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# Serological and Molecular Detection of Latent Viruses in the Apple Germplasm Bank of Santa Catarina

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## HIGHLIGHTS

- RT-PCR, DAS-ELISA and IC-RT-PCR reveals ASGV, ASPV and ACLS latent mixed infection in accessions of the Apple Germplasm Bank of Epagri.

**Abstract:** The Apple Germplasm Bank (AGB) of Santa Catarina Agricultural Research and Rural Extension Company - Epagri, AGB-Epagri, is the largest of the genus *Malus* in Brazil. Twenty-eight main accessions of this bank were virus screened through DAS-ELISA, RT-PCR and IC-RT-PCR during two consecutive reproductive cycles, and each accession showed latent mixed infection by at least two species, among ASGV, ASPV and ACLSV. The combined use of diagnostic methods helped overcome inconsistencies commonly found in apple virus detection and was shown essential for the AGB-Epagri can be safely used as a source of genetic variability and for the exchange of virus-free propagative material.

**Keywords:** apple stem grooving virus (ASGV); apple stem pitting virus (ASPV); apple chlorotic leaf spot virus (ACLSV); *Malus* spp.

## INTRODUCTION

The AGB-Epagri has 387 accessions belonging to different *Malus* species. It is an old collection kept in the field, what prone it to phytosanitary problems arising, such as *Botryosphaeria obtusa* cankers, *Venturia inaequalis* and *Colletotrichum gloeosporioides* leaf spots. Moreover, viruses are suspected to be associated with decline and reduced survival of grafted plants. More than 20 virus and viroid species are reported in apple, among them, *Apple stem grooving virus* (ASGV, genus *Capillovirus*), *Apple stem pitting virus* (ASPV, genus *Foveavirus*), *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*) and *Apple mosaic virus* (ApMV, genus *Ilarvirus*) [1]. The ApMV was previously detected by DAS-ELISA in some accessions of AGB-Epagri causing yellowish leaf spots. However, latent infections of other species were suspected.

Multiple virus infections result in death, plant predisposition to other pathogens, sharp drop in production, and low quality of fruits [2,3,4,5]. Less aggressive infections of latent viruses probably interfere in phenotype

evaluation, like is being experienced in the breeding program for *Glomerella* leaf spot resistance carried out at Epagri.

Considering the AGB-Epagri is an important repository for germplasm exchanges, this work aimed to evaluate the 28 main accessions of this bank for ASGV, ASPV and ACLSV infection. These latent viruses are commonly associated with apple diseases all over the world.

## MATERIAL AND METHODS

One plant per accession of AGB-Epagri was tested from non-fully expanded apical leaves of grown plants in the spring of the years 2015 and 2016. Accessions were selected based on their simultaneous resistance to apple scab (*Venturia inaequalis*) [6] and *Glomerella* leaf spot (*Colletotrichum* spp.) [7]. For double antibody sandwich-enzyme-linked immunosorbent assays (DAS-ELISA), plant extracts were prepared in 20 mL.g<sup>-1</sup> of extraction buffer (137 mM CaCl<sub>2</sub>, 3 mM KCl, 2% PVP K25, 0.05% Tween 20, and 0,02% NaN<sub>3</sub>). Each accession was tested with purified specific antibodies (Bioreba®) for ASGV, ASPV and ACLSV in three replicates. Samples with absorbance readings at 405 nm equal to or greater than twice values of the negative readings were considered positives. Total RNA from leaves was extracted by adsorption on silica [8]. For cDNA synthesis in microcentrifuge tubes, 4 µL of total RNA from each accession, 5 µL of DEPC water and 1 µL of antisense primer (0,4 µM) were added. Samples were incubated at 80 °C for 2 min and on ice for 3 min, following addition of 5 µL of 5x M-MLV reverse transcriptase buffer (Ludwig®), 1 µL dNTPs (2,5 mM each), 0,7 µL RNase inhibitor (Invitrogen®, 40 U.µL<sup>-1</sup>), 1 µL of reverse transcriptase M-MLV (Ludwig®, 200 U) and 7,3 µL of DEPC water for 1h incubation at 37 °C. PCR samples were prepared to a final volume of 15 µL by adding 1,5 µL of 5x *Taq* DNA polymerase buffer (Ludwig®), 0,6 µL MgCl<sub>2</sub> (25 mM), 0,3 µL dNTPs (2, 5 µM each), 1,5 µL of sense and antisense primers (10 µM), 0,2 µL *Taq* DNA polymerase (Ludwig®, 5 U.µL<sup>-1</sup>), 5 µL cDNA and 4,4 µL DEPC water, subjected to amplification conditions of each primer (Table 1). Components of blank control consisted of all reagents except the RNA from plants.

**Table 1.** Primers for ASGV, ACLSV and ASPV detection in 28 accessions of Apple Germplasm Bank-Epagri.

Primer name	Sequence (5' - 3')	Position in genome	Fragment size	Annealing temperature
ASGV 6396 r	CTG CAA GAC CGC GAC CAA GTT T	6373 - 6396	524 bp <sup>9</sup>	60 °C
ASGV 5873 f	CCC GTC GTT GGA TTT GAT ACA CCT C	5873 - 5898		
ASGV 6396 r	CTG CAA GAC CGC GAC CAA GTT T	6373 - 6396	755 bp <sup>10</sup>	54 °C
ASGV 5641 f	ATG AGT TTG GAA GAC GTG CTT C	5641 - 5663		
ACLSV 7233 r	CAG ACC CTT ATT GAA GTC GAA	7213 - 7233	358 bp <sup>11</sup>	54 °C
ACLSV 6875 f	GGC AAC CCT GGA ACA GA	6875 - 6891		
ACLSV 7365 r	CTA AAT GCA AAG ATC AGT CGA C	7343 - 7365	581 bp <sup>2</sup>	55 °C
ACLSV 6784 f	ATG GCA GCA GTT CTG AAT TTG	6784 - 6805		
ASPV 3770 r	GTC AGG TCA AAG ATG CTG AAA	3750 - 3770	291 bp <sup>12</sup>	50 °C
ASPV 3480 f	AGC GGT TGC CTA TTT TTG CTC C	3480 - 3501		
ASPV 9262 r	ATA GCC GCC CCG GTT AGG TT	3750 - 3770	269 bp <sup>12</sup>	52 °C
ASPV 8993 f	CTC TTG AAC CAG CTG ATG GC	3480 - 3501		

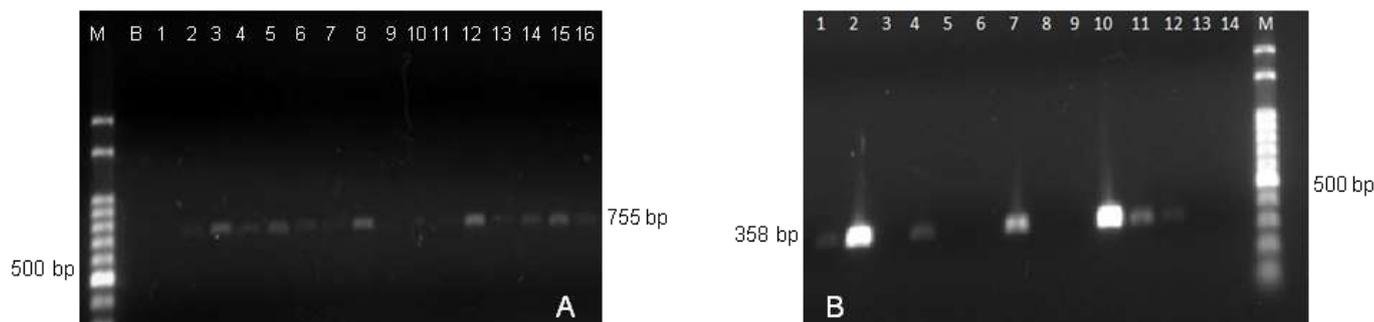
[Ref. 9], [Ref. 10], [Ref. 11], [Ref. 2], [Ref. 12], f- forward, r – reverse.

For immunocapture reverse transcription (IC-RT)-PCR leaves were ground in liquid nitrogen and after in nucleoprotein extraction buffer (Bioreba®), in 5 mL/ 500 mg proportion. Cultivar SCS417 Monalisa simultaneously infected by ASGV, ASPV and ACLSV was used as positive control (Table 2 and 3). Antibodies for each virus were 1:1000 diluted (200 µL) and incubated in PCR tubes (0,2 mL) during 4 h at 37 °C. After PBS-Tween washes and addition of leaf extracts (200 µL), 25 µL of the commercial mix (Qiagen® One Step RT-PCR Kit) were used as previously in the RT-PCR with same described primers (Table 1).

## RESULTS AND DISCUSSION

The ASGV was detected by DAS-Elisa in 23 out of 28 accessions in the reproductive cycle of 2015, and in 18 genotypes of the 2016 cycle. Through molecular methods, detection in year 2015 was only with primers for amplification of 755 bp by IC-RT-PCR, in 23 accessions. Evaluation results of 16 accessions are shown (Fig 1A). For reproductive cycle of 2016, only RT-PCR gave amplifications of 755 and 524 bp, confirming the ASGV infection in respective 25 and 24 accessions. In serological and molecular detection, inconsistencies were observed (Tables 2 and 3). As in DAS-Elisa tests, ASGV was not detected in 'Baronesa' and 'Priscilla' also by PCR. A 755 bp amplification product from accession '21-502-1' was purified and sequenced at the Human Genome Research Center of the University of São Paulo. After alignment with GenBank sequences

(<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), the ASGV from AGB-Epagri was found 98% homolog with coat protein gene of accession LT574875 [13]. Variations in detection of ASGV were observed from year to year in accessions 'Co-op 14', 'Imperatriz', *Malus floribunda*, 'Nova Easygro' and 'Red Free' by DAS-ELISA tests, and in '21-261-75', 'Catarina' and 'Priam' by PCR (Tables 2 and 3).



**Figure 1.** Electrophoresis in 1,2% agarose gel of IC-RT-PCR 755 bp amplifications of ASGV – A : M - 100 bp ladder (Ludwig®), B- blank; 1- '21-361-75'; 2 – '21-373-58'; 3 – '21-555-13'; 4 – '21-300-13'; 5 – '21-300-21'; 6 – '21-379-64'; 7 – '21-503-1'; 8 – 'Akane'; 9 – 'Baronesa'; 10 – 'Catarina'; 11 – 'Coop 14'; 12 – 'Coop 16'; 13 – 'Coop 24', 14 – 'Coop 8'; 15 – 'D1R102T116'; 16 – 'D1R103T245'. IC-RT-PCR 358 bp amplifications of ACLSV – B : M- 100 bp ladder (Ludwig®), 1 – 'Coop 24'; 2 – 'D1R102T116'; 3 – 'D1R103T245'; 4 – 'Florina'; 5 – 'Fred Hough'; 6 – 'Imperatriz'; 7 – 'Liberty'; 8 – 'Mac Free'; 9 – *Malus floribunda*; 10 – 'Nova Easygro'; 11 – 'Priam'; 12 – 'Priscilla'; 13 – 'Red Free' e 14 – 'Sansa'.

**Table 2.** DAS-ELISA detection of ASGV, ASPV and ACLS in apples of the Germplasm Bank-Epagri during reproductive cycles of 2015 and 2016.

Accession (Origin)	ASGV		ASPV		ACLSV	
	2015	2016	2015	2016	2015	2016
21-361-75 (Argentina)	0,556 (+)	0,349 (+)	0,286 (-)	0,765 (+)	0,558 (+)	0,241 (-)
21-373-58 (Argentina)	0,951 (+)	0,457 (+)	0,348 (-)	0,521 (+)	0,387 (+)	0,335 (-)
21-555-13 (Argentina)	0,489 (+)	0,402 (+)	0,695 (+)	0,312 (-)	0,579 (+)	0,854 (+)
21-300-13 (Argentina)	0,576 (+)	0,421 (+)	0,795 (+)	0,688 (+)	0,309 (-)	0,347 (-)
21-300-21 (Argentina)	1,616 (+)	0,627 (+)	0,620 (+)	0,496 (+)	0,976 (+)	3,062 (+)
21-379-64 (Argentina)	0,809 (+)	0,912 (+)	0,493 (-)	0,550 (+)	0,447 (+)	2,840 (+)
21-502-1 (Argentina)	1,156 (+)	0,746 (+)	0,915 (+)	0,634 (+)	1,219 (+)	1,984 (+)
Akane (Japan)	0,975 (+)	0,460 (+)	0,498 (-)	0,765 (+)	0,836 (+)	0,248 (-)
Baronesa (Brazil/Epagri)	0,203 (-)	0,181 (-)	0,300 (-)	0,742 (+)	0,295 (-)	0,192 (-)
Catarina (Brazil/Epagri)	1,361 (+)	0,405 (+)	0,204 (-)	0,817 (+)	0,310 (-)	0,207 (-)
Co-op 8 (U.S)	0,195 (-)	0,190 (-)	0,282 (-)	0,702 (+)	0,308 (-)	0,203 (-)
Co-op 14 (U.S)	0,839 (+)	0,320 (-)	0,555 (+)	0,778 (+)	0,667 (+)	0,252 (-)
Co-op 16 (U.S)	0,192 (-)	0,215 (-)	0,341 (-)	0,250 (-)	0,630 (+)	0,828 (+)
Co-op 24 (U.S)	1,860 (+)	0,413 (+)	0,387 (-)	0,832 (+)	0,832 (+)	0,217 (-)
D1R102T116 (U.S)	1,412 (+)	1,854 (+)	0,369 (-)	0,448 (-)	0,706 (+)	9,999 (+)
D1R103T245 (U.S)	1,185 (+)	0,595 (+)	0,239 (-)	0,764 (+)	0,526 (+)	1,419 (+)
Florina (France)	1,606 (+)	0,428 (+)	0,547 (+)	0,445 (-)	1,448 (+)	1,859 (+)
Fred Hough (Brazil/Epagri)	1,302 (+)	0,582 (+)	0,269 (-)	0,880 (+)	0,457 (+)	0,424 (+)
Imperatriz (Brazil/Epagri)	0,860 (+)	0,243 (-)	0,261 (-)	1,372 (+)	0,441 (+)	0,199 (-)
Liberty (U.S)	0,885 (+)	0,402 (+)	0,323 (-)	0,467 (+)	0,777 (+)	1,421 (+)
Mac Free (U.S)	0,694 (+)	0,366 (+)	0,470 (-)	0,856 (+)	0,647 (+)	0,225 (-)
<i>Malus floribunda</i> (Japan)	0,449 (+)	0,206 (-)	0,570 (+)	0,453 (-)	0,542 (+)	0,267 (-)
Nova Easygro (Canada)	0,756 (+)	0,321 (-)	1,153 (+)	0,991 (+)	0,803 (+)	0,467 (+)
Priam (U.S)	0,189 (-)	0,196 (-)	0,244 (-)	0,459 (+)	0,513 (+)	9,999 (+)
Priscilla (U.S)	0,182 (-)	0,171 (-)	0,321 (-)	0,828 (+)	0,849 (+)	0,509 (+)
Red Free (U.S)	0,786 (+)	0,311 (-)	0,457 (-)	0,711 (+)	0,755 (+)	0,533 (+)
Sansa (Japan)	1,865 (+)	0,363 (+)	0,281 (-)	0,959 (+)	0,789 (+)	0,366 (-)
Monalisa (Brazil/Epagri)	0,457 (+)	0,492 (+)	0,773 (+)	1,166 (+)	0,585 (+)	0,796 (+)
Negative Control (Bioreba®)	0,183	0,168	0,273	0,229	0,169	0,206

Values in table correspond to the average of three readings in Abs<sub>405 nm</sub>. (+) indicates positive reaction whose reading average was higher than twice the value of negative control; (-) indicates negative reaction. Readings were for 120 min after enzyme substrate addition. Blank control was not tested.

Fluctuation in virus titer, the uneven distribution in apple tree, type of tissue, and seasonal variations, would explain virus detection inconsistencies from one year to the next [14]. Moreover, RT-PCR is prone to false negatives due to RNA degradation or inhibitors of the reverse transcriptase or polymerase [15].

The second latent virus, ASPV, was detected by DAS-ELISA in nine out of 28 accessions in the reproductive cycle of 2015, and in 23 accessions of 2016. In the IC-RT-PCR, amplifications of 269 bp were obtained for all 28 accessions, with primers ASPV 9262r and ASPV 8993f, for the reproductive cycle of 2015, and by RT-PCR in the reproductive cycle of 2016. However, results were negative for accessions '21-361-75', 'Akane', 'Florina', *M. floribunda*, 'Priam', 'Red Free', 'Sansa' and 'Monalisa' (Table 3).

Third latent virus, ACLSV, was detected by DAS-Elisa in 24 and 15 accessions, respectively in 2015 and 2016. The 'Co-op 8' accession was identified as the only non-positive for ACLSV, based on molecular and serological performed tests (Table 2 and 3). Variations in detection occurred in accessions '21-361-75', '21-373-58', 'Akane', 'Co-op 14', 'Co-op 24', 'Imperatriz', 'Mac Free', *M. floribunda* and 'Sansa'. On the other hand, in molecular tests, ACLSV was detected in 27 accessions (Table 2 and 3). Amplifications of 358 bp with primers ACLSV 7233r and ACLSV 6875f were obtained by IC-RT-PCR, in 15 and 16 accessions, of respective cycles of 2015 and 2016. Evaluation results of 14 accessions are shown (Fig 1B). However, with the same pair of primers, there was amplification by RT-PCR in 18 accessions of the 2016 cycle.

The ACLSV amplified RT-PCR product was 90% homolog to part of coat protein of isolate ACLSV from Greece (GenBank accession AM292923.1) [16]. With the second pair of primers, ACLSV 7365r and ACLSV 6784f, 581 bp amplifications were obtained in nine of the 28 accessions by RT-PCR, but only for reproductive cycle of 2016. These primers anneal in part of each, coat and movement protein genes. Among ACLSV isolates there is high homology in coat protein, and lower in the movement protein, probably correlating with the common inconsistencies in ACLSV detection [11].

Inconsistencies in detection of ASGV and ASPV were also noticed in the apple germplasm bank of Canada, assigned to virus title fluctuation [1,17]. In general, combined use of virus detection methods in woody plants is most recommended strategy. Escape or false negative and/or positive results intrinsic to each method, can be minimized [13,18,19]. Analysis of 28 accessions of AGB-Epagri showed latent infection by at least two virus species in each accession. This confirmed detection of mixed infections in traditional apple tree cultivars in southern Brazil [10]. There are no reports of insect vectors for ASGV, ASPV and ACLSV. Therefore, rootstocks of unknown sanitary conditions probably are the main factor associated with high rates of infection. Latent virus infection would lead to plant decline and death, depending on virus strain and cultivar susceptibility, primarily as observed in apples grafted on 'Marubakaido', the most common apple rootstock in Brazil associated with ASGV and ACLSV [2,10]. Occurrence of ASGV, ASPV and ACLSV in accessions of AGB-Epagri would not recommend them for germoplasm exchange. However, a clonal cleaning program, permanent viral certification, and progressive reintroduction of accessions, would make them feasible as planting material.

**Table 3.** IC-RT-PCR and RT-PCR detection of ASGV, ASPV and ACLSV in apples of the Germplasm Bank-Epagri during reproductive cycles of 2015 and 2016.

Accession	2015	2016	2016	2015	2016	2016	2015	2016	2016	2016
	ASGV 755 bp	ASGV 755 bp	ASGV 524 bp	ASPV 269 bp	ASPV 269 bp	ASPV 291 bp	ACLSV 358 bp	ACLSV 358 bp	ACLSV 581 bp	ACLSV 581 bp
	IC*	RT-PCR	RT-PCR	IC	RT-PCR	RT-PCR	IC	IC	RT-PCR	RT-PCR
21-361-75	—**	+	+	+	+	—	+	+	—	+
21-373-58	+***	+	+	+	+	+	+	+	—	—
21-555-13	+	+	+	+	+	+	+	+	—	+
21-300-13	+	+	+	+	+	+	—	—	+	+
21-300-21	+	+	+	+	+	+	—	—	+	—
21-379-64	+	+	+	+	+	+	+	+	—	+
21-503-1	+	+	+	+	+	+	+	+	—	—
Akane	+	+	+	+	+	—	—	+	+	—
Baronesa	—	—	—	+	+	+	+	—	+	—
Catarina	—	+	+	+	+	+	—	—	+	—
Coop 8	+	+	—	+	+	+	—	—	—	—
Coop 14	+	+	+	+	+	+	+	+	—	—
Coop 16	+	+	—	+	+	+	—	—	+	+
Coop 24	+	+	+	+	+	+	+	+	+	+
D1R102T116	+	+	+	+	+	+	+	+	+	—
D1R103T245	+	+	+	+	+	+	—	+	+	—
Florina	+	+	+	+	+	—	+	+	—	—
Fred Hough	+	+	+	+	+	+	—	—	+	—
Imperatriz	+	+	+	+	+	+	—	—	+	—
Liberty	+	+	+	+	+	+	+	+	—	—
Mac Free	+	+	+	+	+	+	—	—	+	—
<i>Malus floribunda</i>	+	+	+	+	+	—	—	+	—	+
Nova Easygro	+	+	+	+	+	+	+	+	+	—
Priam	—	—	+	+	+	—	+	+	+	—
Priscilla	—	—	—	+	+	+	+	—	+	+
Red Free	+	+	+	+	+	—	—	+	+	+
Sansa	+	+	+	+	+	—	—	—	+	—
Monalisa	+	+	+	+	+	—	+	—	+	—

\*IC: immunocapture-RT-PCR. \*\*(-): negative, no amplification of expected fragment. \*\*\*(+): positive, expected fragment amplification.

## CONCLUSION

All the twenty-eight accessions of AGB-Epagri are infected by at least two of the main latent virus species that worldwide infect apple trees, ASGV, ASPV and ACLSV.

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