

Evaluation of detection methods for Virus, Viroids and Phytoplasmas affecting pear and apple

Laviña, A., Sabaté, J., Batlle, A.

Dpt. Protecció Vegetal, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Ctra Cabrils s/n. 08348 Cabrils (Barcelona).

Abstract

The RT-PCR technique for the detection of apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV) and pear blister canker viroid (PBCV) was evaluated for health control of fruit plants from nurseries. The technique was evaluated in purified RNA and crude extracts and also in phloem collected in autumn and from young spring shoots. The results obtained for phytoplasma detection with ribosomal and non-ribosomal primers are also presented.

Keywords: Detection, fruit virus, viroids, phytoplasma

Introduction

The virus Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), Apple chlorotic leaf spot virus (ACLSV) and Apple mosaic virus (ApMV), the viroid Pear Blister canker (PBCV) and the phytoplasmas *Candidatus Phytoplasma pyri* (pear decline, PD) and *Ca. P.mali* (apple proliferation, AP), cause significant diseases in *Malus* and *Pyrus* species. The sensitivity of molecular methods to detect these virus and phytoplasmas has been reported by many authors but it is still recommended, that their absence be confirmed by indexing on indicator woody plants.

There is an increasing need for health control in fruit nurseries, due to the large number of diseases caused by viruses, phytoplasmas and other harmful organisms. One explanation why the number of certified plants has not increased, may - depending on the species - be found in the fact that certifying by field indexing can take between 2-4 years: the time needed for symptoms to appear.

In the present work, we established the presence of ASPV, ASGV, ApMV, ACLSV and PBCV by means of RT-PCR and of *Ca. P. pyri* and *Ca. P.mali* by PCR on tree samples from different nurseries and orchards. In addition, the presence of these viruses was evaluated in positive controls of different transmissible diseases, including vein yellows, ring mosaic, red mottle, and russet wart, as was the presence of phytoplasmas in apple samples with chat fruit and rubbery wood symptoms.

Material and methods

Detection of Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus and Apple mosaic virus by RT-PCR: Virus detection was carried out on crude extract and on purified RNA. Total RNA was extracted from different parts of the plant (leaves, petioles and bark) by different extraction methods, using a commercial extraction kit (Rnaseasy Qiagen) according to the manufacturer's instructions. Extraction was performed on young shoots selected in May and from phloem selected in October.

For ASPV, three pairs of specific primers were assayed: those cited by Klerks et al. (2001); those cited in Batlle et al. (2004), and the primers used by Massart et al. (2008). For other viruses, only the primers cited in Massart et al. (2008) were used.

Detection was carried out on samples taken from trees infected with each of these viruses and on hosts of various diseases, such as vein yellows, ring mosaic, red mottle and russet wart. The technique was evaluated in samples from different plant nurseries.

Pear blister canker detection by RT-PCR: The plant material used came from two orchards and positive controls were supplied by the Certification Institute of Zaragoza (Spain). Two pairs of specific primers were assayed: the primer pair cited by Malfitano et al. (2004), and that used by Hassen et al. (2006). The RT-PCR procedure applied was a modified version of the one referred to in Hassen et al. (2006). Four µl of RNA extract was mixed with 0.4 µM complementary primer (Malfitano et al. 2004), heated for 5 min at 100°C and then immediately chilled on ice. The RT-PCR reaction mixture contained 0.4 µM of homologous primer and the Qiagen reaction mix (5 µl of 5 x RT-PCR buffer, 1 µl dNTPs (0.4 µM final), 1 µl enzyme mix and 5 µl RNase-free water).

Phytoplasma detection by PCR: For phytoplasma detection by PCR, samples were collected between October and December, which has been shown to be the best period for the detection in our climate (Garcia et al. 2003). DNA was isolated from approximately 1.0 g of fresh stems, using the phytoplasma-enrichment procedure of Ahrens and Seemüller (1992). The nested-PCR procedure was used with P1/P7 primers in the first step (Smart et al. 1996) and with specific primers for the apple proliferation group fO1/rO1 (Lorenz et al. 1995) in the second step. The presence of phytoplasmas other than the 16Sr-X or AP group, in those samples giving a negative with the specific primers, was determined using the universal primer pair fU5/rU3 (Lorenz et al. 1995). Samples which tested positive in the nested-PCR assay were further analysed by RFLP. Amplified products (10 µl) obtained with the universal primers fU5/rU3 were digested with Tru9I restriction endonuclease following the manufacturer's instructions (MBI Fermentas, Germany). Amplified products obtained with the fO1/rO1 specific primers were digested as above with Ssp I and Rsa I at 37°C. Profiles obtained were compared with those established by Seemüller et al. (1998).

In addition, we evaluated primers developed for the amplification of different fragments of the pear decline genome by Garcia et al. (2004): 7 f/r, which amplified fragments of a gene coding for a thymilate kinase (TMPK), 14f/r for a peptide release (PR), 10 f/r for 50S ribosomal protein L4, 15f/r for DNA polymerase III and 13 f/r (no significant similarity). The primers developed by Danet et al. 2006 that amplify a sequence encoding a surface protein were also evaluated.

Results and discussion

Detection of Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus and Apple mosaic virus by RT-PCR: The best primers for the detection of ASPV, ASGV, ACLSV and ApMV by RT-PCR were those used by Massart et al. (2008). With these primers, it is possible to detect all four viruses, both in purified RNA and in crude extracts, and also in extracts of phloem in autumn and of young shoots in spring. The virus most frequently identified in samples from nurseries was ASPV (Table 1). It was also identified in positive controls for different transmissible diseases: vein yellows, ring mosaic, red mottle, and russet wart. In the latter, ASGV was also detected, indicating that these viruses may be responsible for these diseases either alone, or in synergism with other viruses or pathogenic organisms (Table 2).

Tab. 1 Virus and phytoplasma detection in samples from pear and apple varieties taken from different nurseries

Species	Season	ASPV	ASGV	ApMV	ACLSV	Phytoplasma
Apple, several varieties	Spring	3+/6	0+/6	4+/6	0+/6	0+/6
Pear, several varieties	Spring	3+/6	0+/6	0+/6	0+/6	0+/6
Pear, cv. Conference	Autumn	3+/15	0+/15	0+/15	0+/15	13+PD/15
Pear, several varieties	Winter	0+/5	0+/5	0+/5	0+/5	5+PD/5
Apple, several varieties	Winter	0+/7	0+/7	0+/7	0+/7	3+AP/7

Tab. 2 Virus and phytoplasma detection by RT-PCR and nested PCR in positive controls of different transmissible diseases

Symptom	Virus	Phytoplasma
Ring Mosaic	ASPV	-
Red mottle	ASPV	-
Vein yellows	ASPV	-
Russet wart	ASPV + ASGV	-
Chat fruit	-	positive

Pear blister canker detection by RT-PCR: PBCV was best detected using the primers cited by Malfitano et al. (2004) and the procedure recommended by Hassen et al. (2006). With this method, PBCV was detected in all the infected controls but in no healthy plants.

Phytoplasma detection: For phytoplasma detection in pear and apple trees, both ribosomal and non-ribosomal primers gave good results, the best obtained with PCR using purified DNA and nested PCR with fP1/rP7 primers in the first step and fO1/rO1 primers in the second step. For *Ca. P. pyri* detection, good results were obtained using a single PCR amplification with some of the non-ribosomal primers previously developed (Garcia-Chapa et al. 2004), which amplified small fragments. Nevertheless, more comparative analyses is necessary before these primers can be recommended instead of those normally used. Some of the primers designed for *Ca. P. pyri* sequences also amplified the *Ca. P. prunorum* phytoplasma (European stone fruit yellows, ESFY) (Table 3). Furthermore, the *Imp* primers developed by Danet et al. (2006 and 2008), amplifying a fragment of a gene encoding a membrane surface protein, also gave good results, both in simple and in nested-PCR (Table 3).

Tab. 3 Phytoplasma detection by PCR with different primers

Sample	Phytoplasma detected	Primers						Imp PD (A)	Imp PD(B)
		13 f/r 197 pb	7f/r 165 pb	14f/r 231 pb	10 f/r 127 pb	15 f/r 264 pb			
Pear 1	PD	+	+	+	+	-	-	+	
Pear 2	PD	+	+	+	+	-	+	+	
Pear 3	PD	+	+	+	+	-	+	+	
C. roseus	PD	+	-	-	+	-	+	-	
C. roseus	PD	+	-	-	-	-	+	+	
Pear 4	PD	+	-	-	+	-	-	-	
Pear 5	PD	+	-	-	-	-	-	-	
Pear 6	PD	+	-	-	-	-	-	-	
Pear 7	PD	+	-	-	+	-	+	+	
<i>Prunus cerasifera</i> 1	ESFY	+	+	+	+	-	+	-	
<i>Prunus cerasifera</i> 2	ESFY	-	-	+	-	-	-	-	

As in the case of virus detection, the presence of phytoplasmas in samples of pear and apple from different plant nurseries was analysed. In contrast to virus detection, the detection of phytoplasmas in crude extracts is very erratic and, on the basis of our results, it cannot be recommended at present. A phytoplasma belonging to the 16Sr-XII group was detected in apple plants with symptoms of chat fruit (Table 2).

Discussion

RT-PCR for the specific detection of ASPV, ASGV, ACLSV, ApMV and PBCV and PCR for specific detection of the phytoplasmas *Ca. P. Pyri* and *Ca. P. mali* can be used throughout the year in woody hosts. RT-PCR appears to be a sensitive method and avoids the need for field indexing of the virus and viroid. It is important to point out that, in samples from many of the pear and apple trees that had supposedly been submitted to a process of sanitary control, virus and phytoplasmas were detected by RT-PCR and PCR. The process may not have been carried out correctly or it is possible that errors may occur when using woody indicators, due to the irregular distribution of phytoplasmas. Results obtained in the detection of *Ca. P. pyri* (PD) and *Ca. P. mali* (AP) show that PCR can be used in certification programs for early detection of these pathogens and that this method is more sensitive than the field indexing technique. There is increased sensitivity when tests are carried out between October and December, which is when phytoplasma accumulation in the phloem is at its peak (Garcia et al. 2003).

The implication of some of the viruses and the phytoplasmas tested in other pear and apple diseases has already been discussed. If this implication could be fully ascertained, the use of RT-PCR to detect viruses and PCR to detect phytoplasmas could significantly reduce the time required for certification.

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