

RT-PCR-ELISA for Detection of *Apple stem grooving virus* in Apple Trees

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ABSTRACT

A method to detect *Apple stem grooving virus* (ASGV) based on reverse transcription polymerase chain reaction (RT-PCR) was developed using primers ASGV4F-ASGV4R targeting the viral replicase gene, followed by a sandwich hybridisation, in microtiter plates, for colorimetric detection of the PCR products. The RT-PCR was performed with the Titan™ RT-PCR system, using AMV and diluted crude extracts of apple (*Malus domestica*) leaf or bark for the first strand synthesis and a mixture of Taq and PWO DNA polymerase for the PCR step. The RT-PCR products is hybridised with both a biotin-labelled capture probe linked to a streptavidin-coated microtiter plate and a digoxigenin (DIG)-labelled detection probe. The complex was detected with an anti-DIG conjugate labelled with alkaline phosphatase. When purified ASGV was added to

extracts of plant tissue, as little as 400 fg of the virus was detected with this method. The assay with ASGV4F-ASGV4R primers specifically detected the virus in ASGV-infected apple trees from different origins, whereas no signal was observed with amplification products obtained with primers targeting the coat protein region of the ASGV genome or with primers specific for *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV). The technique combines the power of PCR to increase the number of copies of the targeted gene, the specificity of DNA hybridization, and the ease of colorimetric detection and sample handling in microplates.

Additional keywords: colorimetric detection of ASGV, *Prunus armeniaca*.

RESUMO

RT-PCR-ELISA para a detecção do *Apple stem grooving virus* em macieira

Um método foi desenvolvido para a detecção do *Apple stem grooving virus* (ASGV), baseado na transcrição reversa do RNA viral e na polimerização em cadeia do DNA (RT-PCR), usando os oligonucleotídeos iniciadores ASGV4F-ASGV4R, localizados na região do gene da RNA polimerase, sendo os produtos de amplificação detectados por hibridização "sandwich", em placas de poliestireno, através de reação colorimétrica. A RT-PCR foi realizada com a "Titan™ RT-PCR System", complexo enzimático formado pela AMV, responsável pela transcrição do RNA viral em cDNA e as DNA polimerases PWO e Taq, usadas para a etapa de PCR, utilizando-se para isso extrato bruto de folhas e casca do caule de macieira (*Malus domestica*). A detecção dos produtos de amplificação RT-PCR foi realizada hibridizando esses produtos com uma sonda de captura marcada com biotina, ligada à placa de ELISA previamente

sensibilizada com estreptavidina, e uma sonda de revelação marcada com digoxigenina. O complexo foi detectado utilizando-se um conjugado fosfatase alcalina marcada com anti-DIG. Foi avaliada a sensibilidade desse método utilizando-se uma série de diluições de vírus purificado, tendo sido possível detectar até 400 fg de vírus. A especificidade do método foi demonstrada usando-se os oligonucleotídeos iniciadores ASGV4F-ASGV4R e macieiras de diferentes origens infetadas com ASGV. Não foram reveladas colorimetricamente as amplificações observadas em gel de agarose, quando essas mesmas plantas foram testadas utilizando-se oligonucleotídeos iniciadores para o ASGV escolhidos na região que codifica para capa protéica e amplificações provenientes de material infetado com o *Apple chlorotic leaf spot virus* (ACLSV) e *Apple stem pitting virus* (ASPV). Esta técnica combina o desempenho da PCR, a especificidade da hibridização molecular e as facilidades do teste de ELISA.

INTRODUCTION

Apple stem grooving virus (ASGV) is a member of the genus *Capillovirus*. This virus infects apple (*Malus domestica* Borkh) trees, usually without causing obvious symptoms, and is distributed worldwide. It also infects apricot (*Prunus armeniaca* L.), cherry (*P. serotina* Ehrh.) and pear (*Pyrus communis* L.) trees (Lister, 1970; Sawamura *et al.*, 1988;

Takahashi *et al.*, 1990; Kinard *et al.*, 1996). Control of the disease is by planting virus-free stock. The standard detection technique for ASGV relies on graft bioassays, which are time-consuming, although the use of the newly selected *Malus* clones (Howell *et al.*, 1996) allows detection within two months. Serological (ELISA) detection is reliable during a short period in the year, but it does not work well with dormant woody tissues (Kinard *et al.*, 1996). The use of reverse transcription

polymerase chain reaction (RT-PCR) for the detection of ASGV has been described (Kinard *et al.*, 1996; Mackenzie *et al.*, 1997; Kummert *et al.*, 1998) using an amplification step followed by electrophoresis and the detection of the amplicons by ethidium bromide staining. The amplified products cannot be reliably detected using ethidium bromide when the virus titer is low. Moreover, the specificity of that identification protocol relies on the assessment of the size of amplified products, and false positive results are possible.

Recently, sensitive assays for the detection of viral infections have been based on colorimetric (Lassner, 1995; Poljak & Seme, 1995; Adams *et al.*, 1996; Lage *et al.*, 1996) or chemiluminescent (Zammatteo *et al.*, 1995; Schoen *et al.*, 1996) detection of hybridized PCR products. These approaches combine the specificity of DNA probe hybridization, the sensitivity of colorimetric or luminescent detection, and the ease of sample handling in microplates.

We earlier reported a RT-PCR method for diagnosis of ASGV using primers specific for the putative RNA polymerase region of the virus (Marinho *et al.*, 1998a,b). The detection of the amplified products was based on UV fluorescence on ethidium bromide staining of agarose gels (Kummert *et al.*, 1998) or on a colorimetric technique based on direct labelling of PCR products with digoxigenin (Daniels *et al.*, 1998). Although sensitive, the latter system can easily give rise to false positives since any amplified product is labelled.

This paper describes a fast, sensitive and specific colorimetric assay for detecting amplified ASGV products. It includes hybridisation of the amplicons, first with a DIG-labelled detection probe and then with a biotin-labelled capture probe that is bound to a streptavidin-coated microtiter plate.

MATERIALS AND METHODS

Virus isolates and host plants

The ASGV isolates 10311 and 10771 were obtained from the Gorseme Experimental Station, Belgium, transferred by mechanical inoculation and multiplied in *Nicotiana glutinosa* L. and *Chenopodium quinoa* Willd. Isolates 10291 and 10392 were maintained in apple trees in the field. Isolates of *Apple chlorotic leafspot virus* (ACLSV) (91300) and *Apple stem pitting virus* (ASPV) (91325), maintained in *C. quinoa* and in apple trees in the field, respectively, were occasionally used as control.

Template preparation for RT-PCR

Clarified plant extracts: The extraction protocol of Marinho *et al.* (1998a) was used. Fifty mg of apple leaves or bark were ground in a 1.5 ml polypropylene tube containing 1 ml of TE buffer (50 mM Tris, pH 8.0, 10 mM EDTA). The homogenate was vortexed, centrifuged at 10,000 g for 10 min at 4 °C, and 1 µl of supernatant was transferred to the RT-PCR mixture.

Partially purified virions: The purification method of Sequeira & Lister (1969) was used with minor modifications. Frozen leaves of *C. quinoa* infected with ASGV (isolate 10771) were ground in three volumes of 0.01 M Tris buffer, pH 8.0, containing 0.2%

of 3, 3' diaminodipropylamine and 20 mM iodoacetic acid. Bentonite (40 mg/ml) was added at 10 mg/g of leaf tissue after filtration of the homogenate. The mixture was shaken for 10 min, stored at 4 °C for 10 min, and centrifuged at 1,500 g for 10 min. The supernatant was filtered through cheesecloth and then submitted to centrifugation on a 20% sucrose cushion at 80,000 g for 2 h. Pellets were resuspended in 1 ml 0.05 M Tris, pH 8.0, containing 0.005 M MgCl₂. After a second cycle of clarification (3,000 g for 10 min) and centrifugation on a 20% sucrose cushion (100,000 g for 90 min), the final pellets were resuspended in the same buffer and the suspension clarified as before. The virions concentration was estimated by light absorbance at 260 nm using an extinction coefficient of 2.5 cm²mg⁻¹. An aliquot of 1.0 µl of purified virus or purified virus diluted in extracts of healthy *Malus* leaves was directly transferred to the RT-PCR mixture.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Specific primers were designed from the nucleotide sequence of ASGV (P-209) published by Yoshikawa *et al.* (1992) and from partial sequences of four European ASGV isolates. The upstream primer ASGV4F (5'-GTT CAC TGA GGC AAA AGC TGT C-3') site is located between nucleotides 3918 and 3940, and the downstream primer ASGV4R (5'-CTT CCG TAC CTC TTC CAC AGG AC-3') site is complementary to nucleotides 4491 to 4469, in the putative viral RNA polymerase-encoding region (Marinho *et al.*, 1998a) and about 300 nucleotides downstream from the variable region described by Magome *et al.* (1997). The primers give an amplification product of 574 bp.

The primers designed by Kinard *et al.* (1996) and those reported by MacKenzie *et al.* (1997) target the putative coat protein gene of ASGV, giving rise to amplicons of 420 and 524 bp, respectively.

The TitanTM one tube RT-PCR System (Boehringer Mannheim, Germany) was used according to manufacturer's protocols. Thermal cycling was performed in a TRIO-thermoblock cyler (Biometra, Göttingen, Germany) for 30 min at 50 °C for the reverse transcription step, and with the following parameters for PCR: initial template denaturation at 94 °C for 2 min; 35 cycles, of 94 °C for 30 s (denaturation), 62 °C for 1 min (annealing), and 72 °C for 2 min (DNA synthesis); and final elongation of 72 °C for 15 min.

Detection of the PCR products

Electrophoresis: Amplified products (10 µl) were separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA). The PCR products were stained with 0.1% ethidium bromide and photographed with Polaroid films.

Southern blotting: For the preparation of the digoxigenin-labelled probe, the amplified products obtained from ASGV isolate 10311 using primers ASGV1F-ASGV1R (Kummert *et al.*, 1998) were recovered using a QIAEX gel extraction kit (Qiagen GmbH, Germany) and cloned into the pCRTM II vector using the TA Cloning Kit (Invitrogen, Netherlands). The recombinant

plasmids were used for PCR synthesis of the DNA probe and direct incorporation of digoxigenin through the PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Germany). The probe was 560 bp long, corresponding to nucleotides 3925 to 4485 of ASGV-RNA.

For the detection, PCR products were electrophoresed in 1% agarose and transferred to a positively-charged nylon membrane (Boehringer Mannheim, Germany). The digoxigenin-labeled probe (100 ng/ml) was hybridized to the membrane and detected with the PCR DIG Luminescent Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Colorimetric detection in microtiter plates: The capture and detection probes were designed according to the sequences of four ASGV isolates. The absence of an unsuitable secondary structure in the sequence of the probe was confirmed by computer analysis (Oligo 4.0 Soft Primers, Biosciences, Inc., Plymouth, USA). The capture probe (BIO-probe), referred to as ASGV-5C1 (Biotine-TTC ACT GAG GCA AAA GCT GGT CAA ACC TT), was prepared by labelling an extended ASGV4F primer sequence with biotin at its 5' terminus. The detection probe (DIG-probe), referred to as ASGV-SR2 (AGA AGA AAA GGG GAA AAA GCA GCA AGA C-DIG), was prepared by labelling a selected nucleotide sequence, homologous to an internal fragment in the amplicon, located between nucleotides 4419 and 4447, with digoxigenin at its 3' end. The colorimetric detection of the PCR products is schematically outlined in Figure 1. An aliquot of 10 µl of PCR product were added to 40 µl of the denaturation solution (Boehringer Mannheim, Germany) to denature the double-stranded DNA and the mixture incubated at room temperature for 10 min. The denatured PCR product (50 µl) was then added to 450 µl of hybridization solution containing 100 ng/ml of the DIG-labelled detection probe (ASGV-SR2), and the hybridization was carried out at 52 °C for 3 h with shaking (step A). In step B, the Bio-probe (ASGV-5C1) at 100 ng/ml in PBS-Tween was added to each well of a microtiter plate (MTPs) (200 µl per well) that was precoated with streptavidin. The plates were then incubated for 1 h at 37 °C. The MTPs were then washed four times with 250 µl of PBS-Tween, and 200 µl of the hybridized PCR products was added to each of two wells. The hybridization was carried out at 37 °C for 1.5 h with shaking (step C). The MTPs were then washed with PBS-Tween, and 200 µl of conjugate buffer (PBS-Tween plus 0.5% blocking reagent) containing 1:3,000 dilution of Anti-Digoxigenin-AP Fab fragments (Boehringer Mannheim, Germany) was added to each well. The MTPs were incubated at 37 °C for 30 min with shaking, washed as before, and 200 µl of substrate buffer (10 mM diethanolamine-HCl, pH 9.5) containing 0.7 mg/ml of enzyme substrate (p-nitrophenyl phosphate-PNPP) was added (step D). The optical density (OD) was measured at 405 nm in a Multiskan Plus colorimeter after incubation of the MTPs for 1 to 16 h at room temperature. The PCR reaction mixture containing water in place of template (blank) was used as a negative control in each assay. The cut-off value of scoring a positive sample was two standard

deviations above the means for the blank PCR mixtures (sample without DNA). Reagents for denaturation and hybridization steps and MTPs were purchased from Boehringer Mannheim, Germany.

RESULTS

Sensitivity of the colorimetric hybridization assay for detection of ASGV amplification products

The Titan™ one tube RT-PCR system and the primers ASGV4F-ASGV4R were used to amplify ASGV viral sequences directly from diluted plant sap of either ASGV-infected herbaceous hosts (isolates 10311 and 10771) and from leaves or bark of apple trees (isolates 10291 and 10392). The amplification products were then visually detected in ethidium bromide stained gels, by Southern blotting or in the colorimetric assay.

When successive dilutions of 1:5 through 1:500,000 of the RT-PCR amplification products were tested with the different systems, the virus was detected in dilutions of 1:50,000 (Figure 2 B-C) using the colorimetric assay and Southern blotting, and in dilutions of about 1:500 in the ethidium bromide stained gels (Figure 2-A).

The sensitivity of the assay to detect virus in crude extracts was also estimated using a serial, 20-fold dilution of

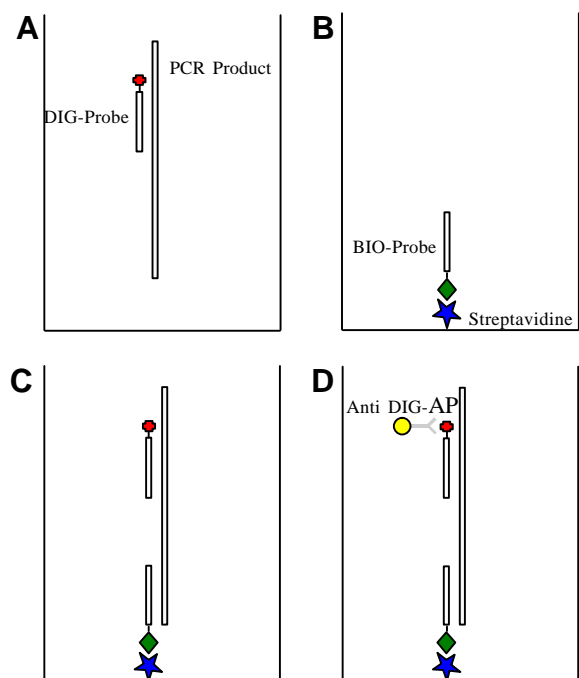


FIG. 1 - Steps of colorimetric RT-PCR assay for *Apple stem grooving virus* (ASGV) detection. **A.** Denaturation of the amplicon and hybridization to the digoxigenin (DIG)-labelled detection probe; **B.** Biotin-labelled capture probe (BIO-probe) bound on plate coated with streptavidin; **C.** Hybridization of the amplicon/digoxigenin labelled probe complex with the biotin labelled capture probe; **D.** DIG detection by anti-DIG alkaline phosphatase (AP) conjugate, reaction of AP with its substrate (PNPP) and colour development.

purified virus (starting with a virus concentration of 0.8 µg/µl) in crude juice of healthy *Malus* sp. leaves (diluted 1: 200 in TE buffer, pH 8.0). The detection limit of the amplified product in agarose gels was about 40 pg of the purified virus (Figure 3-A), whereas the colorimetric hybridization assay (Figure 3-B) and Southern blotting (results not shown) detected as little as 400 fg of purified virus.

Specificity of the colorimetric hybridization assay for detection of ASGV

In order to determine the specificity of the colorimetric assay for the detection of the ASGV amplified product, the test was performed on crude extracts from leaves or bark tissues of plants infected with different isolates of ASGV, on healthy plants and on RNA preparations from plants infected with ASGV (isolate 10392), ACLSV or ASPV. The plant extracts or RNA preparations were submitted to the Titan one tube RT-PCR amplification in the presence of the specific primers ASGV4F-ASGV4R or primers for detection of ACLSV (isolate 91300) and ASPV (isolate 91325). The RT-PCR products obtained from extracts of ASGV-infected plants amplified with the primers targeting the coat protein gene (Kummert *et al.*, 1998; Sawamura *et al.*, 1988) were used as checks for the colorimetric detection assay.

No significant absorbance was measured (OD_{405} values ≤ 0.1) with the processing of extracts of healthy plants (sample 10) or plants infected with ACLSV or ASPV (samples 8 and 9). Also, no significant absorbance was seen with the ASGV amplicons using the primers targeting the coat protein gene (samples 3, 4, 5, 6), even though these PCR products showed the correct band size when analyzed by gel electrophoresis (Figure 4). In contrast, the amplification products obtained with the specific primers ASGV4F-ASGV4R resulted in OD_{405} values of ≥ 1.0 for all of the tested ASGV-infected samples.

DISCUSSION

Validation of diagnostic tests for quality control of plant material is a classic statistical problem of hypothesis testing. Two types of mistakes may occur: (i) a false positive occurs when a healthy plant is claimed to be infected, and (ii) a false negative occurs when the test concludes that the pathogen is absent in an infected plant. Any quality control program must assure the grower that the probability of either mistake will be confined to a small and predetermined range. This objective of "quality assurance" can be achieved by a proper sample size and by an assay technique exhibiting adequate sensitivity and specificity. Moreover, the diagnostic test must also be of practical use for routine application on a large number of samples.

The aim of PCR is to produce detectable amounts of the target DNA sequence. Therefore, the sensitivity and the specificity of the whole procedure rest on the detection steps and on the amplification itself, which is dependent on the template, primers, and the parameters of thermocycling.

We showed that the specific primers ASGV4F and

ASGV4R (Marinho *et al.*, 1998a) designed to amplify a 574 bp fragment located in the putative RNA polymerase gene of ASGV can be used in a one step RT-PCR procedure with crude extracts of apple trees. However, the detection of the ASGV amplification products was based on ethidium bromide staining in agarose gels (Kummert *et al.*, 1998). This diagnosis is based on the size of the amplicon and does not totally discount the possibility of false positives due to unspecific amplification of non-target sequences. Moreover, this detection method is much less sensitive than Southern blotting and time-consuming for processing large numbers of the samples.

Novel strategies for detection of amplified products in routine tests have been published recently. Schoen *et al.* (1996) have shown that the fluorescence of a probe (Taq Man™ probe) that hybridizes specifically to the target PCR product results in a sensitive and specific detection of amplicons. However that technique requires a luminescence spectrometer. On the

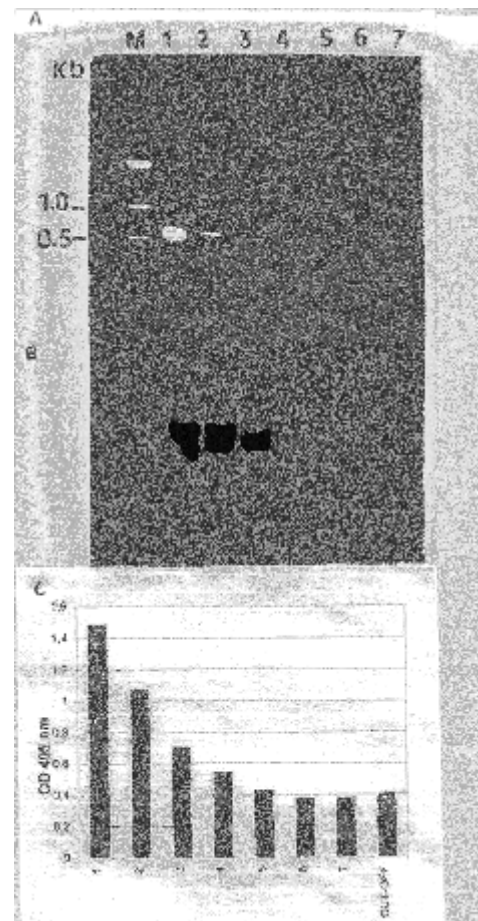


FIG. 2 - Detection of successive dilutions (from 1:5 to 1:500,000) of RT-PCR amplified products obtained from *Chenopodium quinoa* leaves infected with *Apple stem grooving virus* (ASGV) isolate 10311 by agarose gel electrophoresis (A), Southern blotting (B), and colorimetric hybridization assay (C). Lanes 1 to 6 correspond to the dilutions of RT-PCR products; lane 7, blank (no template added); Lane M, size of markers in Kb. The cut-off value of the colorimetric assay was defined as two standard deviations above the mean for the blank PCR mixtures.

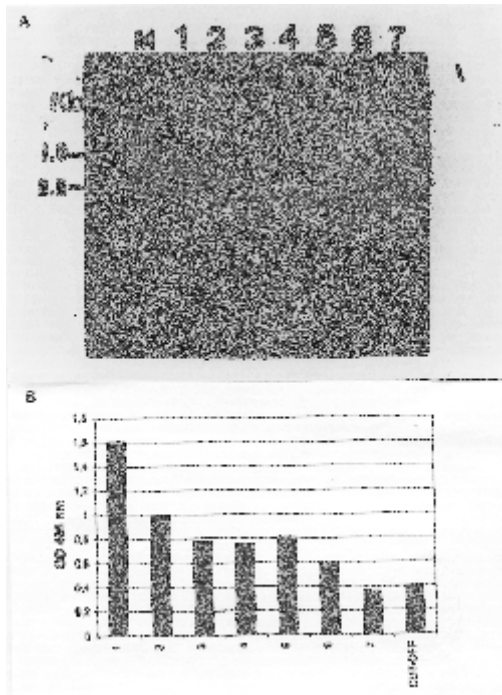


FIG. 3 - Detection of the amplified products of a serial, 20-fold dilutions of the purified *Apple stem grooving virus* (ASGV) by agarose gel electrophoresis (A) and colorimetric hybridization assay (B). Lane 1 to 6, serial, 20-fold dilutions of purified ASGV isolate 10771 (starting virus concentration $0.8 \mu\text{g}/\mu\text{l}$) prepared in crude extracts of healthy leaves of *Malus* sp. Lane 7, blank (no template added). Lane M, size of markers in Kb. The cut-off value was defined as two standard deviations above the mean for the blank PCR mixtures.

other hand, colorimetric detection of amplification products using PCR-ELISA only requires equipment widely used for conventional enzyme immunoassays in routine clinical microbiology laboratories and, therefore, represents an attractive alternative.

In the simplest PCR-ELISA protocol, reported by Adams *et al.* (1996) for the detection and typing of *Human papillomavirus* (HPV), two primers are labelled with biotin and digoxigenin, respectively. Although sensitive for detection of HPV, this method can easily result in false positives since any amplified product will be labelled. The capture of the amplicons by a specific probe bound to the micro titer plate introduces an additional level of specificity for colorimetric detection.

Two possible means to detect captured amplification products have been described. In the first, the amplicons can be directly labelled during PCR by incorporation of biotin dUTP or digoxigenin dUTP, or by using 5'-labelled primer (Poljak & Seme, 1995; Adams *et al.*, 1996; Daniels *et al.*, 1998). In the second, an internally-labelled detection probe hybridises to a specific region of the target amplicon (Lage *et al.*, 1996). The first alternative is not as specific, as non-target amplicons will also be labelled. Such a false positive was observed with the primer pair ASGV1F-ASGV1R when a DIG dUTP-labelled amplification product and the forward primer labelled with biotin were used for detection of ASGV. In this case, a positive signal

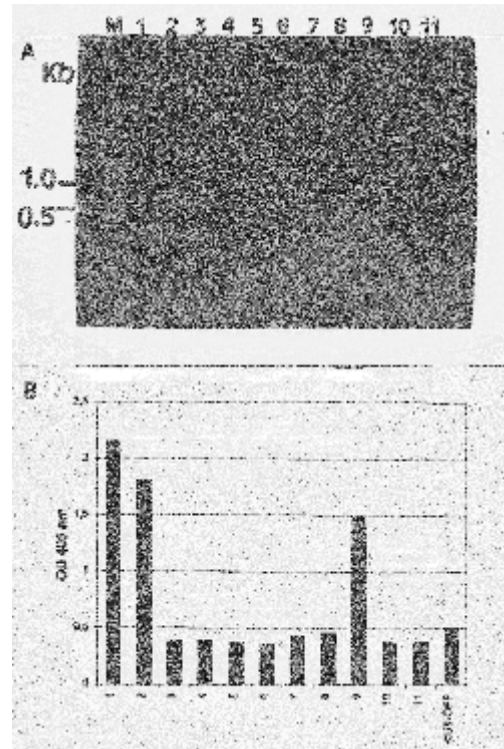


FIG. 4 - Specificity of the colorimetric hybridization assay for the detection of *Apple stem grooving virus* (ASGV)-amplified products. Lanes 1 and 3, crude extracts from *Nicotiana glutinosa* leaves infected with isolate 10311; lanes 2 and 4, crude extracts from bark tissues of *Malus* sp infected with isolate 10392; lane 5, total RNA preparation from *Chenopodium quinoa* leaves infected with isolate 10771; lanes 6 and 9, total RNA preparation from *Malus* sp leaves infected with isolate 10392; lanes 7 and 8, total RNA preparations from *Malus* leaves infected with ACLSV and ASPV isolates, respectively; lane 10, crude extracts from *C. quinoa* healthy leaves; lane 11, blank (no template added). For samples 1, 2, 9, 10 and 11, ASGV4F-ASGV4R primers were used for RT-PCR. For samples 3, 4, 5 and 6, the primers used amplified the coat protein gene. For samples 7 and 8, ACLSV and ASPV specific primers were used, respectively. Lane M, size of markers in Kb. The cut-off value was defined as two standard deviations above the mean for the blank PCR mixtures.

was observed for a sample of ACLSV-infected plant material (Daniels *et al.*, 1998).

In order to improve the specificity of the detection step, we adopted the second strategy using a DIG-labelled detection probe corresponding to a region of the amplicon that is specific to the ASGV genome. Although the ASGV4F-ASGV4R primers and amplification conditions used in the RT-PCR offer a high degree of specificity, our colorimetric hybridization protocol provides the additional assurance needed for a routine diagnostic test. The specificity of this system was demonstrated by using amplification products obtained with primers targeting another region of the ASGV genome or with specific primers for ACLSV and ASPV, which did not give colorimetric signals, although amplification products were observed in gels using ethidium bromide staining.

Three systems to detect the RT-PCR products were

compared for sensitivity. The dilution threshold limits found for colorimetric hybridization and gel electrophoresis followed by Southern blotting were 100 times lower than that found for ethidium bromide staining of agarose gels. Similar results were reported by Lage *et al.* (1996) for the colorimetric hybridization assay in the detection of amplified *Helicobacter pylori* DNA.

In conclusion, the colorimetric hybridization assay described in this paper is a very sensitive assay for the detection of ASGV in apple trees. The assay combines the specificity of DNA hybridization with the ease and speed of colorimetric detection. Partial automation is possible, and nearly 100 samples can be processed simultaneously in one micro titer plate. Thus the protocol is well suited to the routine testing of planting material for a quality control program.

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