Scientia Horticulturae 125 (2010) 68–72

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Short communication

Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR

J. Jarošová, J.K. Kundu*

Department of Virology, Crop Research Institute, Drnovská 507, Prague 6, 16106, Czech Republic

ARTICLE INFO

Article history:
Received 23 March 2009
Received in revised form 11 February 2010
Accepted 11 February 2010

Keywords:
PPV
PDV
PNRSV
Detection
Multiplex RT-PCR

ABSTRACT

A protocol of multiplex RT-PCR in a one-tube system for the detection of the most common stone fruit trees viruses [e.g., plum pox virus (PPV), prune dwarf virus (PDV), and *Prunus* necrotic ringspot virus (PNRSV)], including the internal control of NADH dehydrogenase subunit 5 (*nad*5) gene are described here. The method specificity was tested on more than 80 different samples with various isolates and strains of the viruses. It showed that the targeted viruses produced the expected amplicons, whereas all other related viruses produced only the *nad*5 internal control amplicon. The method sensitivity was evaluated by comparing it with Simplex RT-PCR with the same primers; no significant differences in detection limits were recorded. Furthermore, the competitiveness of the primers in the assay was tested by serial RNA dilutions of samples with mixed and single infections. The least competitive was the internal control *nad*5 gene primer pair; therefore, there is a reduced risk of false negatives as all the other primers tend to be more efficient in the given primer cocktail than in the primers for internal control.

1. Introduction

Prunus spp. are affected by many viruses, and most frequently occur in the genera *Ilarvirus*, *Potyvirus,* and *Trichovirus*. The following viruses are the most important for stone fruit trees: plum pox virus (PPV), *Prunus* necrotic ringspot virus (PNRSV), prune dwarf virus (PDV), and apple chlorotic leaf spot virus (ACLSV) (Németh, 1986). Currently, these pathogens may be detected by biological indexing (Di Terlizi, 2000; Bertozzi et al., 2002; Gentit, 2006), immunological methods (Clark and Adams, 1977; Cambra et al., 1994; Spiegel et al., 1998; Mekuria et al., 2003), molecular hybridization (Palkovics et al., 1994; Saade et al., 2000), and reverse transcription polymerase chain reaction (RT-PCR) (Wetzel et al., 1991, 1992; Scott et al., 1992; Olmos et al., 1997; Rosner et al., 1998; Spiegel et al., 1999). In all these assays, usually one pathogen is detected per assay (Hadidi et al., 2004). Procedures that allow simultaneous detection and/or identification of different viruses are desirable for routine diagnosis because they require less time, labor, and cost than single RT-PCR. Several multiplex systems are available for routine detection of multiple viruses and viroids (Grieco and Gallitelli, 1999; Bertolini et al., 2001; Ito et al., 2002; Menzel et al., 2002; Ragozzino et al., 2004; Roy et al., 2005) in fruit trees. However, the efficiency of multiplex assays is variable with respect to combination and number of tested viruses or viroids (Sánchez-Navarro et al., 2005).

We describe here a protocol of multiplex RT-PCR in a one-tube system for the detection of the most common viruses (e.g., PNRSV, PDV, and PPV) in stone fruit trees. The detection specificity of the assay was also performed by sequence analysis of the PCR fragments.

2. Materials and methods

2.1. Virus source

Leaf samples (three–five leaves per sample) were collected randomly from plum (*Prunus domestica* L.), cherry (*P. avium* (L.) L.), sour cherry (*P. cerasus* L.), Nanking cherry (*P. tomentosa* Thunb.), peach (*P. persica* (L.) Batsch), and blackthorn (*P. spinosa* L.) trees known or suspected to be PPV-, PDV-, and PNRSV-positive grown in research and commercial orchards and in the wild in the Czech Republic. Two PDV isolates from Romania, BN51125-2/8 and BN51125-2/4 from *P. domestica,* were used for comparison with the Czech isolates.

2.2. Oligonucleotides

Current PDV and PNRSV coat protein nucleotide sequences from the GenBank database were aligned using the program Clustal W version 1.7 (Thompson et al., 1997) and used to identify oligonucleotide primer sequences that may be used for universal detection of PDV and PNRSV. Additional primers used in this study include the PPV-RR and F3 primers described by Varga and James (2005, 2006) and *nad5* mRNA-specific primers Nad5-F and Nad5-R (mRNA coding mitochondrial gene of higher plants encoding subunit 5 of the NADH ubiquinone oxidoreductase complex) described by

* Corresponding author. Tel.: +420 233022410; fax: +420 233310636.
E-mail address: jiban@vurv.cz (J.K. Kundu).

0304-4238/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2010.02.011

Table 1
Primer used in this study.

Primer name	Sequence (5′-3′)	Nucleotide position	Fragment size	Target	Reference
PPV-RR	CTCTTCTTGTGTTCCGACGTTTC	9475-9497[a]	345 bp	PPV	Varga and James (2005)
F3	GGAATGTGGGTGATGATGG	9153-9171[b]			Varga and James (2006)
PDVdpR	CCTTTAATGAGTCCGT	1572-1557[c]	220 bp	PDV	This study
PDVdpuF	CCGAGTGGATGCTTCACG	1353-1370[c]			This study
PNcpR	CTTTCCATTCGGAGAAATTCG	1821-1801[d]	425 bp	PNRSV	This study
PNcpinF	GAGTATTGACTTCACGACCAC	1396-1416[d]			This study
Nad5-R	CTCCAGTCACCAACATTGGCATAA	968-987 and 1836-1838[e]	181 bp	Plant	Menzel et al. (2002)
Nad5-F	GATGCTTCTTGGGGCTTCTTGTT	1973-1995[e]			

[a] Genome position on PPV accessions D (X16415) and M (M92280).
[b] PPV Fantasia, accession AY912056.1.
[c] PDV ch-137 accession L28145.
[d] PNRSV PV32 accession Y07568.
[e] nad5 accession D37958.

Menzel et al. (2002) as an internal control. Primers are described in Table 1.

2.3. RNA preparation

RNA was isolated from the leaves of all the above mentioned plants by using a commercially available extraction kit, RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with a modification according to Mekuria et al. (2003). Two hundred milligrams of either fresh or frozen leaf tissue was ground into a fine powder in liquid nitrogen, mixed with 2.0 ml of extraction buffer containing 4.4% (w/v) PVP-40 (Sigma, MO, USA) and 1% (w/v) sodium metabisulphite, and briefly vortexed. Five hundred microliters of the homogenate was mixed with 60 μl of 20% (w/v) sarkosyl (*N*-lauroyl-sarcosine, Sigma) and incubated at 70 °C with agitation for 10 min. The contents were then transferred to a QIA shredder mini column and centrifuged at 14,000 rpm for 5 min. The column flowthrough (350 μl) was mixed with 315 μl of 95% ethanol, and the remainder of the protocol was carried out according to the manufacturer's instructions. RNA was stored at −20 °C (or at −80 °C for long-term storage). The quality and quantity of isolated RNA were determined by spectrophotometry at 260, 230, and 280 nm.

2.4. One-step RT-PCR for multiplex virus detection

One-step-RT-PCR was performed with the One-Step-RT-PCR kit (Qiagen) as described by Kundu (2003). The One-Step-RT-PCR mixture containing 5 μl of the 5× Qiagen One-Step-RT-PCR buffer, 10 nM of each dNTP, 1 μl of the Qiagen One-Step-RT-PCR enzyme mixture, 1 μl of Q solution, and 6 pM of reverse and forward primers (Table 1) were prepared in 2 μl of RNA and the mixture was adjusted to 25 μl with RNase-free water. The reaction was carried out in a thermocycler (MJ Research) as follows: a RT step at 50 °C for 30 min and an initial PCR activation step at 95 °C for 15 min, then 33 cycles of 94 °C for 30 s (denaturation), 51 °C for 45 s (annealing), and 72 °C for 80 s (extension). After the last cycle, a final extension step at 72 °C for 10 min was added.

The PCR products were analyzed in 2% agarose gel electrophoresis; staining was done by SYBR Green (Invitrogen, CA, USA). PCR fragments were also analyzed by sequencing of amplicons using reverse and forward primers of each virus of several isolates. Sequences were analyzed by using software Clustal W version 1.7 (Thompson et al., 1997), Sequencher 4.8 (Gene Codes Corporation, MI, USA) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

3.1. Virus detection by simple and multiplex RT-PCR

A multiplex RT-PCR was developed for the identification of PPV, PDV, and PNRSV in stone fruit tree tissues. Included in the method were the PPV-specific primers developed by Varga and James (2005, 2006), which resulted in a 345 bp amplicon; universal primers used to amplify a 181 -p fragment of the *nad*5 mRNA, serving as an internal positive control for the RT-PCR (Menzel et al., 2002); and novel primers designed to amplify a 425-bp fragment of the coat protein gene characteristic of PNRSV and a 220-bp fragment of the coat protein gene of PDV (Fig. 1A). The primers in this reaction were chosen among many candidates because of their specificity and ability to produce only the targeted amplicons (Fig. 1A). The thermal gradients of annealing temperature were run for some samples (the T_a between 48 and 63 °C) and the efficiency of the assay was confirmed to be optimal between 50 and 55 °C. However, the reaction is so robust that the targeted sequences amplified under all tested temperatures—nevertheless, the products were weaker under annealing temperatures higher than 55 °C.

The method specificity was tested on more than 80 different samples with various isolates and strains of the viruses and showed that the targeted viruses resulted in the expected amplicons (Fig. 1B), while all other related viruses produced only the *nad*5 internal control amplicon (data not shown). In the study, three PPV strains common in Europe were included—PPV-D (Dideron), PPV-M (Marcus), and PPV-Rec (Recombinant) (Candresse et al., 1998; Glasa et al., 2004). A simple one-step-RT-PCR was carried out for each of the tested samples and for each of the tested viruses to compare the specificity of the reaction. The primers used in the multiplex RT-PCR were used in the simple RT-PCR. There were no differences recorded for specificity and robustness of these two methods. There were an equal number of positive samples detected by both methods (data not shown).

The specificity of the detection assay was further proved by sequence analysis of PCR fragments of some isolates of detected viruses. The Genbank accession numbers of the sequences of the virus isolate are as follows: PPV (isolates cz1cp = FJ842715, cz2cp = FJ842716, cz3cp = FJ842717, cz4cp = FJ842718, cz6cp = FJ842719, z8cp = FJ842720); PDV (isolates cz1cp–cz9cp = FJ842698–FJ842706); and PNRSV (isolates cz1cp–cz8cp = FJ842707–FJ842714).

3.2. Multiplex RT-PCR with/or without internal control

Multiplex RT-PCR including and excluding internal control was carried out and compared. Serial dilution tests compared the sensitivity of multiplex RT-PCR including and excluding the internal control. The exclusion of the internal control from the reaction did not have any effect on the sensitivity of PDV detection. On the other hand, the sensitivity of PPV and PNRSV detection was even increased by the addition of the internal control primers into the reaction mix—in this case, on average, the differences in RNA dilutions, in which the viruses were detected, varied from 10 to 100 times (Fig. 1C and D). The multiplex RT-PCR method can therefore

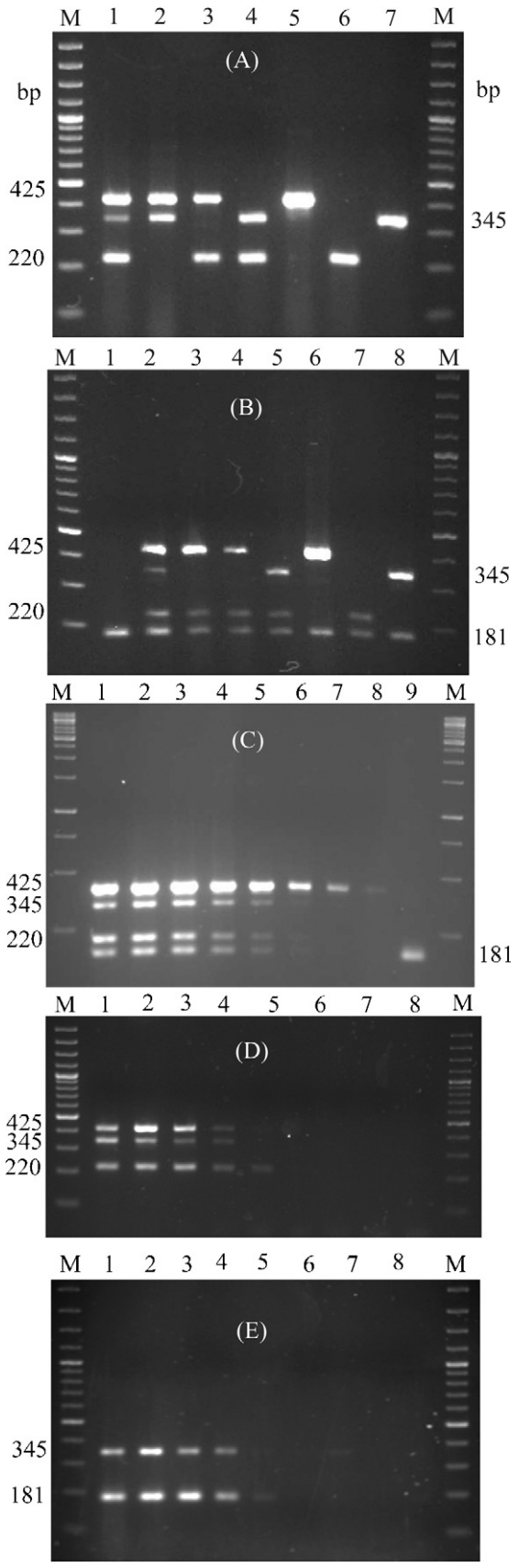

Fig. 1. (A–E) One-step-multiplex RT-PCR for the detection of PDV, PPV, and PNRSV. (A) One-step multiplex RT-PCR without the *nad*5 gene as an internal control. Lane 1: mixed infected plant with PPV, PDV, and PNRSV; lane 2: mixed infected plant with PPV and PNRSV; lane 3: mixed infected plant with PNRSV and PDV; lane 4: mixed infected plant with PDV and PPV; lane 5: plant infected with PNRSV; lane 6: plant infected with PDV; lane 7: plant infected with PPV; lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada). (B) One-step multiplex RT-PCR with the *nad*5 gene as an internal control. Lane 1: sample of a healthy plant; lane 2: mixed infected plant with PPV, PDV, and PNRSV; lanes 3 and 4: mixed infected plant with PNRSV and PDV; lane 5: mixed infected plant with PDV and PPV; lane 6: plant infected with PNRSV; lane 7: plant infected with PDV; lane 8: plant infected with PPV infection; lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada). (C) Sensitivity of one-step multiplex RT-PCR assay using serially diluted plant RNA with the *nad*5 gene as an internal control. Lanes 1–8: serially diluted RNA samples of mixed PPV, PDV, and PNRSV-infected plum (*P. domestica*), when RNA was diluted 10-fold – total RNA concentration 50 ng in the reaction (lane 1) to 100 million-fold – total RNA concentration 5 fg in the reaction (lane 8). Lane 9: a healthy plant sample. Lane M: DNA ladder 1 kb (Fermentas Int., Burlington, Canada). (D) Sensitivity of one-step multiplex RT-PCR assay using serially diluted plant RNA extracts and without the *nad*5 gene as an internal control. Lanes 1–8: serially diluted RNA samples of mixed PPV, PDV, and PNRSV-infected plum (*P. domestica*), when RNA was diluted 10-fold – total RNA concentration 50 ng in the reaction (lane 1) to 10 million-fold – total RNA concentration 50 fg in the reaction (lane 7). Lane 8: a healthy plant sample. Lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada). (E) Sensitivity of one-step simlex RT-PCR assay with PPV primers using serially diluted plant RNA with the *nad*5 gene as an internal control. Lanes 1–8: serially diluted RNA samples of PPV-infected plum (*P. domestica*), when RNA was diluted 10-fold – total RNA concentration 50 ng in the reaction (lane 1) to 10 million-fold – total RNA concentration 50 fg in the reaction (lane 7). Lane 8: A healthy plant RNA. Lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada).

be used both with and without the internal control with more or less the same results. The only exceptions were samples of *P. tomentosa*, where amplification of the internal control was unsatisfactory. Furthermore, artificially mixed RNAs from different infections (single as well as mixed infections) were tested in the reaction with the same positive results (data not shown).

3.3. Sensitivity of the multiplex RT-PCR

The sensitivity of the reaction was tested by serial dilutions of plant RNA extracts in water used for the RT-PCR amplification. In the multiplex PCR reactions, some primers often tended to be more efficiently "aggressive" than others, resulting in the production of their own amplicon to the disadvantage of the other primers and targeted sequences. In our assay, we have confirmed the competitive character of multiplex PCR by serial RNA dilutions. When RNA from samples infected with all three viruses was diluted 10-fold to 100 million-fold, internal control and PDV were detectable in RNA diluted up to 1 million-fold, whereas PNRSV was detectable in all performed RNA dilutions (Fig. 1C). The serial dilution was tested on different samples with similar results—the internal control was always the first amplicon to disappear from the electrophoresis gel visualization.

Serial dilution tests were carried out for simple reactions and compared with the multiplex reaction. No significant differences were recorded for the detection capability of the individual assays. In the case of PPV, the sensitivity of the reaction was usually even enhanced by adding more primers into the reaction mix. In multiplex reactions, the PPV was detectable in RNA dilutions up to 10^{-5}, the simple reaction with PPV primers produced visible amplicons only in dilutions up to 10^{-4} for all tested samples (Fig. 1E).

The assay was compared with the multiplex RT-PCR assay published by Sánchez-Navarro et al. (2005). The Czech isolates of PDV were not detectable by the RT-PCR assay according to Sánchez-Navarro et al. (2005) neither in the simple nor in the multiplex reactions in our hands (Fig. 2A and B).

4. Discussion

The detection of viruses by rapid and reliable techniques is still one of the most demanding tasks in plant virology. In this report, we describe the development of a one-step multiplex RT-PCR assay for common stone fruit virus detection, which is rapid, highly sen-

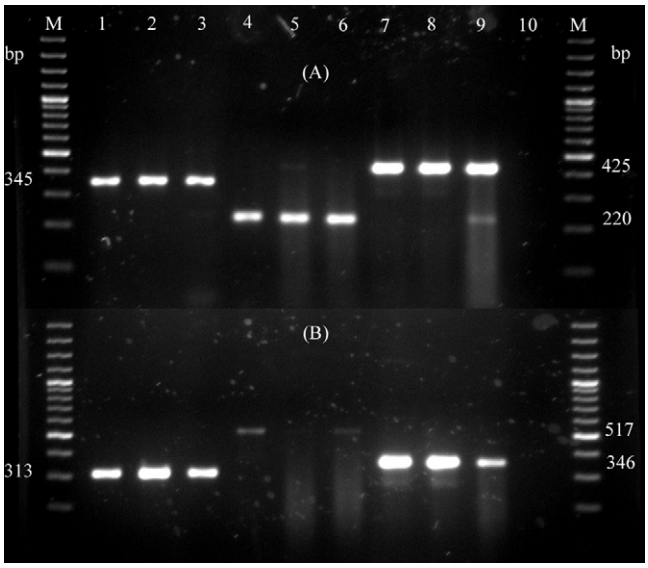

Fig. 2. (A and B) Comparison of one-step RT-PCR multiplex assay using primers described in this paper (A) versus primers described in Sánchez-Navarro et al. (2005) (B) for the detection of PDV, PPV, and PNRSV.
Lanes 1–3: PPV-infected plants, lanes 4–6: PDV-infected plants, lanes 7–8: PNRSV-infected plants, lane 9: PDV and PNRSV mixed infected plant, lane 10: healthy plant, lane M: DNA ladder 100 bp (Fermentas Int., Burlington, Canada).

sitive, and specific. It thus appears to be useful for virus typing in research or for routine diagnostic application. So far, to our knowledge, two multiplex RT-PCR methods for stone fruit virus detection have been published – Sánchez-Navarro et al. (2005) developed a simultaneous detection of eight viruses of stone fruit trees – PNRSV, PDV, PPV, apple mosaic virus (ApMV), American plum line pattern virus (APLPV), apple chlorotic leaf spot virus (ACLSV), apricot latent virus (ApLV), and plum bark necrosis stem pitting associated virus (PBNSPaV) and Saade et al. (2000) reported an assay for detection of PNRSV, PDV, and ApMV. Sánchez-Navarro et al. (2005) reported a decrease in sensitivity when the primer cocktail contained more than five different pair primers and reported that the sensitivity of the reaction was influenced by the number of different primer pairs, instead of the total amount of primers present in the cocktail. The method was applied under our conditions and found to be inefficient in detecting the Czech PDV isolates (Fig. 2). The downside of Saade's assay in terms of routine diagnosis is the absence of PDV detection possibility as it is the most important and frequent stone fruit virus in Europe.

Undoubtedly, multiplex PCR can be less sensitive than a single reaction with the same primers. The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products primarily because of the formation of primer dimers. These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or reduce such nonspecific interactions (Elnifro et al., 2000). There are the ways to overcome these problems. The first is careful primer selection. The specificity of these primers is vital, particularly with respect to their capacity to partially anneal to any part of the other co-infected viruses' genomes in question. Furthermore, these primers should be designed so that they cannot create self-dimers. Second, the conditions under which the reaction is run are of great importance. The annealing temperatures of all primers should be similar so that high specificity can be achieved. Annealing temperature that is too low allows amplification of unspecific products; annealing temperature that is too high reduces the chance of the targeted product to amplify. The same is true for annealing times. Finally, the concentration of individual primers might be able to solve the problem of the "predaceous" primers by decreasing their concentration and increasing the concentration of the less aggressive primers. In our study, we concentrated on careful primer selection and all primers producing unspecific amplicons in PCR reaction were excluded from our study. The sensitivity of the reaction, when compared with simple reaction with the same primers, was not lowered. Furthermore, due to the absence of unspecific products, the recommended final annealing temperature settled at 51 °C as reaction specificity there seems not to require temperatures higher than this.

Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously (Elnifro et al., 2000). In our study, this was tested by serial RNA dilutions of a sample infected by all three viruses and samples infected with single infections. The least competitive primer pair is the pair for the *nad5* gene. Therefore, when the assay was run with the internal control, and the internal control was positive, it is very probable that the primers for targeted viruses did not fail to amplify the present sequences. This is due to the fact that all the other primers seemed to be more competitive and therefore more capable of amplifying the targeted sequence than the primers for the *nad5* gene. On the other hand, there is a risk of internal control being false negative, thereby leading to inaccurate conclusions. When the concentration of the virus template is high, the more competitive primers for the given virus can use up most of the chemicals such as nucleotides and therefore stop the *nad5* primers from amplifying the targeted sequence successfully. In our experience, apart from the samples of *P. tomentosa* mentioned above, all the tested samples of ordinarily diluted good-quality RNA have always produced amplicons for internal control in the multiplex RT-PCR reaction, and thus, when there is a negative result of the *nad5* gene presence, it is highly probable that it is due to the low quality of template RNA. The assay when used with the internal control can efficiently and quickly provide the desired information. In the case of *P. tomentosa* samples, the unsatisfactory amplification of the internal control is probably due to a slightly different genome as, in some cases, the product was weak but visible and, in some cases, the product was missing completely. Therefore, when testing the samples of *P. tomentosa*, we recommend using the assay without the internal control.

In conclusion, the multiplex PCR method described here is found useful for the diagnosis of virus pathogens in stone fruits as a complement to ELISA detection, which often remains the method of choice for these three viruses. Nevertheless, the assay is especially indicated for routine diagnosis as it is time- and money-saving, and, as we have shown in this paper, is also highly specific, robust, and sensitive. When used with the internal control, it can serve as a reliable diagnosis tool.

Acknowledgements

We wish to express our gratitude to J. Suchá, L. Talacko from the Research and Breeding Institute of Pomology Holovousy Ltd. and Dr. Ioan Zagrai from Statiunea de Cercetare-Dezvoltare pentru Pomicultura (SCDP) Bistrita, Romania for providing the samples of known isolates of PDV. We are also deeply grateful to Dorothy Mogg for proofreading the manuscript. This work was supported by a grant from the Ministry of Agriculture, Czech Republic (Project no. QG60123).

References

Bertolini, E., Olmos, A., Martínez, C.M., Gorris, T.M., Cambra, M., 2001. Single-step multiplex RT-PCR simultaneous and colourimetric detection of six RNA viruses in olive tress. J. Virol. Methods 96, 33–41.

Bertozzi, T., Alberts, E., Sedgley, M., 2002. Detection of *Prunus necrotic ringspot virus* in almond: effect of sampling time on the efficiency of serological and biological indexing methodologies. Aust. J. Exp. Agr. 42, 207–210.

Cambra, M., Asensio, M., Gorris, M.T., Pérez, E., Camarasa, E., García, J.A., Moya, J.J., López-Abella, D., Vela, C., Sanz, A., 1994. Detection of *Plum pox potyvirus* using monoclonal antibodies to structural and non-structural proteins. Bull. OEPP/EPPO Bull. 24, 569–577.

Candresse, T., Cambra, M., Dallot, S., Lanneau, M., Asensio, M., Gorris, M.T., Revers, F., Macquaire, G., Olmos, A., Boscia, D., Quiot, J.B., Dunez, J., 1998. Comparison of monoclonal antibodies and polymerase chain reaction assays for the typing of isolates belonging to the M and D serotypes of *Plum pox potyvirus*. Phytopathology 88, 198–204.

Clark, M.F., Adams, A.N., 1977. Characteristics of the micro-plate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475–483.

Di Terlizi, B., 2000. Biological diagnosis of virus and virus-like diseases: a special reference to stone fruit certification. Options Méditerranéennes, serie B/n 19: Stone fruit viruses and certification in the Mediterranean: problems and prospects. pp. 151–169.

Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E., 2000. Multiplex PCR: optimization and application in diagnostic virology. Clin. Microbiol. Rev. 13, 559–570.

Gentit, P., 2006. Detection of *Plum pox virus*: biological methods. Bull. OEPP/EPPO Bull. 36, 251–253.

Glasa, M., Palkovics, L., Komínek, P., Labonne, G., Pittnerová, S., Kúdela, O., Candresse, T., Šubr, Z., 2004. Geographically and temporally distant natural recombinant isolates of *Plum pox virus* (PPV) are genetically very similar and form a unique PPV subgroup. J. Gen. Virol. 85, 2671–2681.

Grieco, F., Gallitelli, D., 1999. Multiplex reverse transcriptase-polymerase chain reaction applied to virus detection in globe artichoke. J. Phytopathol. - Phytopathol. Z. 147, 183–185.

Hadidi, A., Czosnek, H., Barba, M., 2004. DNA microarrays and their potential applications for the detection of plant viruses, viroids, and phytoplasmas. J. Plant Pathol. 86, 97–104.

Ito, T., Ieki, H., Ozaki, K., Iwanami, T., Nakahara, K., Hataya, T., Ito, T., Isaka, M., Kano, T., 2002. Multiple citrus viroids in citrus from Japan and their ability to produce exocortis-like symptoms in citron. Phytopathology 92, 542–547.

Kundu, J.K., 2003. A rapid and effective RNA release procedure for virus detection in woody plants by reverse transcription-polymerase chain reaction. Acta Virol. 47, 147–151.

Mekuria, G., Ramesh, S.A., Alberts, E., Bertozzi, T., Wirthensohn, M., Collins, G., Sedgley, M., 2003. Comparison of ELISA and RT-PCR for the detection of *Prunus necrotic ring spot virus* and *Prune dwarf virus* in almond (*Prunus dulcis*). J. Virol. Methods 114, 65–69.

Menzel, W., Jelkmann, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99, 81–92.

Németh, M., 1986. Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees. Martinus Nijhoff Publishers, The Netherlands and Akademini Kiado, Hungary, p. 841.

Olmos, A., Cambra, M., Dasí, M.A., Candresse, T., Esteban, O., Gorris, M.T., Asensio, M., 1997. Simultaneous detection and typing of *Plum pox potyvirus* (PPV) isolates by heminested-PCR and PCR-ELISA. J. Virol. Methods 68, 127–137.

Palkovics, L., Burgyan, J., Balazs, E., 1994. Sensitive non-radioactive nucleic acid hybridization assay for *Plum pox virus* detection. Res. Virol. 145, 387–392.

Ragozzino, E., Faggioli, F., Barba, M., 2004. Development of a one tube step RT-PCR protocol for the detection of seven viroids in four genera: *Apscaviroid*, *Hostuviroid*, *Pelamoviroid* and *Pospiviroid*. J. Virol. Methods 121, 25–29.

Rosner, A., Shilboleth, Y., Spiegel, S., Krisbai, L., Kölber, M., 1998. Evaluating the use of immunocapture and sap-dilution PCR for the detection of *Prunus necrotic ringspot virus*. Acta Hortic. 472, 227–233.

Roy, A., Fayad, A., Barthe, G., Brlansky, R.H., 2005. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. J. Virol. Methods 129, 47–55.

Saade, M., Aparicio, F., Sánchez-Navarro, J.A., Herranz, M.C., Myrta, A., Di Terlizzi, B., Pallas, V., 2000. Simultaneous detection of the three ilarviruses affecting stone fruit trees by nonisotopic molecular hybridization and multiplex reverse-transcription polymerase chain reaction. Phytopathology 90, 1330–1336.

Sánchez-Navarro, J.A., Aparicio, F., Herranz, M.C., Minafra, A., Myrta, A., Pallas, V., 2005. Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. Eur. J. Plant Pathol. 111, 77–84.

Scott, S.W., Bowman-Vance, V., Bachman, E.J., 1992. The use of nucleic acid probes for the detection of *Prunus necrotic ringspot virus* and *Prune dwarf virus*. Acta Hortic. 309, 79–83.

Spiegel, S., Rosner, A., Tam, Y., Zilkah, S., Faingersh, E., Rotbaum, A., Krizbai, L., 1998. Detection of *Prune dwarf virus* in sweet cherry in Israel. Acta Hortic. 472, 249–256.

Spiegel, S., Tam, Y., Maslenin, L., Kolber, M., Nemeth, M., Rosner, A., 1999. Typing *Prunus necrotic ringspot virus* isolates by serology and restriction endonuclease analysis of PCR products. Ann. Appl. Biol. 135, 395–400.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Varga, A., James, D., 2005. Detection and differentiation of *Plum pox virus* using real time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. J. Virol. Methods 123, 213–220.

Varga, A., James, D., 2006. Use of reverse transcription loop-mediated isothermal amplification for the detection of *Plum pox virus*. J. Virol. Methods 138, 184–190.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., Dunez, J., 1992. A highly sensitive immunocapture polymerase chain reaction method for *Plum pox potyvirus* detection. J. Virol. Methods 39, 27–37.

Wetzel, T., Candresse, T., Ravelonandro, M., Dunez, J., 1991. A polymerase chain reaction assay adapted to *Plum pox virus* detection. J. Virol. Methods 33, 355–365.