

The phytosanitary status of the National Collection of fruits and nuts of Afghanistan and the private Mother Stock Nurseries: a virus survey

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Abstract: The horticultural industry is a vital component of the agriculture sector of Afghanistan, the primary engine of the country's recovering economy which engages approximately 80% of the working population. This sector was thriving in the 1970s, but is today incapable of competing in the international market. To recover and develop the horticulture of the country, the European Community (EC) supports the PHDP (Perennial Horticulture Development Project), to provide true to type/ecotype and healthy planting materials, and the Plant Biotechnology Laboratory, to ensure the health status of local germplasm. This laboratory started screening the health status of the Afghan Germplasm National Collections in order to ensure the multiplication of not only the best-selected varieties or ecotype, but also to avoid production and distribution of virus-infected trees. Inspection for symptoms and sample collection for viral diseases was carried out in all the National Collection fields, including cherry, pear, peach, plum, apricot, almond, apple, grape and citrus plants, located in different areas of the country. Stone fruit plants infected by *Apple chlorotic leaf spot virus* or *Prunus necrotic ringspot virus* have been identified in the National Collection experimental farms located in different provinces of Afghanistan. Moreover, many grape plants included in the National Collection located in Herat and Kandahar resulted infected by *Grapevine fanleaf virus*, but only few imported plants by *Grapevine leafroll associated virus 1*, *Grapevine leafroll associated virus 3* or *Grapevine virus A*. Finally, in Jalalabad (Nangarhar province) citrus plants showing vein flecking, yellowing and plant decline symptoms were found to be infected by *Citrus tristeza virus*. Some of the identified viral isolates have been characterized molecularly, amplifying a fragment corresponding to the coat protein gene from a selection of positive samples. The presence of those viruses in different accessions of the national collection is of concern for Afghan horticulture. Implementation of the certification schemes is therefore necessary to quarantine the production and for the employment of virus-free propagating material.

1. Introduction

A variety of horticultural products is grown in Afghanistan with the potential to be (re)developed into valuable export commodities and brands. The primary engine of the Afghan economy is its agriculture industry, which engages approximately 80% of the working population. Fruit trees, such as peach, plum, apricot or almond and grape are therefore of significant economic importance to the country, with importing or local exchange of plant material for propagation or commercial growing of these fruits

likely. The absence of certain viruses is an essential pre-requisite for virus-free certification of plant material in general in many parts of the world (Golino and Savino, 2005; Barba *et al.*, 2015). In Afghanistan, there is a tremendous increase in fruit tree nursery production and regulation of phytosanitary certification of plant propagation material is therefore of major importance.

In 2005 the Ministry of Agriculture, Irrigation and Livestock (MAIL) stated in the agriculture master plan that the rejuvenation of the horticulture sector would be a government priority. Thriving in the 1970s, this sector has become incapable of competing in the international market. Three interconnected problems have been identified for the industry: 1) the existing orchards are run-down and production is

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dwindling in quantity and quality; 2) nursery owners lack access to quality inputs and services; and 3) the regulatory framework to develop and support a competitive horticulture industry is minimal.

To support and develop the horticultural industry of the country, a nursery certification system was started in 2006 from the complete collection Afghanistan's fruit and nut trees crop varieties by the Perennial Horticulture Development Project (PHDP) funded by European Union (EU). One of the main causes for the decrease in production is deleterious pests and diseases, especially plant viruses and virus-like diseases present in Afghanistan.

Virus infection in plants is particularly difficult to detect as viral infection may be sectorial or latent, with symptoms masked or even absent under certain conditions. Virus-free certification is currently implemented using conventional techniques for virus detection in vegetal material such as biological indexing, serological or molecular assays. Biological indexing requires, in many instances, grafting in greenhouse facilities which is expensive and time consuming. Serological assays and molecular techniques, particularly ELISA and RT-PCR, have been tested in several laboratories and shown to be efficient for detection and quantification of virus infection in pome and stone fruits (Gambino and Gribaudo, 2006; Faggioli *et al.*, 2013). In a country such as Afghanistan, the establishment of a laboratory where sensitive, reliable and specific techniques for routine detection of selected viruses of fruit trees can be performed is therefore essential for virus-free certification.

Many viral diseases can affect fruit trees, resulting in latent or symptomatic infection. According to their effect on the cultivated host, presence of the most important viruses in propagation plant material is regulated at international level. In particular, *Plum pox virus* (PPV) is the causal agent of Sharka, the most damaging virus disease of stonefruits. Some strains of *Apple chlorotic leafspot virus* (ACLSV) can also induce serious diseases in stone fruits. *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) are of considerable economic significance in stonefruits as well, while *Citrus tristeza virus* (CTV) is considered to be the most destructive virus of citrus crops. *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) are spread worldwide and able to cause significant losses in mixed infections (Hadidi *et al.*, 2011; Barba *et al.*, 2015).

Arabis mosaic virus (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine fanleaf virus* (GFLV), *Grapevine*

virus A (GVA) and "grapevine leafroll-associated viruses" (GLRaVs) such as *Grapevine leafroll associated virus 1, 2 and 3* (GLRaV-1, GLRaV-2 and GLRaV-3) are of great economic impact in grape and their absence in propagation material is an essential requirement in the certification schemes of most countries (Martelli, 2014).

National collection centres were established in six agro-ecological zones in Afghanistan to collect germplasm. After registration and cataloguing, the mother stock nurseries were established from the same planting material to ensure the availability of high quality and healthy planting material to nursery growers and farmers.

Theoretical and practical knowledge are essential to develop a complete profile for viral diseases in order to improve the quality of planting material within the national fruit tree germplasm (Rizzo *et al.*, 2012, 2015). We therefore developed an in-depth study regarding the detection and identification of viral pathogens through the use of advanced and sensitive methodologies and techniques to characterize identified plant viral pathogens and to develop control strategies for the management of diseases.

These efforts are aimed at improving the quality of planting material and will ensure access to quality and certified planting material for nursery growers and orchard owners for the establishment of new orchard/motherstock nurseries, as well as for the rehabilitation of existing orchards. Screening agricultural products according to the protocols and regulations of global plant quarantine will also positively affect the import and export of Afghan agricultural entities. Following the standards of international sanitary and phytosanitary rules and regulations is the only way to compete in the international market.

2. Materials and Methods

Plant material

Plant material was collected from the National Collection Centres (NCCs) and Mother Stock Nurseries (MSNs) from 2009 to 2015. In total, more than 12,000 samples were collected from different species and tested for different viruses. In particular, almond, apricot, cherry, peach and plum samples were tested for the presence of ACLSV, ApMV, PDV, PNRSV and PPV; apple and pear samples for ACLSV, ApMV, ASGV and ASPV; citrus samples for CTV; and grape samples for ArMV, GVA, GFkV, GFLV, GLRaV-1, GLRaV-2 and GLRaV-3.

About 32% of the fruit tree samples were collected from the six NCCs located in Kabul (apricot and plum), Balkh (apricot and almond), Kunduz (almond), Herat (grape and plum), Kandahar (grape and pomegranate) and Nangarhar (citrus and pomegranate). Moreover, samples were also collected from MSNs (26%) and from private orchards (nearly 40%) (Fig. 1).

Each year stone fruit samples were collected during the vegetative stage in spring or summer, between March and September, according to the climatic conditions of each location. Similarly, apple and pear samples were collected in May, June, July or September; citrus in March, May or November; and grape in dormant stage in January, October or December.

The collecting method consisted of sampling leaves (in the case of stone and pome fruits), shoots

or canes (grape) or leaves and flowers (citrus) homogeneously distributed around the canopy of the plant. All collected material was maintained under refrigeration (below 10°C) during transport from the field to the laboratory, then kept at -20°C until analyses.

Serological and molecular analyses

All samples were ground in "Universal" extraction bags (12x15 cm) using the HOMEX 6 homogenizer (Bioreba) then analysed by DAS-ELISA using reagent kits from Bioreba (Reinach, Switzerland) specific for each virus assayed and following the manufacturer's instructions.

Samples that resulted positive by DAS-ELISA test were subsequently analysed by RT-PCR reaction using specific primer pairs (Table 1).

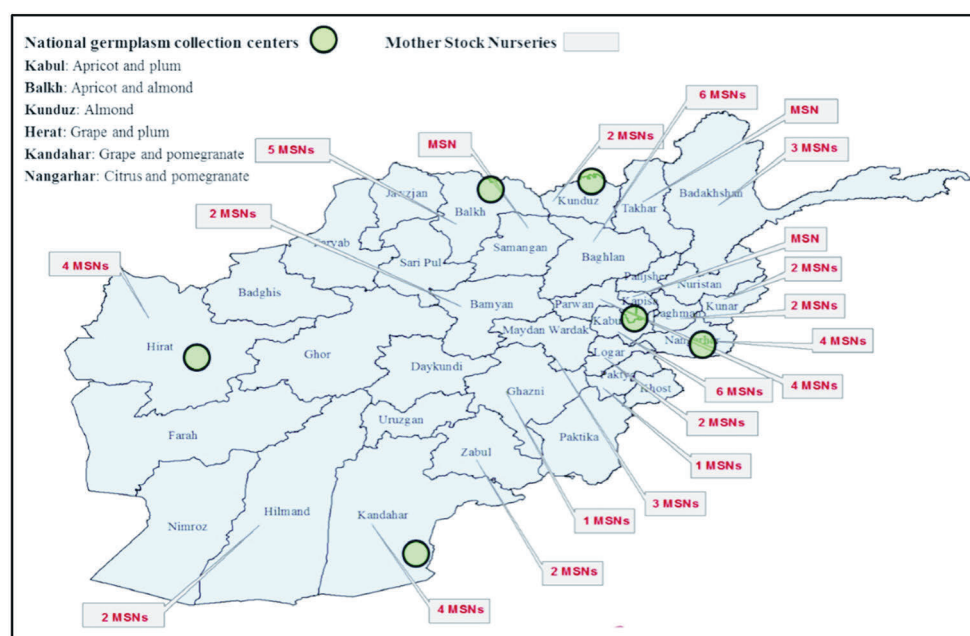


Fig. 1 - Location of the six national collection centres and of the 57 mother stock nurseries in Afghanistan.

Table 1 - List of primer pairs, size of amplicons, and RT-PCR conditions used to confirm viral infection in all samples resulting positive to the DAS-ELISA test

Virus	Primers	Primers sequences (5' - 3')	Size of amplif. (bp)	Cycling (temp./time)*			N° of cycles	Reference
				Denat.	Anneal.	Exten.		
ACLSV	Sense	TTCATGGAAGACAGGGGCAA	677	94°C/20 s	58°C/20 s	72°C/20 s	35	Menzel <i>et al.</i> , 2002
	Antisense	AAGTCTACAGGCTATTTATTATAAGTCTAA						
PNRSV	MG1	ATGGTTTGCCGAATTTGC	675	94°C/60 s	55°C/45 s	72°C/60 s	35	Glasa <i>et al.</i> , 2002
	MG2	ACTCTAGATCTCAAGCAGGTC						
GFLV	M3	ATGCTGGATATCGTGACCCTGT	118	94°C/10 s	54°C/10 s	72°C/45 s	35	Gambino and Gribaudo, 2006
	M4	GAAGGTATGCCTGCTTCAGTGG						
CTV	CTVF	TAATGGACGACGAACAAAGA	655	94°C/40 s	56°C/40 s	72°C/60 s	30	Rehman <i>et al.</i> , 2012
	CTVR	CCAAGTGCCTGACATTAGT						

* PCR amplifications were performed at 94°C for 5 min for initial denaturation and 72°C for 5 min for final extension.

Total RNAs were extracted from each sample using CTAB RNA extraction method, as previous reported (Ratti *et al.*, 2004). Complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, USA) in a final volume of 5.0 μ l. RNA (1.0 μ l), mixed with M-MLV 5x buffer, 0.5 μ l 10 mM dNTPs, 1.0 μ l 10nmol/ml specific reverse primer, 0.25 μ l of 200 U/ μ l M-MLV and 2.25 μ l of nuclease-free water, then incubated at 4 2°C (or 37°C for ACLSV and PNRSV) for 1 h. For PCR amplification, 5.0 μ l of cDNA template, 5.0 μ l of 5x Green GoTaq Buffer, 1.5 μ l of 25 mM MgCl₂, 1.0 μ l 10 nmol/ml of specific forward primer, 0.5 μ l of 10 mM dNTPs and 0.2 μ l of 5 U/ μ l GoTaq Polymerase (Promega, USA) were mixed in a total volume of 25 μ l. The number of cycles and cycling conditions for each primer set are given in Table 1. The amplified PCR products were analysed in 1% agarose gel stained by ethidium-bromide and visualized under UV light after electrophoresis.

3. Results

DAS-ELISA analyses

All apple and pear plants analysed (180 samples) resulted free from ACLSV, ApMV, ASGV and ASPV. Symptoms such as yellowing, leaf distortion and puckering were observed in some stone fruit plants during collection of the 5521 samples that were tested by DAS-ELISA (Fig. 2). Among them, 23 (0.4%) samples collected from several MSNs in Kabul, Khandahar, Parwan, Herat and Baghlan provinces resulted positive to ACLSV. In particular, there were two plants of European plum (Clone 364/local name Alu BokharaShalili), one cross of Myrobalan and Japanese plum (4031/Grangej Zard), 17 plants of peach (five from 804/Turki Sorkh, five from 812/Dir Ras and seven from 811/TurkiZard), one plant of almond (159/Sattarbai Bakhmali) and two plants of apricot (373/Shakarpara and 4037/Aqa Banu). Two (0.03%) almond samples, both collected from the NCC in Balkh province, resulted infected by ApMV; in was interesting that one of them also tested positive to PNRSV. Analyses against PNRSV evidenced 38 samples (0.6%), collected from NCCs or MSNs located in Balkh, Herat, Kabul, Khandaharand Paktya as infected by the virus (13 almond, five plum, nine peach, and 11 cherry plants). Finally, one plum sample (cv. Alu Bokhara Shalili), from a MSN in Kabul, resulted infected by PDV while none of the stone fruit samples tested resulted positive to PPV (Tables 2-6 and Fig. 3).



Fig. 2 - Yellowing observed in an apricot accession during sample collection in a National Collection Centre.

Among the 895 grape samples analysed, one plant from the NCC in Kandahar (cv. Shir Ahmadi Herat) resulted infected by GVA and only one sample of the cv. Pizzutello bianco, imported from Italy and collected in the NCC in Herat, tested positive to GLRaV-1 (it also resulted as infected by GLRaV-3). Moreover, GLRaV-3 was detected in two samples (cv. Pizzutello bianco and Uva Palieri both imported from Italy), collected from the NNC in Herat. Regarding GFLV, all the 62 samples which resulted infected by the virus (6.9%) belong to Afghan cultivars, with the exception of the cv. Regina dei vigneti imported from Italy. All grape samples tested resulted negative to ArMV, GFkV and GLRaV-2 (Table 7) (Fig. 3).

In addition, the Plant Biotechnology Laboratory analyzed 5400 citrus plants and 318 of them (5.9%) resulted infected by CTV. In particular, among the positive samples, eight plants, showing vein clearing and flecking, yellowing and plant decline symptoms (Fig. 4), were collected from the NCC in Jalalabad and belong to six different accessions: Kumquat cv.

Table 2 - Almond samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
PPV	North	174	0	0	0	205	0	174	0	53	0	82	0	78	0	766	0
	Central	2	0	0	0	24	0	136	0	103	0	69	0	42	0	376	0
	South	0	0	0	0	0	0	0	0	47	0	0	0	0	0	47	0
PDV	North	174	0	0	0	205	0	174	0	53	0	82	0	78	0	766	0
	Central	2	0	0	0	24	0	136	0	103	0	69	0	42	0	376	0
	South	0	0	0	0	0	0	0	0	47	0	0	0	0	0	47	0
PNRSV	North	174	6 (Balkh)	0	0	205	5 (Balkh)	174	0	53	0	82	0	78	0	766	11
	Central	2	1 (Herat)	0	0	24	0	136	0	103	0	69	0	42	1 (Herat)	376	2
	South	0	0	0	0	0	0	0	0	47	0	0	0	0	0	47	0
ApMV	North	174	0	0	0	205	0	174	0	53	0	82	0	78	0	766	0
	Central	2	0	0	0	24	0	136	0	103	0	69	0	42	0	376	0
	South	0	0	0	0	0	0	0	0	47	0	0	0	0	0	47	0
ACLSV	North	0	0	0	0	205	0	174	0	53	0	82	0	78	0	592	0
	Central	0	0	0	0	24	0	136	0	103	0	69	0	42	1 (Herat)	374	1
	South	0	0	0	0	0	0	0	0	47	0	0	0	0	0	47	0
Total		176	7	0	0	229	5	310	0	203	0	151	0	120	2	1189	14

Northern regions: Badakhshan, Baghlan, Balkh, Faryab, Jowzjan, Kunduz, Samangan, Sar-e Pol and Takhar. Central regions: Badghis, Bamian, Farah, Ghor, Herat, Kabul, Kapisa, Kunar, Laghman, Logar, Nangarhar, Nurestan, Panjshir, Parwan and Wardak. Southern regions: Daykundi, Ghazni, Helmand, Kandahar, Khost, Nimruz, Orüzgān, Paktia, Paktika, Zabol.

Table 3 - Apricot samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
PPV	North	0	0	0	0	0	0	68	0	0	0	40	0	80	0	188	0
	Central	278	0	833	0	0	0	221	0	141	0	69	0	48	0	1590	0
	South	0	0	0	0	0	0	0	0	29	0	0	0	0	0	29	0
PDV	North	0	0	0	0	0	0	68	0	0	0	40	0	80	0	188	0
	Central	278	0	833	0	0	0	221	0	141	0	69	0	48	0	1590	0
	South	0	0	0	0	0	0	0	0	29	0	0	0	0	0	29	0
PNRSV	North	0	0	0	0	0	0	68	0	0	0	40	0	80	0	188	0
	Central	278	0	833	0	0	0	221	0	141	0	69	0	48	0	1590	0
	South	0	0	0	0	0	0	0	0	29	0	0	0	0	0	29	0
ApMV	North	0	0	0	0	0	0	68	0	0	0	40	0	80	0	188	0
	Central	278	0	833	0	0	0	221	0	141	0	69	0	48	0	1590	0
	South	0	0	0	0	0	0	0	0	29	0	0	0	0	0	29	0
ACLSV	North	0	0	0	0	0	0	68	0	0	0	40	0	80	2 (Baghlan)	188	2
	Central	0	0	0	0	318	0	221	0	141	0	69	0	48	0	797	0
	South	0	0	0	0	0	0	0	0	29	0	0	0	0	0	29	0
Total		278	0	833	0	318	0	289	0	170	0	109	0	128	2	2125	2

See legend of Table 2 for provinces included in Northern, Central and Southern regions.

Table 4 - Cherry samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
PPV	North	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	0
	Central	0	0	0	0	0	0	1	0	93	0	58	0	35	0	187	0
	South	0	0	0	0	0	0	0	0	18	0	0	0	0	0	18	0
PDV	North	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	0
	Central	0	0	0	0	0	0	1	0	93	0	58	0	35	0	187	0
	South	0	0	0	0	0	0	0	0	18	0	0	0	0	0	18	0
PNRSV	North	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	0
	Central	0	0	0	0	0	0	1	0	93	6 (Kabul)	58	1 (Kabul)	35	3 (Herat)	187	10
	South	0	0	0	0	0	0	0	0	18	1 (Paktya)	0	0	0	0	18	1
ApMV	North	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	0
	Central	0	0	0	0	0	0	1	0	93	0	58	0	35	0	187	0
	South	0	0	0	0	0	0	0	0	18	0	0	0	0	0	18	0
ACLSV	North	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	0
	Central	0	0	0	0	0	0	1	0	93	0	58	0	35	0	187	0
	South	0	0	0	0	0	0	0	0	18	0	0	0	0	0	18	0
Total		0	0	0	0	0	0	8	0	111	7	58	1	35	3	212	11

See legend of Table 2 for provinces included in Northern, Central and Southern regions.

Table 5 - Peach samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
PPV	North	0	0	0	0	0	0	75	0	41	0	9	0	65	0	190	0
	Central	4	0	0	0	267	0	374	0	186	0	21	0	13	0	865	0
	South	0	0	0	0	0	0	0	0	75	0	0	0	0	0	75	0
PDV	North	0	0	0	0	0	0	75	0	41	0	9	0	65	0	190	0
	Central	4	0	0	0	267	0	374	0	186	0	21	0	13	0	865	0
	South	0	0	0	0	0	0	0	0	75	0	0	0	0	0	75	0
PNRSV	North	0	0	0	0	0	0	75	0	41	0	9	0	65	0	190	0
	Central	4	0	0	0	267	0	374	7 (Herat)	186	1 (Kabul)	21	0	13	0	865	8
	South	0	0	0	0	0	0	0	0	75	1 (Kandahar)	0	0	0	0	75	1
ApMV	North	0	0	0	0	0	0	75	0	41	0	9	0	65	0	190	0
	Central	4	0	0	0	267	0	374	0	186	0	21	0	13	0	865	0
	South	0	0	0	0	0	0	0	0	75	0	0	0	0	0	75	0
ACLSV	North	0	0	0	0	0	0	75	0	41	0	9	0	65	0	190	0
	Central	0	0	0	0	267	0	374	0	186	17 (Kabul)	21	0	13	0	861	17
	South	0	0	0	0	0	0	0	0	75	0	0	0	0	0	75	0
Total		4	0	0	0	267	0	449	7	302	19	30	0	78	0	1130	26

See legend of Table 2 for provinces included in Northern, Central and Southern regions.

Table 6 - Plum samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
PPV	North	0	0	0	0	0	0	48	0	31	0	0	0	45	0	124	0
	Central	160	0	0	0	190	0	168	0	140	0	31	0	15	0	704	0
	South	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PDV	North	0	0	0	0	0	0	48	0	31	0	0	0	45	0	124	0
	Central	160	0	0	0	190	0	168	0	140	0	31	0	15	0	704	0
	South	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PNRSV	North	0	0	0	0	0	0	48	0	31	0	0	0	45	0	124	0
	Central	160	5 (Herat)	0	0	190	0	168	0	140	0	31	0	15	0	704	5
	South	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
ApMV	North	0	0	0	0	0	0	48	0	31	0	0	0	45	0	124	0
	Central	160	0	0	0	190	0	168	0	140	0	31	0	15	0	704	0
	South	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACLSV	North	0	0	0	0	0	0	48	0	31	0	0	0	45	0	124	0
	Central	0	0	0	0	190	0	168	0	140	2 (Kabul)	31	0	15	0	544	2
	South	0	0	0	0	0	0	0	0	33	1 (Kandahar)	0	0	0	0	33	1
Total		160	5	0	0	190	0	216	0	204	3	31	0	60	0	861	8

See legend of Table 2 for provinces included in Northern, Central and Southern regions.

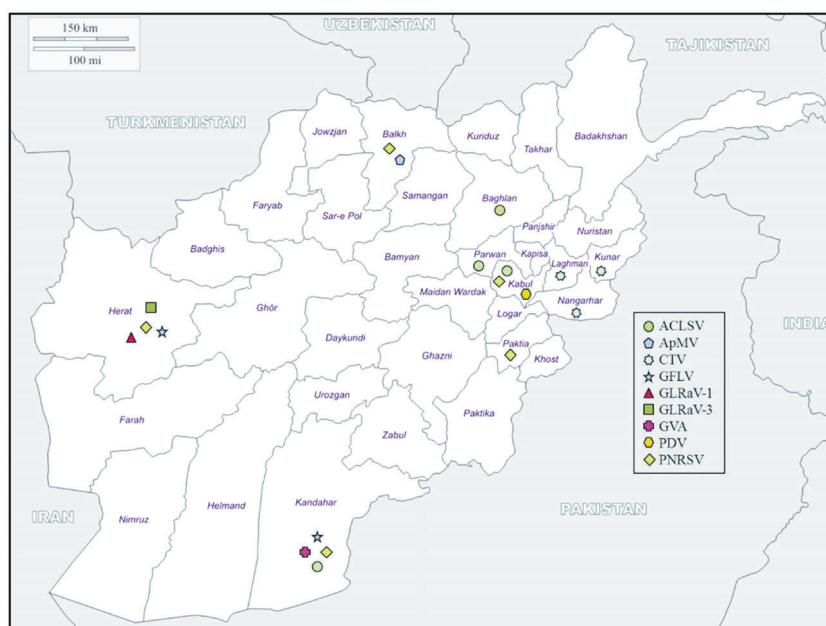


Fig. 3 - Distribution, among Afghan provinces, of samples resulting positive to ACLSV, ApMV, CTV, GFLV, GLRaV-1, GLRaV-3, GVA, PDV or PNRSV.

Margarita (isolates J4 and J8), Orange cv. Mahali (J61), Rough lemon cv. Mahali (J101) and Mandarin Group cvs. Fruter (J76), Tangelo Mapo and Clementine Di Nules. The other 312 plants positive to CTV were collected from private orchards located in the provinces of Kunar, Laghman, and Nangarhar (Table 8, Fig. 3).

RT-PCR and molecular characterization

Samples from stone fruit plants which resulted infected by ACLSV, or PNRSV following DAS-ELISA technique were analysed also by RT-PCR reaction. The results obtained confirmed the presence of each viral agent assayed in the samples tested.

In particular, regarding ACLSV isolates, a 677-long nucleotide fragment, corresponding to partial coat protein gene, was amplified from all ELISA-positive samples by RT-PCR using ACLSV sense and antisense primers (Menzel *et al.*, 2002). Eleven of the identified isolates of ACLSV were then characterized molecularly. Sequence analysis revealed similarity ranging from 83.6 to 100.0% within ACLSV isolates detected in Afghanistan. Blast analysis showed that sequences of two peach isolates, 812/Dir Ras, shared the highest nucleotide similarity (95.8 and 96.2%) with the GenBank ACLSV isolates Apr 3 from Jordan (AJ586631). Moreover, the same Afghan isolates showed nucleotide identity between 95.0 and 95.7% with isolates S4, PP23, PP63, and HL2 (JN849008, GU327991, GU328003 and GQ334211, respectively) from China. Sequence analysis of isolate 364/Alu



Fig. 4 - Vein clearing and yellowing symptoms observed in citrus accessions collected from the National Collection Centre in Jalalabad (Nangarhar province).

Table 7 - Grape samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
GFLV	Herat	0	0	0	0	156	19	117	13	271	22	0	0	0	0	544	54
	Kandahar	0	0	0	0	0	0	0	0	268	0	0	0	0	268	0	
ArMV	Herat	0	0	0	0	156	0	117	0	271	0	0	0	0	544	0	
	Kandahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
GVA	Herat	83	0	0	0	156	0	117	0	271	0	0	0	0	627	0	
	Kandahar	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	
GFkV	Herat	83	0	0	0	156	0	117	0	271	0	0	0	0	627	0	
	Kandahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
GLRaV-1	Herat	83	0	0	0	156	0	117	1	271	0	0	0	0	627	1	
	Kandahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
GLRaV-2	Herat	83	0	0	0	156	0	117	0	271	0	0	0	0	627	0	
	Kandahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
GLRaV-3	Herat	83	0	0	0	156	0	117	2	271	0	0	0	0	627	2	
	Kandahar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Total		83	0	0	0	156	19	117	16	539	23	0	0	0	895	58	

Table 8 - Citrus samples tested and resulting positive by DAS-ELISA during the first seven years of activity of the Plant Biotechnology Laboratory based in Kabul.

Virus	Region	2009		2010		2011		2012		2013		2014		2015		Total	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
CTV	Nangarhar	0	0	193	6	263	2	1276	153	1554	110	131	0	492	0	3909	271
	Laghman	0	0	0	0	0	0	180	9	200	4	0	0	296	3	676	16
	Kunar	0	0	0	0	0	0	318	21	246	9	0	0	249	1	813	31
Total		0	0	193	6	263	2	1774	183	2000	123	131	0	1037	4	5398	318

Bokhara Shalili, 804/Turki Sorkh and 811/Turki Zard proved that they are 99.6 to 100.0% identical with each other and share high (94.3 to 94.7%) nucleotide identity with the Iranian isolate T12Ja (KM586376).

Lower nucleotide similarity (86.2%) was shown by one 804/Turki Sorkh isolate with the isolate 274-Chal (FN391009) from Greece. Finally, analysis of the sequence of isolates 804/Turki Sorkh, 811/Turki Zard and 812/Dir Ras revealed a high degree of identity (86.2 to 88.4%) with the corresponding nucleotide sequences of the isolate SK-92-pl (HQ398253) from Slovakia.

RT-PCR reaction performed on samples infected by PNRSV, according to DAS-ELISA results, amplified a fragment of 675 nucleotides in length when PNRSV specific primer pair MG1/MG2 was used (Table 1).

Regarding grape samples, specific PCR products of 118 nucleotides were also observed on DAS-ELISA positive samples, using specific primers pair M3/M4 against GFLV as reported in Table 1.

Some of the citrus samples tested positive by DAS-ELISA were also tested using primers CTVF and CTVR allowing amplification of specific PCR products 655 nucleotides in length (Table 1). Moreover, six CTV isolates collected at the Jalalabad station were characterized. Sequence analysis revealed high similarity, ranging from 91.1 to 99.8%, within the CTV isolates detected. In accordance with the previously defined phylogenetic groups (Zemzami *et al.*, 2002), nucleotide sequences of Afghan CTV isolates cluster in Group 1 (J4 and J8), Group 4 (J61 and J76) and Group 5 (J101). In particular, J4 and J8 isolates show, respectively, 99.4 and 99.2% identity with reference isolate T36 (M76485) from the USA (Florida). Furthermore, in Group 4, isolate J61 and J76 were more similar to ANO-1 isolate (DQ211658) from Egypt (98.5 and 98.0% identity, respectively) than to isolate 443-4 (AY791844) from Croatia (97.4 and 97.5%, respectively). Finally, isolate J101, in Group 5, shows 95.6% identity with isolates C268-2 (AY750770) and C269-6 (AY750775) from Argentina.

4. Discussion and Conclusions

To improve Afghanistan's agricultural sector, the EC supports the PHDP (Perennial Horticulture Development Project), for true to type/ecotype and healthy planting materials, and the Plant Biotechnology Laboratory, to ensure the health status of local germplasm. The laboratory started

screening the health status of the Afghan Germplasm National Collection in order to ensure the multiplication of not only the best-selected varieties or ecotype, but also to avoid production and distribution of virus-infected fruit trees. Symptom inspection and sample collection for viral diseases was carried out in all the National Collection fields, including peach, plum, apricot, almond, apple, grape and citrus plants, located in different areas of the country.

During the first seven years of its activity, the Plant Biotechnology Laboratory based in Kabul, played a key role by satisfying the increasing need for certified propagation plant material to establish new plantations in Afghanistan. Moreover, it contributed to protecting the country from undesirable introduction of pathogens due to increasing exchanges of propagation plant material among different countries and between different continents.

Until now, the Plant Biotechnology Laboratory has verified the absence of PPV (the most devastating virus for stone fruit) in the Afghan germplasm and detected and verified the presence of several important plant viruses in stone fruits, citrus and grapevines. Further activity will be addressed to confirming, by RT-PCR, infection by ApMV, PDV, GVA, GLRaV-1 and GLRaV-3 in almond, plum and grape accessions which resulted positive by DAS-ELISA, using specific primer pairs (Parakh *et al.*, 1995; Menzel *et al.*, 2002; Goszczynski and Jooste, 2003; Osman *et al.*, 2008).

The Plant Biotechnology Laboratory first reported ACLSV occurrence in peach, plum, almond and apricot plants in Afghanistan. According to the preliminary results of sequence analysis, due to the relative low identity with other ACLSV isolates present in GenBank, our mother stock nurseries appear to be infected by several Afghan isolates of the virus, but further studies will be addressed to understanding if ACLSV has been introduced into the country through infected propagation material. In any case, the presence of ACLSV in important cultivars of peach and plum is a worrying aspect of the Afghan certification program.

Similarly, our results identified, for the first time, PNRSV, ApMV, and PDV infected plants in Afghanistan. Even if the latter two viruses were detected by DAS-ELISA test, the presence of the viruses in accessions of the National Collection field is cause for worry for Afghan horticulture as all three viruses spread through infected propagating material and nursery productions (Mink, 1992). Moreover, PNRSV and PDV are also seed- and pollen-transmit-

ted and can be spread by pollinating insects (Kelley and Cameron, 1986; Aparicio *et al.*, 1999; Glasa *et al.*, 2002; Amari *et al.*, 2007) while seed transmission of ApMV is known only in hazelnuts (Cameron and Thompson, 1985). A comparative sequence and phylogenetic analysis will be performed (Zindović *et al.*, 2015) to show if clustering of various isolates is associated or not with geographic and host origin.

Regarding the detection of viruses in grape, according to our results the presence in the NCC in Kandahar of GVA and GLRaV-1, and GLRaV-3 in Herat seems to be restricted to only a few accessions. Early detection of these viruses by the Plant Biotechnology Laboratory avoided multiplication and therefore the spread of infected grape material in Afghanistan. In contrast, the noticeable presence of GFLV (nearly 7%) in local cultivars maintained at the NCC in Herat suggests the presence of the virus in Afghan germplasm of grape for some time. This hypothesis should be confirmed by studying the genetic variability of Afghan isolates in order to investigate the relationship among their geographical origin, sequence variability, and grapevine cultivar (Meng *et al.*, 2006; Vigne *et al.*, 2009; Terlizzi *et al.*, 2015).

Finally, our analyses identified, for the first time, CTV infected plants in Afghanistan (Rehman *et al.*, 2012). The presence of a dangerous viral disease such as CTV in many citrus trees (nearly 6% of the assayed plants) from NCCs, MSNs and private orchards represents a serious problem for the Afghan citrus industry.

High sequence identity with many isolates from USA, Egypt, Croatia, and Argentina together with the large spread of the virus in Afghan citrus-cultivating areas suggests the introduction of the virus into the country a long time ago. The presence of this virus seems to be, therefore, endemic; further investigations showed that infected plants did not exhibit quite as dramatic symptoms as those usually found in other citrus cultivation areas (e.g. Spain, Brazil, Argentina etc.) where the virus is extremely dangerous and causes in some cases plants death (Rocha-Peña *et al.*, 1995). A specific research program is in progress to investigate if a mild strain of CTV is present in the eastern region (Nangarhar, Kunar, Laghman) environment, if tolerant/resistant sour orange rootstock clones exist, or if the environmental (climate and soil conditions) influences the infectivity of CTV.

Due to the economic importance of these crops, implementation of the certification schemes is therefore necessary in Afghanistan in order to guarantee

the production and employment of virus-free propagating material.

It is well known that data regarding the presence and spread of different plant viruses are important for individual countries, their neighbours, and for the whole agricultural community where different viruses can be spread by vegetative propagation via plant material. A continuous survey is therefore necessary to protect Afghanistan from the introduction and/or spread of dangerous pests. As previously experienced, most effective management programs require prompt removal of infected trees and replacement with healthy planting material from certified nurseries (Hadidi *et al.*, 2011; Pallas *et al.*, 2012). Establishment of rigid phytosanitary controls related to the importation of propagation material, as well as the eradication of infected trees, is therefore of great importance in Afghanistan.

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