affecting pome fruit trees, including *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*), *Apple mosaic virus* (ApMV, genus *Ilarvirus*), *Apple stem pitting virus* (ASPV, genus *Foveavirus*) and *Apple stem grooving virus* (ASGV, genus *Capillovirus*) have been described. Mixed infection by these viruses can induce significant yield reductions (Schmidt, 1972; Zahn, 1996).

The use of healthy propagation plant material is an effective way of controlling the viral diseases. To this aim a voluntary certification programme for pome fruit propagation material has been initiated in Tunisia, and distributed material belonging to the category *virus tested* needs to be free from the main viruses, such as ACLSV, ApMV, ASPV and ASGV. Preliminary investigations, reported in thius paper, have been undertaken to assess plant health status of pome fruit in this country.

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Materials and methods

Field surveys and sample collection

Field surveys were carried out in the spring 2009 and 2010. Samples were collected from the two main Tunisian mother blocks, located at Kairouan (centre of Tunisia) and at Jendouba (northern Tunisia). A total of 248 samples were randomly collected from trees of apple (111 samples), pear (106 samples) and quince (31 samples).

Twelve apple varieties were assessed, including four local varieties (Zina, Aziza, Chahla and Boutabgaya) and eight introduced varieties (Anna, Llor-

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ka, Richared, Red spur, Golden spur, Starkrimson, Golden Delicious and Starkiliest). For pear, samples were collected from four local varieties (Meski ahrech, Meski arteb, Meski bouguedma and Anbri) and nine introduced varieties (Williams, Wilder, General Lechlerc, Parkham triumph, Dr. Guyot, Marguerite, Alexandrine, Passe crassane and Claps). For quince, all the samples were collected from the variety Geant de Vranja.

Leaf samples were collected from around the canopy of each tree and stored at 4°C until assayed for viruses.

Serological analysis

Leaf extracts were obtained by macerating leaf tissues in the PBS-buffer (V:V). All the collected samples were tested by DAS-ELISA (Clark and Adams, 1977) for the presence of ApMV, and DAS-Simultaneous ELISA (Flegg and Clark, 1979) for the presence of ACLSV, using specific polyclonal antibodies from Loewe-Germany. The samples were considered to be positive for the viruses when the absorbance values at 405 nm were greater than three times the average obtained for healthy samples.

Molecular analysis

RT-PCR was used to assay all the collected samples for the presence of ACLSV, ApMV and ASPV, using the specific sets of primers outlined in Table 1.

Total nucleic acids (TNAs) were extracted from 0.5 g leaf veins from each tree sample, macerated in grinding buffer (4 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5.0 and 2.5% w/v PVP-40), containing 0.2% sodium metabisulfite and purified according to Foissac *et al*. (2001). Reverse transcription was performed using *Moloney Murine Leukaemia Virus* (MMLV) reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). The synthesized cDNA was submitted to PCR amplification using 0.25 μL (5 unit μL^{-1}) Taq polymerase (Promega Corporation, , Madison, WI, USA), 0.5 μL of 10 μM primer (sense), 0.5 μL of 10 μM primer (anti-sense) in a final volume of 25 μL. Amplification was carried out in a thermocycler (Applied Biosystems, USA), through a preliminary denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, annealing at 55°C for 45 sec, and 72°C for 1 min, and a final extension step at 72°C for 7 min.

Amplified products were electrophoresed in 1.2% agarose gel in TBE buffer and stained with ethidium bromide. The gels were visualized by ultraviolet light illumination and the sizes of the expected bands were determined by comparison with DNA molecular marker 100bp (Promega Corporation).

Sequencing and sequence analysis

To confirm the identity of ASPV in apple, pear and quince, five isolates were chosen for sequencing. The RT-PCR products were purified with the ExoASP-IT purification kit. Direct sequencing was performed with the same primers used for RT-PCR on 3730×L DNA analyzer (Applied Biosystems, USA) automated sequencer.

The sequences obtained were compared with those of the published homologous gene of ASPV isolates using the BLAST program, while nucleotide and amino acid alignments were performed using the CLUSTAl-X program.

Results

Field surveys

All of the 248 tested trees of apple, pear and quince accessions were individually inspected. Some trees showed symptoms which indicated virus infection. These included leaf yellow mosaic on some apple varieties and leaf vein yellowing on some pear varieties. However, most of the surveyed pome fruit trees did not show any disease symptoms.

Serological assays

The ELISA tests indicated that 92 of the 248 samples were infected by at least one virus. The total infection rate was 51% for pear, 34% for apple, and no virus was detected in quince trees (Table 2). The ELISA results indicated a large number of trees (34%) were infected with ACLSV (Table 2).

For pear, ACLSV was the most widespread virus with a mean infection rate of 45%. The virus was detected in all the sampled trees of the variety Williams, whereas it was absent in the varieties Claps, Alexandrine and Passe crassane.

ApMV was detected in 8% of the samples. The greatest level of ApMV occurred in cv. General Lechlerc (40%).

Table 1. Sets of DNA primers used for RT-PCR detection of pome fruit-infecting viruses.

Virus	Genus	Amplicon length (Pb)	Primer sequence (from 5' to 3')	Reference
ACLSV	Trichovirus	677	Sense: TTCATGGAAAGACAGGGGCAA Antisense: AAGTCTACAGGCTATTTATTATAAGTCTAA	Menzel *et al*., 2002
ASPV	Foveavirus	370	Sense: ATGTCTGGAACCTCATGCTGCAA Antisense: TTGGGATCAACTTTACTAAAAAGCATAA	Menzel *et al*., 2002
ApMV	Ilarvirus	450	Sense: CGTAGAGGAGGACAGCTTGG Antisense: CCGGTGGTAACTCACTCGTT	Hassan *et al*., 2006

Table 2. Numbers and proportions of apple, pear or quince samples tested and shown to contain ACLSV and ApMV, as indicated by DAS-ELISA.

Host	Samples tested	ACLSV		ApMV		Total	
		Infected	%	Infected	%	Infected	%
Apple	111	37	33	2	2	38	34
Pear	106	48	45	9	8	54	51
Quince	31	0	0	0	0	0	0
Total	248	85	34	11	4	92	37

Regarding pear varieties, only three (Claps, Alexandrine and Passe crassane) were free from the tested viruses, whereas two varieties (Williams and Meski Arteb) were totally infected. In the other tested varieties the infection rate ranged from 17% in Marguerite to 86% in Parkham triumph.

For apple, the infection rate of ACLSV was 33%, and was particularly significant in the varieties Boutabgaya (100%), Starkiliest (80%) and Golden spur (66.6%). ApMV was present in 2% of the sampled trees and occurred in the two native varieties Zina and Chahla, with an infection rate of 9% for each variety. No virus was detected in the samples of varieties Anna, Richared or Starkrimsen, and all the samples of the native variety Boutabgaya were infected. For the other varieties, the infection level ranged from 7% in the native variety Aziza to 67% in the varieties Llorka and Golden spur.

Molecular assays

All the samples were tested using RT-PCR, and 31% of were shown to be free of the studied viruses, while 26% had mixed infections. Apple trees were the most infected, with 80% of samples with virus infections, followed by pear (75% infected) and quince (10% infected) (Table 3). ASPV was to be the most widespread virus with 46% infection rate (Table 3).

Apple was the species most infected with ASPV (61%). This virus was identified in 100% of the samples from the varieties Chahla, Boutabgaya and Golden spur. However, ASPV was absent from the varieties Zina, Starkiliest and Golden Delicious.

The local apple varieties showed high ASPV infection rates (72%) compared to imported varieties (53% infection).

For pear, ASPV was detected in 41% of the tested samples; it was present in all the sampled trees of varieties Meski bouguedma and Wilder. In contrast the varieties Alexandrine and Marguerite were negative for ASPV. For quince, ASPV was detected in 10% of the sampled trees of the variety-Geant de Vranja.

The second most prevalent virus was ACLSV (39%), occurring in pear with an infection rate of 48% (Table 3). The greates incidence of this virus was

Table 3. Numbers and proportions of apple, pear or quince samples tested and shown to contain ACLSV, ApMV and ASPV, as indicated by RT-PCR.

Host	Samples tested	ACLSV		ApMV		ASPV		Total	
		Infected	%	Infected	%	Infected	%	Infected	%
Apple	111	46	41	15	14	68	61	89	80
Pear	106	51	48	11	10	43	41	80	75
Quince	31	0	0	0	0	3	10	3	10
Total	248	97	39	26	10	114	46	172	69

in the varieties Williams (100%), General Lechlerc (80%) and Parkham triumph (86%). The infection rates in the local varieties ranged from 18% in Meski arteb to 62% in Meski bouguedma.

ACLSV was also present in 41% of the tested apple trees. Greatest infection rates of this virus were in the local variety Boutabgaya (100%) as well as in the imported varieties Starkiliest (100%) and Llorka (73.3%). Four apple varieties (Anna, Richared, Starkrimsen and Zina) were free of ACLSV.

The ApMV prevalence (10%) was less than the other viruses. This virus was detected both in local and imported varieties of pear, while only the local varieties of apple were infected.

Mixed virus infections were common, with 26% of the tested samples having mixed infections. The mixed infections included ASPV + ACLSV (23% of samples), ACLSV + ApMV (2%) and ASPV + ApMV (1%).

The identity of ASPV in apple, pear and quince was confirmed by sequencing. The sequence identities of the five Tunisian isolates with ASPV reference sequences in GenBank ranged from 80 to 93% at nucleotide level.

The two ASPV isolates from pear (MTN-116 and CTN-118) showed the greatest identities of 89 and 91% with the isolates from pear ARG-2 (GQ356782) and GNKVII/34 (AF345893) respectively. Isolates from apples (ATN-159 and ChTN-162) showed the greatest similarities 93 and 92% with the isolates from apple YL07 (EU665492) and E13ASP (FJ970958) respectively.

The presence of ASPV in quince was confirmed, and the isolate GVTN-144 showed 89% identity with the isolates 405ASP (FJ970955) and E13ASP (FJ970958).

Discussion

In this study, three of the most important pome fruit viruses were detected for the first time in Tunisia using laboratory assays. These results indicate a high prevalence of pome fruit viruses in the main Tunisian mother plots, which were used as sources of budwood propagation material for the commercial orchards. Similar results of a high virus infection rates on apple and pear have been previuosly recorded from other Mediterranean countries, including Bosnia and Herzegovina (Lolić *et al*., 2010), Morocco (Afechtal *et al*., 2010) and Turkey (Çağlayan *et al*., 2006).

The high incidence of ASPV is in line with previous studies on pome fruit trees, reported from different Mediterranean countries (Myrta *et al*., 2004; Lolić *et al*., 2010).

The low ApMV distribution in comparison with ASPV and ACLSV, has been reported in other Mediterranean countries, Europe and United State of America (Cameron and Thompson, 1985; Desvignes *et al*., 1992; Myrta *et al*., 2004; Çağlayan *et al*., 2006; Ismaeil *et al*., 2006; Lolić *et al*., 2010).

The absence of an efficient certification programme of plant propagating material and the selection of clones based on visual observation is likely to have favoured the occurrence and spread of these viruses.

ELISA was less reliable than RT-PCR for virus detections, which could be due to the host species and low virus titre, as previously reported (Desvignes *et al*., 1992; Boscia *et al*., 1999).

Among the tested trees, 22 apple, 26 pear and 28 quince trees were negative for the three viruses when tested by ELISA and RT-PCR, and later also to

Apple scar skin viroid (ASSVd), Apple dimple fruit viroid (ADFVd) and Pear blister canker viroid (PBCVd) (Unpublished data). The apple accessions of the varieties Zina, Richared, Red spur and Golden Delicious; the pear accessions of the varieties Meski ahrech, Anbri, Alexandrine, Dr. Guyot, Marguerite, General Lechlerc and Claps, and the quince variety Geant de Vrenja, will be tested for ASGV and the negative lines will be used as primary sources for the ongoing pome fruit virus certification programme in Tunisia.

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