*Phytopathol. Mediterr.* (2005) 44, 189–194

# Studies on parameters influencing the performance of reverse transcriptase polymerase chain reaction (RT-PCR) in detecting *Prunus necrotic ringpot virus* (PNRSV)

Mustafa Usta, Hikmet Murat Sipahioglu and Bulent Polat

Yuzuncu Yil University, Agricultural Faculty, Department of Plant Protection, 65080 Van, Turkey

**Summary.** In order to have a more detailed understanding of the various factors influencing a reverse transcriptase polymerase chain reaction (RT-PCR), a number of important parameters such as $Mg^{+2}$, primer, enzyme concentration and others were optimized for the detection of *Prunus necrotic ringspot virus* (PNRSV). Using a PNRSV isolate with a pair of primers, complementary DNA of viral genome as template, and an appropriate enzyme together with magnesium chloride, the following optimal conditions were identified: primer concentration between 0.2 and 0.0002 pmol $\mu l^{-1}$ and 0.06–2 units $\mu l^{-1}$ for *Taq* DNA polymerase enzyme for a 50 $\mu$l reaction volume when other parameters were optimum; magnesium chloride concentration less than 2.5 mM; dNTP concentration between 1 and 10 mM. The optimum cDNA amount should be ~360 ng for a 50 $\mu$l reaction mixture. When these optimized concentrations and/or values of the main PCR parameters were brought together for a new RT-PCR, a clear and a reliable PNRSV detection having no background was performed from both growth-chamber and field-grown PNRSV-infected plants.

**Key words:** optimization, PCR parameters, detection.

## Introduction

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR is a major development in analyses of DNA and RNA because it has both simplified existing technology and enabled a rapid development of new techniques which would not otherwise have been possible (Taylor, 1991). The PCR technique enables the specific amplification and hence detection of target DNA sequences from complex mixtures of nucleic acid. A combination of short, specific primers and thermostable DNA polymerases are used to amplify the target sequence through repeated cycles of denaturation, annealing, and DNA synthesis at high temperatures, allowing an exponential increase in the amount of the DNA of interest. By the addition of a reverse-transcription (RT) step PCR can also be applied to cDNA generated from RNA templates (Seal and Coates, 1998).

*Prunus necrotic ringspot virus* (PNRSV) is the most common virus of cultivated species of *Prunus* and some other Rosaceous plants. It occurs in many strains and forms, some of which cause serious diseases in fruit trees (Nyland *et al*., 1976). The virus is transmissible through grafting and also by pollen and seeds (Gilmer and Way, 1961; Marenaud

Corresponding author: M. Usta
Fax: +90 43 22251867
E-mail: mustafausta@yyu.edu.tr

and Llacer, 1976; Gella, 1980). Since the virus has many strains, in some species, the effect may depend on the particular virus strain involved (Mink, 1992).

Reliable detection of PNRSV is required in certification schemes aimed at guaranteeing virus-tested propagation material to the fruit growing industry and preventing entry of imported PNRSV-infected plant materials (Stain *et al*., 1987). PCR is a well-established, highly sensitive diagnostic tool that has been shown to be superior for certain plant viruses over the commonly used serological methods (Vunsh *et al*., 1990). Despite such assays, detection is still severely hampered by the uneven distribution of viruses in infected trees (Nemeth, 1986).

In the last decade, numerous studies have appeared reporting the reliability of the RT-PCR technique in the detection of PNRSV (Spiegel *et al*., 1996; Malinowski, 1997; Rosner *et al*., 1997; Mekuria *et al*., 2003). Most of these studies have focused on enhancing the detection sensitivity of RT-PCR (Navarro *et al*., 1998; Helguera *et al*., 2001a; Helguera *et al*., 2001b).

Although PCR is now a routine technique in many laboratories, there are still many problems in getting good, reproducible amplifications (Seal and Coates, 1998). It is already clear however that no single protocol will be appropriate to all situations (Innis and Gelfand, 1990). In diagnostic laboratories the use of PCR is limited by its cost, and sometimes by the lack of an adequate test sample volume.

Though the RT-PCR is basically simple, successful performance depends on a number of factors. Numerous papers and manuals discuss in detail the conditions influencing the quality of PCR in general, but relatively little has been published about important experimental factors and difficulties frequently encountered with RT-PCR. This study presents a brief overview of some critical PCR parameters and strategies for optimizing RT-PCR reaction in detecting PNRSV.

## Materials and methods

### Plant and virus source

A cherry isolate of PNRSV was used as the reference isolate. The virus was transmitted to two healthy *Prunus mahaleb* plants. One of the plants was maintained outside and the other was kept in a growth chamber. Inoculated plants served as a virus source during the trials. Leaf tissues of one-year-old shoots of systemically infected *P. mahaleb* were used in the RT-PCR assays.

### Plant total RNA extraction and synthesis of complementary DNA (cDNA)

Total RNA was recovered with silica based method according to Foissac *et al*., (2000). Oligonucleotide primer sequences (Primer I: 5'-TACCTCTAGATCTCAAGCAG-3', Primer II: 5'-GAGCTCTGGTCCCACTCAGG-3') reported by Spiegel *et al*., (1999) were used to perform RT-PCR to detect PNRSV. The amplified fragment was 616 bp in length. The sequence of reverse complementary primer I was used for viral cDNA synthesis.

### PCR amplification of cDNA

One $\mu$l of cDNA was mixed with 24 $\mu$l of the amplification mixture containing 2.5 $\mu$l of the 10× reaction buffer (200 mM Tris-HCL, pH 8.4, 500 mM KCl) 1.5 $\mu$l of $MgCl_2$ (25 mM), 0.5 $\mu$l dNTPs (10 mM), 0.5 $\mu$l of each primer (100 pmol $\mu l^{-1}$), 0.2 $\mu$l of *Taq* DNA polymerase (Cat. No. M1661, Promega Corporation, Madison, WI, USA) and 17.8 $\mu$l of RNase free sterile water. Initial denaturation was at 94°C for 3 min and followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min. Ten $\mu$l of amplified PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining. While one of the PCR parameters was increased stepwise the others were kept constant to determine the optimal PCR parameters.

## Results

A series of PCR parameters and strategies to optimize detection of PNRSV by RT-PCR were studied. The standard conditions were principally used to provide the starting ones for designing new RT-PCR amplification to detect PNRSV. A prerequisite for the amplification of a complementary DNA (cDNA) of viral RNA was the titration of the magnesium ion ($Mg^{+2}$) concentration. Every parameter was kept constant and only the concentration of $Mg^{+2}$ was allowed to vary. In all $Mg^{+2}$ concentrations tested, ranging from 1.0 mM to 4.0 mM, frag-

ments of the expected size were obtained (Fig. 1a). $Mg^{+2}$ concentration clearly affected both the quality and the quantity of the amplified fragments. The optimum $MgCl_2$ concentration was between 1.25 and 2.5 mM (Fig. 1a).

At cDNA template quantities between 36 and 360 ng 50$\mu l^{-1}$ reaction mixture a slight difference was observed; however, below 36 ng the amount of PCR products was decreased. As appears from Fig. 1b at least 360 ng of cDNA was needed for a better amplification yield.

Primer concentration significantly influenced the PCR products. The most conspicuous result was obtained when the primers were diluted to 1:500,000 corresponding to a concentration of 0.0002 pmol $\mu l^{-1}$ concentration. In other words, the quantity of amplified fragments increased when the primer concentration was gradually decreased. As seen in Figure 2a the standard primer concentration (lane 1) increased the probability of spurious priming and caused the generation of primer dimers (white arrow at lane 1). A substantial surplus of primer therefore even led to a reduction in the amplification yield. The optimum primer concentration was thus between 0.0002 and 0.2 pmol $\mu l^{-1}$.

In order to minimize misincorporations, equivalent concentrations of four dNTPs were used in the trials. The lowest dNTP concentration very strongly reduced PCR yields (Fig. 2b). With the lower concentrations of dNTPs it was not possible to obtain a considerable specificity in the PCR system. The best results were at 10 mM of each dNTP. Concentrations of dNTP lower than 10 mM allowed PCR amplification but with visibly lower amounts of products.

Optimum enzyme concentration was between 0.0625 and 2 units $\mu l^{-1}$. As can be seen in Figure 3, in lane 1 the high enzyme concentration increased the amount of non-specific background products (conspicuous in lane 1, Fig. 3) while enzyme concentrations that were too low resulted in insufficient amount of the desired products (lanes 7 and 8).

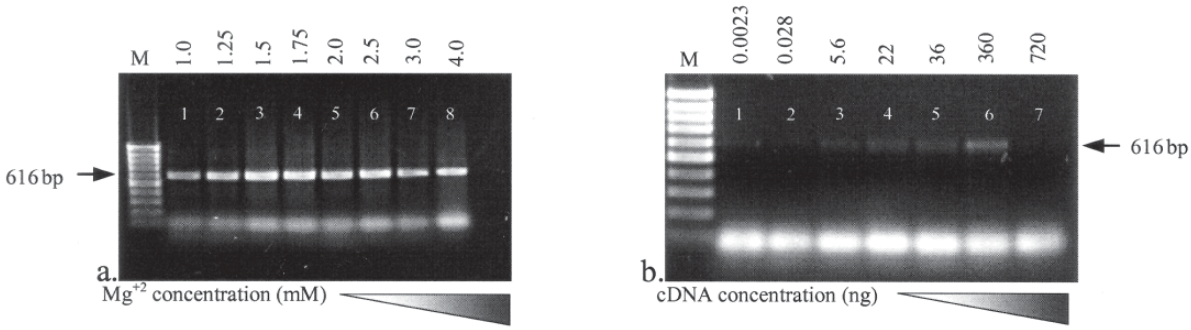


Fig. 1. $Mg^{+2}$ concentrations (a) and amount of cDNA (b) as template in RT-PCR reactions. M, molecular size markers.

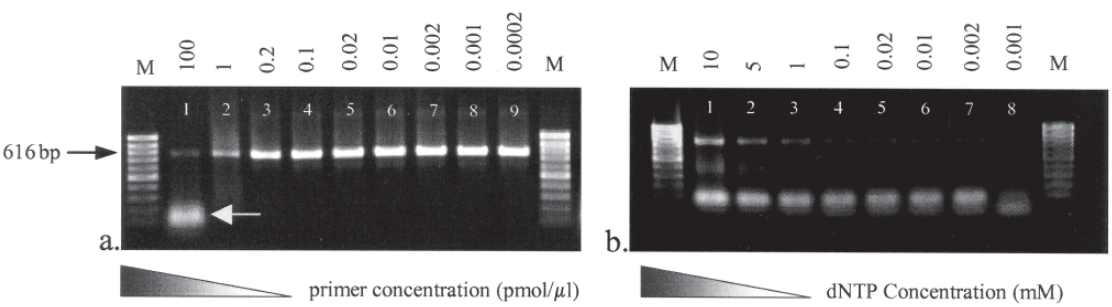


Fig. 2. Electrophoretic analysis of RT-PCR products deriving from different primer (a) and dNTP concentrations (b). M, molecular size markers.

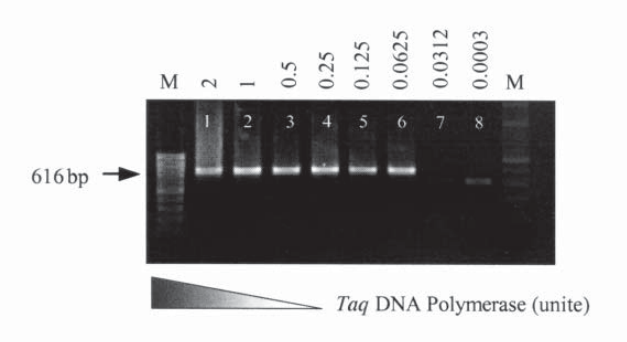


Fig. 3. Electrophoretic analysis of RT-PCR products deriving from different *Taq* DNA polymerase concentrations. M, molecular size markers.

To determine the sensitivity of the optimized concentrations (Table 1) in the RT-PCR system, PNRSV-infected growth-chamber and field-grown *P. mahaleb* plants were simultaneously used in the amplification of viral genome. As shown in Fig. 4a and b the corresponding PCR products of the correct size were detected in both the field and growth-chamber plants. The success of the optimized concentrations in the RT-PCR tests was verified by obtaining satisfactory amplification products from both conditions (Fig. 4a and b).

## Discussion

To optimize the RT-PCR method in the detection of PNRSV leaf samples of *P. mahaleb* were chosen. These samples had previously been used in standard two-step RT-PCR reactions. It has recently been demonstrated that RT-PCR is a useful and rapid diagnostic tool for the management of the PNRSV in stone-fruit orchards (Malinowski, 1997; Rosner *et al*., 1997; Mekuria *et al*., 2003). The optimized, relatively simplified RT-PCR allowed the reliable detection of PNRSV with high yield. There is obviously no single set of conditions that can be applied to all PCR amplifications. Therefore, in trying different concentrations of the main PCR parameters, the RT-PCR detection of PNRSV was optimized. Optimized parameters

Table 1. Optimum concentrations of PCR parameters obtained throughout the RT-PCR reactions.

| Main parameters | Concentrations or amounts | |
|---|---|---|
| | Standard | Optimized |
| dNTP | 10 mM | 10 mM |
| Primers | 100 pmol $\mu l^{-1}$ | 0.001 pmol $\mu l^{-1}$ |
| $MgCl_2$ | 1.5 mM | 1.75 mM |
| cDNA | 700–800 ng | ~360 ng |
| *Taq* DNA polymerase enzyme | 2 units | 0.5–1 unit |

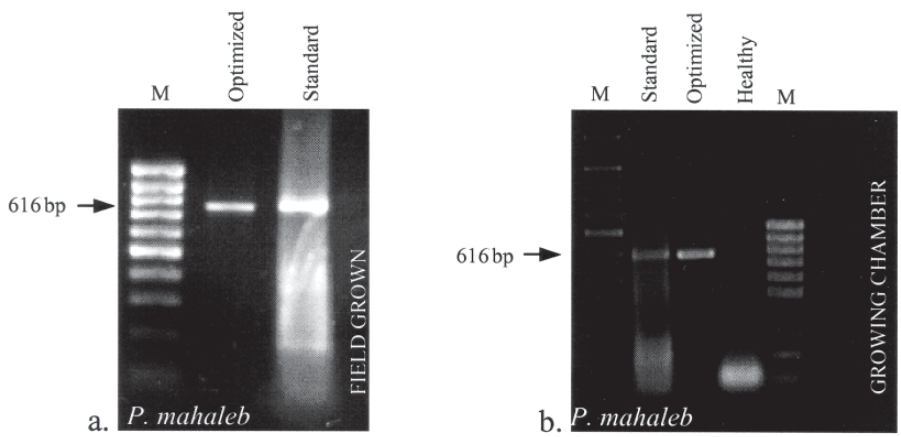


Fig. 4. Optimized PCR amplification to detect PNRSV from field (a) and growth-chamber (b) grown plants.

proved to be a valuable and cost effective-tool for monitoring the emergence of PNRSV in its host plants.

The optimization of $Mg^{+2}$ is a critical factor since *Taq* DNA polymerase is magnesium-dependent. In addition, *Taq* DNA polymerase, the template DNA, the primers and dNTP all bind $Mg^{+2}$. Therefore the optimal $Mg^{+2}$ concentration will depend on the dNTP concentration, the specific template DNA, and the sample buffer composition. Excessive $Mg^{+2}$ stabilizes the DNA double strand and prevents complete denaturation of DNA, reducing yield. Excessive $Mg^{+2}$ can also stabilize spurious annealing of the primers to incorrect template sites, decreasing specificity (Markoulatos *et al*., 2002). Although gradually increasing the $Mg^{+2}$ concentration can improve the reaction, for optimal PCR yield a concentration of 1.25–2.5 mM $Mg^{+2}$ is recommended. Markoulatos *et al*. (2002) reported that the optimal combination of $Mg^{+2}$, amount of template DNA, primer, dNTP and *Taq* DNA polymerase enzyme concentrations was essential in any PCR to obtain highly specific amplification products. It should be noted that the primer, dNTP and *Taq* DNA enzyme concentration seem to be much more important in obtaining a specific high yield of PCR products.

A concentration of primer and *Taq* DNA polymerase enzyme lower than the standard concentration is recommended to avoid the formation of primer dimers and a dirty background. This will ensure a relatively clear background in the ethidium bromide stained gel lanes. In our experiments, it was found that when the enzyme concentration was too high, nonspecific background products accumulated and when it was too low, the amount of desired products was insufficient. The recommended concentration range for *Taq* DNA polymerase was between 0.5 and 1 unit per 50 $\mu$l reaction when other parameters were optimal. However, Innis and Gelfand (1990) reported that the enzyme requirements may vary with individual target templates or primers. A primer concentration over 100 pmol $\mu l^{-1}$ tends to reduce the amplification yield. In practical terms, as was the case with primer concentration, an increase in cDNA copies was by a decrease in the detection of the viral agent.

The results indicate that optimized PCR parameters detected PNRSV with high reliability using a two-step RT-PCR reaction; and gave greater sensitivity that did the standard RT-PCR. In general, the optimized concentrations specifically, sensitively and efficiently amplified the viral genome of PNRSV, which is considered one of the characteristics of a qualitative PCR reaction.

## Acknowledgements

The study was financially supported by a grant from The Research Fund of Yuzuncu Yil University-YYUBAP (Project No. ZF-2002-46) and The Scientific and Technical Research Council of Turkey-TUBITAK (Project No. TOGTAG-2990). The authors thank YYUBAP and TUBITAK for their financial support.

## Literature cited

Foissac X., L. Savalle-Dumas, P. Gentit, M.J. Dulucq and T. Candresse, 2000. Polyvalent detection of fruit tree Tricho, Capillo and Faveaviruses by Nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). *Acta Horticulturae* 357, 52–59.

Gilmer R. and R. Way, 1961. Pollen transmission of Necrotic ringspot and Prune dwarf viruses in sour cherry. In: *Proceedings of 4th Symposium of Virus Diseases and Fruit Trees in Europe, Tidsski, Planteueaul* 65, 111–117.

Helguera P.R., D.A. Ducasse, D.M. Docampo and S.F. None, 2001b. Enhanced detection of Prune dwarf virus in peach leaves by immunocapture- reverse transcription-polymerase chain reation (IC-RT-PCR) nested polymerase chain reaction. *Journal of Phytopathology* 149, 1–3.

Helguera P.R., R. Taborda, D.M. Docampo and D.A. Ducasse, 2001a. Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of Prunus necrotic ring spot virus in the peach. *Journal of Virological Methods* 95, 93–100

Innis M.A and D.H. Gelfand, 1990. *Optimization of PCRs*. In: *PCR Protocols*. (Innis A.I., Gelfand D.H., Sninsky J.J., White T.J., ed.), Academic Press, San Diego, CA, USA, 3–12.

Malinowski T., 1997. Silicacapture RT-PCR: Application for the detection of several plant viruses. *4th International Symposium of the European Foundation for Plant Pathology*, 445–448.

Marenaud C. and G. Llacer. 1976. Etude de la diffusion de virus de type ILAR (taches annularies necrotiques) dans un verger de ceriser (*Prunus avium*). *Annales Amèlioration des Plantes* 26(2), 357–363.

Markoulatos P., N. Siafakas and M. Moncany, 2002. Multiplex polymerase chain reaction: A practical approach. *Journal of Clinical Laboratory Analysis* 16, 47–51.

Mekuria G., S.A. Ramesh, E. Alberts, T. Bertozzi, M. Wirthensohn, G. Collins and M. Sedgley, 2003. Comparison of ELISA and RT-PCR for the detection of Prunus necrot-

ic ring spot virus and Prune dwarf virus in almond (*Prunus dulcis*). *Journal of Virological Methods* 114, 65–69.
Mink G.I., 1992. Prunus necrotic ringspot virus. *Plant Diseases of International Importance, Volume III, Disease of Fruit Tree Crops*, 335–356.
Nemeth M., 1986. *Virus Mycoplasma and Rickettsia Diseases of Fruit Trees*. Akademiai Kiado, Budapest, Hungary, 840 pp.
Nyland G., R.M. Gilmer and J.D. Moore, 1976. *Fruit disease and noninfectious disorders of stone fruits in North America*. USDA. *Agriculture Handbook,* Government Printing Office, Washington, DC, USA, 437, 104–132.
Rosner A., Maslenin L. and S. Spiegel, 1997. The use of short and long PCR products for improved detection of prunus necrotic ringspot virus in woody plants. *Journal of Virology Methods* 67, 135–141
Saiki R.K., Scharf S.J., Faloona F., Mullis K.B., Horn G.T., H.A. Erlich and N. Arnheim, 1985. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350–1354.
Sanchez-Navarro J.A., Aporicio F., Rowhani A. and V. Pallás, 1998. Comparative analysis of ELISA, non radioactive molecular hybridization and PCR for the detection of Prunus necrotic ringspot virus in herbaceous and Prunus hosts. *Plant Pathology* 47, 780–786.
Seal S. and D. Coates, 1998. Detection and quantification of plant viruses by PCR. In: *Plant Virology Protocols From Virus Isolation to Transgenic Resistance* (G.D. Foster, S.C. Taylor, ed.), Humana Press, 81, 469–486.
Spiegel S., Scott S.W., Bowman-Vance V., Tam Y., Galiakparov N.N. and A. Rosler, 1996. Improved detection of prunus necrotic ringspot virus by the polymerase chain reaction. *European Journal of Plant Pathology* 102, 681–685.
Spiegel S., Tam Y., Maslenin L., Kolber M., Nemeth M. and A. Rosner, 1999. Typing prunus necrotic ringspot virus isolates by serology and restriction endonuclease analysis of PCR products. *Annals of Applied Biology* 135(1), 395–400.
Stain A., S. Leavy and G. Lobenstein, 1987. Detection of prunus necrotic ringspot virus in several rosaceous hosts by enzyme-linked immunosorbent assay. *Plant Pathology* 36, 1–4.
Taylor R.G., 1991. Polymerase chain reaction: basic principles and automation. *PCR 1 A Practical Approach*. In: (McPherson M.J., Quirke P., Taylor R.G., ed.), The Practical Approach Series, Vol. 1, Oxford University Press.
Vunsh R., A. Rosner and A. Stain. 1990. The use of polymerase chain reaction (PCR) for the detection of bean yellow mosaic virus in gladiolus. *Annales Applied Biology* 117, 561–569.

*Accepted for publication: May 5, 2005*