The first survey of pome fruit viruses in Morocco

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Abstract

Considering the limited information on the presence and incidence of pome fruit virus and viroid diseases in Morocco, a preliminary assessment of the presence of pome fruit viruses in Morocco was carried out. Twenty orchards and nurseries were surveyed in the regions of Midelt, Meknès and Azilal. A total of 100 samples (apples and pears) were collected and tested. Biological indexing was made in a acclimatised greenhouse using the following indicators: *Malus pumila* cvs. ʻSpy 227', ʻRadiant' and ʻR 12740 7A', and *Pyrus communis* cv. ʻLA/62'. All samples were also tested by ELISA for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV). The prevailing viruses infecting apple were ACLSV (71%) and ASPV (58%), whereas ASGV was found in 12 tested trees. The same viruses were present, but less frequently, in pear: ACLSV (61%), *Pear* Vein Yellows Virus (PVYV) (25%) and ASGV (18%). Only four apple trees were found to be infected by ApMV. Additional RT-PCR testing confirmed the high incidence of ACLSV and ASPV.

This was the first report of the presence of pome fruit viruses in Morocco, indicating the high infection rate worsened by the recent report of the presence of fire blight (*Erwinia amylovora*) in the country. Moreover, a total of 168 apples and 81 pears were sampled and tested for pome fruit viroids *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) by tissue printing hybridization. No viroids were detected.

Keywords: pome fruit, viruses, viroids, Morocco, ELISA, Tissue printing hybridization, PCR.

Introduction

Pome fruit crops are mainly grown in the regions; Khénifra-Midelt, the Middle and High Atlas (Azrou-Ifrane), the Saïs plain (Meknès-Fès), Haouz Marrakech and Gharb. Among all rosaceous fruit trees, apple ranks first in terms of production (372,400 t) and second in terms of surface (25,700 ha) after almond (MADRPM, 2002). The area occupied by fruit tree nurseries covers 120 ha, which allows an annual production from 600,000 to 700,000 plants (all fruit species included); however most of the nurseries do not have an appropriate infrastructure for producing certified trees. No sanitary selection has ever been done in the country for pome fruit crops, whereas the sanitary status of the stone fruit industry was recently assessed by Bouani et al. (2004).

Materials and methods

Field Survey: Field inspections and sample collection were carried out in November-December 2006 from a total of 277 trees grown in commercial orchards and nurseries located in Meknès, Midelt, and Azilal regions. Inspected trees varied in age and were almost all imported cultivars in addition to two local cultivars, one of apple and one of pear. Leaf samples collected from different parts of the canopy of the trees were used for printing leaf petioles onto nylon membranes to perform viroid detection. One-year-old bud sticks, 20-30 cm in length, from 70 apple and 30 pear trees were collected (Table 1), labelled and stored at 4°C for about two months and then used for woody indexing. Visual inspection of specific symptoms of virus and viroid infections was also carried out during field surveys.

Tab. 1 Crops, locations, cultivars and number of trees sampled during the survey.

Crop	Location	Cultivar	Sample	
			Budsticks	Leaves
Apple	Meknès	Galaxy	4	29
		Golden Smoothee	9	39
		Crips Pink	4	30
		Mondial Gala	5	10
		Fuji	5	10
		Red Chief	5	10
		Golden Delicious	3	10
	Midelt	Starking Delicious	5	10
		Golden Delicious	3	10
		Royal Gala	5	10
		Delicious	3	-
		Golden	3	-
		Stark	3	-
		Ramboothee	5	-
	Azilal	Stark	1	-
		Golden Delicious	1	-
		Local variety (Lhlou)	6	-
Pear	Meknès	William Rouge	5	25
		William Blanc	6	10
		Cascade	6	15
		Dr Jules Guyot	7	31
	Azilal	Local variety	6	-
Total	-	-	100	249

Biological indexing assay: The collected dormant cuttings were individually tested on woody indicators in three replicates and kept under a screenhouse for nine months of observations. Apple samples were tested on Malus pumila cvs. 'R 12740 7A', 'Spy 227' and 'Radiant', and pear samples on Pyrus communis cv. 'LA/62'. The indicator plants were double chip-budded, positive and negative controls were included in the testing of each pathogen. Visual observations were made weekly from April to June, after the appearance of the first symptoms

ELISA: All collected samples were tested by DAS-ELISA (Clark and Adams, 1977) for the detection of the Apple stem pitting virus (ASPV) and the Apple mosaic virus (ApMV), and by simultaneous (cocktail) DAS-ELISA (Flegg and Clark, 1979) for the detection of the Apple chlorotic leaf spot virus (ACLSV) and the Apple stem grooving virus (ASGV). ELISA was done using extracts from young leaves of the indicator 'R 12740 7A' for apple and of 'LA/62' for pear. Serological reagents were commercial kits produced by Loewe (Germany) and Bioreba (Switzerland).

RT-PCR: A limited number of representative samples (12 apples and 3 pears) were also tested by RT-PCR for the presence of ACLSV and ASPV. Total RNA purification from leaves was carried out using RNeasy Plant Mini Kit, according to the manufacturer's instructions (Qiagen S.p.A, Italy). Complementary DNA was synthesized using total RNA as a template. 6 µl of total RNA were mixed with 1 µl/µg of antisense primer (Table 2) in a final volume of 8.6 µl, denatured for 5 min at 95°C, and then put for 5 min on ice. Reverse transcription was done using 4 µl 5X of AMV buffer (Promega), 1 µl of 10 mM dNTPs (each) (Roche), 0.4 µl 100 U/µl AMV (*Avian myeloblastosis virus*) reverse transcriptase in a final volume of 20 µl. The synthesis was carried out at 39°C for 1h.

Tab. 2 Sequences, positions and expected size of RT-PCR product for each primer as reported by Menzel *et al.*, (2002).

Virus	Primer sequence in 5'-3' orientation	Primer position	Product size
ACLSV	TTCATGGAAAGACAGGGGCAA	6860-6880	677 bp
	AAGTCTACAGGCTATTTATTATAAGTCTAA	7507-7536	
ASPV	ATGTCTGGAACCTCATGCTGCAA	8869-8895	370 bp
	TTGGGATCAACTTTACTAAAAAGCATAA	9211-9238	

The PCR reaction mixture contained 5 µl of cDNA, 5 µl 10X PCR buffer (provided with the hotStart *Taq* polymerase, QIAGEN), 0.5 µl of $MgCl_2$ (25 mmol/L), 1 µl dNTP mixture (each dNTP 10 mmol/l), 1 µl of 10 µM each complementary and homologous DNA primers (Table 2), and 0.4 µl HotStart *Taq* polymerase (5U/ µl, QIAGEN). The cycling parameters were: activation of the HotStart *Taq* polymerase at 95°C for 15 min followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. The final extension step was

at 72°C for 7 min. PCR products were separated by electrophoresis in 2% agarose gels in TAE buffer, stained with ethidium bromide, and visualized under UV light.

Tissue printing hybridization: In total, 249 leaf samples (168 apples and 81 pears) were tested by tissue printing hybridization (TPH) for the presence of *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) as recently reported by Lolic et al., (2007). Imprints of a fresh cut end of the leaf petiole were carried out onto a nylon membrane; the imprinted membranes were covered with a plastic envelope and exposed to ultraviolet light for 2-3 min to fix the nucleic acid. The DIG-labelled riboprobes were synthesized as suggested by the manufacturer's instructions (Roche Diagnostics) using T3 or T7 RNA polymerase and recombinant plasmids containing monomeric ASSVd, ADFVd and PBCVd cDNA insert that were linearized with appropriate restriction enzymes. The pre-hybridization step was done at 68°C by using the pre-hybridisation solution (DIG Easy Hyb granules substrate, Roche), then the specific synthesized cRNA probe was added and the hybridization step was performed overnight. After washing with SSC solution containing SDS, the membranes were treated with RNase A (1 µg/ml), then incubated with antidigoxigenin antibody (Roche Diagnostics) and the chemiluminescent substrate CDP-Star.

Results

Field surveys: During the field surveys, almost 300 apple and pear trees were individually inspected in commercial orchards and nurseries located in the regions of Mèknes, Midelet and Azilal. No symptoms were observed in the surveyed orchards and nurseries, which is in line with previous reports on pome fruit viruses that are normally latent (Desvignes, 1999).

Biological indexing: The 'SPY 227' indicator within 2-3 months from graft-inoculation showed symptoms associated with ASPV infections consisting of epinasty of leaves, with downward curled blades; chlorotic spots were also observed in this indicator and were associated with ACLSV, while characteristic symptoms associated with ASPV were also observed in 'Radiant' indicator that showed epinasty of leaves. The chlorotic spots and leaf deformation (sickle-shaped leaf) that appeared on the 'R 12740 7A' apple indicator were associated with the presence of ACLSV, whereas some inoculated apple indicator plants showed a sudden decline and died. The pear indicator 'LA/62' developed rings and mottling on the leaves, which were associated with the presence of ACLSV.

The biological indexing evidenced a first attempt on the sanitary status of the tested trees; in fact, a total of 51 out of 70 tested apple plants proved to be infected by ACLSV (infection rate of 72.8%). 42 out of 70 apple samples were proved to be infected with ASPV. For pear, 50% of the tested plants were proved to be infected by ACLSV (Table 3).

Tab. 3 Viruses of pome fruits detected by biological indexing on woody indicators in Morocco.

	ACLSV R12 740 7A / LA 62		ASPV SPY 227 / Radiant	
Species	**Infected/Tested**	**Infection rate (%)**	**Infected/Tested**	**Infection rate (%)**
Apple	51/70	72.8	42	60
Pear	15/30	50	nt	-

nt: not tested

ELISA tests: The ELISA tests showed for ACLSV a 37.1% infection rate in apple, whereas the infection by this virus was higher in pear (63.3%). ASPV was detected in 25.7% of the tested apple samples and Pear vein yellows virus (PVYV) was detected in 26.6% of tested pear samples. Regarding ASGV, it was found in 11.4% of the apple and in 20% of pear samples. As for ApMV, 4 apples were positive (5.7% incidence) but no pear was infected (Table 4). ELISA kits failed to detect some viruses which were already detected by previous biological indexing, probably, due to the low virus titer or to the presence of inhibitors such as polysaccharides, or phenolic compounds in tissue extracts from woody plants as reported by Kinard et al. (1999).

Tab. 4 Viruses of pome fruits detected by ELISA.

	Samples			Viruses detected			
Species	**Tested**	**Infected**	**Infection rate (%)**	**ACLSV**	**ASPV/PVYV**	**ASGV**	**ApMV**
Apple	70	42	60	26	18	8	4
Pear	30	21	70	19	8	6	0
Total	100	63		45	26	14	4

RT-PCR: A limited number of representative samples (12 apples and 3 pears) were also tested by RT-PCR for the presence of ACLSV and ASPV. Out of the twelve tested apple cultivars, eight were infected by ACLSV and six were infected by ASPV. As for pear, two out of the three tested cultivars were infected by ACLSV and ASPV. RT-PCR assays were in line with those of biological indexing and both techniques were more sensitive than ELISA. Therefore,

for large scale virus surveys of pome fruits, it is necessary to integrate different diagnostic techniques in order to obtain more accurate results.

Tissue printing hybridization: A total of 168 apple trees and 81 pear trees were sampled and their leaves were collected from different parts of the canopy and used for imprinting petioles onto nylon membranes. All the trees were assayed by TPH for ASSVd, ADFVd and PBCVd infections. No hybridization signals were detected in the membranes for any of the three viroids on the tested trees (data not shown).

Discussion

The sanitary status of pome fruit crops in Morocco was preliminarily evaluated, showing a relatively high rate of virus infection. The presence of four viruses (ACLSV, ASPV, ASGV and ApMV) was detected and the most frequent virus was ACLSV (66%).The infection rate for ACLSV detected by woody indicators on 'R 12740 7A' was 72.8%, much higher than that for ELISA (37.1%), but the woody indicators were less sensitive than RT-PCR (91.66%). In general, ELISA was less reliable than biological indexing in the greenhouse. This low reliability of ELISA for pome fruits has already been reported (Desviges et al., 1992; Nemichinov et al., 1995; Boscia et al., 1999). The large scale survey on pome fruit viroids (ASSVd, ADFVd and PBCVd) by tissue printing hybridization (TPH) did not reveal their presence in the tested samples from Morocco. It was concluded that the three viroids do not occur in Morocco, or at least not in the surveyed areas and orchards.

Recent data obtained in other countries where no sanitary selection has ever been carried out in pome fruits showed a high virus infection level (Myrta et al., 2004, Ismaeil et al., 2006, Lolic., 2006). This study showed that virus infection is widespread in the Moroccan pome fruit industry. The degraded sanitary status of pome fruit trees calls for the enforcement of a national certification program for the propagating material of pome fruit and for measures to prevent the importation of virus-infected material into Morocco.

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