Modified RNA extraction from field woody plants for the routine detection of PDV and PNRSV in cherry by RT-PCR *

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[**Abstract**] An efficient and effective procedure for the extraction of high-quality RNA from woody plants without the use of phenol, organic solvents, or alcohol precipitation is described, which is based on silica capture. The method described has been successfully used for the detection of PDV and PNRSV in cherry by RT-PCR assay using DNA primers for the viral coat protein region. The expected sizes of the amplified products were 172 and 449 bp. Samples from bark, leaves and buds were used. Detection of viral RNA in samples of total plant RNA prepared using this method was found to be as sensitive as the methods previously described using the commercially available Qiagen's RNeasy extraction kit.

[**Key words**] total RNA extraction; silica; cherry; PDV; PNRSV; RT-PCR detection

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The reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive amplification procedure that has been used to detect the presence of plant viruses with RNA genomes[1]. The advantages of the PCR technique include high specificity, the theoretical sensitivity to detect a single target molecule in a complex mixture, and high sample throughput. In comparison with enzyme-linked immunosorbant assay (ELISA), it is more sensitive[2~5]. This characteristic is especially important for tree viruses that have an erratic distribution within a plant and are present in low concentrations[6]. Also in situations where good quality antisera are not available, PCR primers with any desired degree of selectivity can be synthesized at a much lower comparable cost than that associated with the development of monoclonal or polyclonal antibodies. With the vast increase in nucleotide sequence data available in genebanks on the world wide web, it is possible to design primers specific for the detection of a large number of viruses.

Prunus necrotic ringspot ilarvirus (PNRSV) which infects all *Prunus* species in its many strains, is a major virus disease of sweet cherry worldwide and, alone or together with Prune dwarf ilarviru (PDV), causes severe losses on sweet cherry[7,8].

The chief limiting factor in the application of the PCR technique in the routine diagnosis lies in the preparation of good quality nucleic acid, free of PCR inhibitors. Tissues from woody plants, especially when field-grown, such as many tree fruit varieties of *Malus*, *Prunus* and *Pyrus* origin[9], can contain higher amounts of phenolic compounds and polysaccharides. Most standard nucleic acid extraction procedures can not remove contaminating plant polysaccharides or polyphenolic compounds, which can have direct inhibitory effects on subsequent PCR amplification[10,11]. Attempts to overcome these limitations included the development of more elaborate extraction methods, which employ polyvinylpyrrolidone (PVP) or cation-exchange resins to chelate polyphenolics. The use of specialized polymeric matrices, which irreversibly absorb

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inhibitor compounds, together with polyethylene glycol (PEG) precipitation as an alternate to ethanol precipitation of nucleic acids, has been described for the RT-PCR detection of PDV [12].

The RNA silica capture method was first explored by Boom [13] and applied to blood test on medical. Qiagen's RNeasy kits is also based on silica gel membrane of spin column, which combine the advantage of guanidinium thiocyanate lysis with purification of silica capture.

In this paper, a modified silica capture method for RNA isolation from infected cherry trees was developed in order to detect virus in woody tissue. This method represents an inexpensive and simple means for obtaining sufficient nucleic acid from tree leaves and bark of a quality appropriate for RT-PCR.

1 Materials and methods

1.1 Plant materials

All samples were obtained from known uninfected (negative control) and naturally infected woody hosts (sweet cherry trees) maintained in the field at the Institute for Plant Protection in Fruit Crops, Dossenheim, Germany.

Leaves, buds and barks were collected at various stages during the growing season. To account for the possible uneven distribution of virus within one tree, samples from at least four different branches of the same tree were obtained. Samples were transported at room temperature, then stored at 4 ℃ for a while or on ice for a maximum of 3-4 days, or stored at -80 ℃ until they were extracted up to 1 year later.

1.2 Preparation of total RNA extraction

(1) Modified silica capture method: Refered to Boom's[13] method and modified as follow: Approximately 0.3 g tissue was placed in a sample bag (Bioreba, South Bend, IN) and, with a hand-held homogenizer (Bioreba, South Bend, IN), homogenized with 5 mL grinding buffer composed of 4.0 mol/L guanidine thiocyanate, 0.2 mol/L sodium acetate (NaAc), pH 5.2, 25 mmol/L EDTA, 1.0 mol/L KAc and 2.5 g/L PVP-40 (The buffer could be stored at 4 ℃ for several days). 500 μL of the ground plant material was then transferred to a 1.5 mL eppendorf tube to which was added 100 μL 10% N-lauryl sarkosyl and 5 μL 2-mercaptoethanol. The mixture was incubated at 70 ℃ with intermittent shaking for 10 min, placed on ice for 5 min, and then centrifuged at 13 000 r/min for 10 min, 300 μL was then transferred to a new tube to which was added 150 μL EtOH, 300 μL 6 mol/L NaI and 25 μL resuspended silica, incubated at room temperature for 10 min with intermittent shaking and then centrifuged at 6 000 r/min for 1 min. The pellet was resuspended in 500 μL wash buffer (10 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EDTA, 50.0 mmol/L NaCl, 50% ethanol) and centrifuged at 6 000 r/min for 1 min. The wash step was repeated two times, and the pellet allowed drying for several minutes at room temperature before resuspending in 150 μL H_2O. The mixture was incubated at 70 ℃ for 4 min, centrifuged at 13 000 r/min for 3 min, and the supernatant transferred to a new tube and stored at -20 ℃. The NaI solution was prepared by first dissolving 0.75 g Na_2SO_3 in 40 mL water then 36 g NaI. Solution was stored in dark bottle at 4 ℃. Silica was prepared by adding 60 g silica particles (Sigma S5631) to 500 mL distilled H_2O. The silica was well mixed and the allowed to settle for 24 h. The upper 470 mL of supernatant was discarded, 500 mL of distilled H_2O added the suspension mixed well and allowed to settle for another 5 h, The upper 440 mL of solution was then discarded and the remaining 60 mL slurry was adjusted to pH 2.0 with HCl, autoclaved and stored in a dark bottle at room temperature or aliquoted into 1.5 mL eppendorf tubes for storage at 4 ℃ for several months.

(2) Qiagen's RNeasy plant mini kits method: As a control, after the first centrifuged, 300 mL of the supernatant was transferred to a QIAShredder column of Qiagen's RNeasy kits and worked according to manufacture's recommended to finish RNA purification.

1.3 Oligonucleotide Primer sequences

A amplification primers are designed from the

nucleotide sequence of the coat protein coding region of the PDV [12] and PNRSV [14].

For PDV detection, primers PDV1984 (5′-TAG TGC AGG TTA ACC AAA ACG AT-3′) and PDV1812 (5′-ATG GAT GCG ATG GAT AAA GT-3′) amplifying a 172 bp fragment, were used.

For detection of PNRSV, the primers are: 5′-ACG CGC AAA AGT GTC GAA ATC TAA A-3′ and 5′-TGG TCC CAC TCA GAG CTC AAC AAA G-3′. Amplified DNA size is 449 bp.

The primers are synthesized by Life Technologies GmbH, Karlsruhe, Germany. Stock solution (100 μmol/L) and aliquots of diluted working solutions (10 μmol/L) of primers were stored at -20 ℃.

1.4 Viral cDNA synthesis and PCR amplification (RT-PCR)

5 μL of total RNA extraction by silica capture was added to a eppendorf tube containing 6 μL sterilized PCR water, 0.5 μL of each olige (dT) and random hexamers (Tecnologies GmbH, Karlsruhe, Germany). Denatured at 70 ℃ for 10 min, chilled on ice for 2 min, then add 4 μL of 5X first strand buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L $MgCl_2$), 2 μL of 0.1 mol/L DDT, 1 μL of 10 mmol/L dNTP's (2.5 mmol/L of each dGTP, dATP, dCTP, dTTP), incubate at 37 ℃ for 10 min then add: 1 μL MMLV reverse transcriptase (200 U/μL superscript Ⅱ, Life Technologies GmbH) The reaction mixtures were vortexed for 10 sec and then incubated at 42 ℃ for 50 min, then stop reaction by incubating at 70 ℃ for 10 min. cDNA was stored on ice if about to do PCR or at -20 ℃.

PCR tube contained 5 μL of 10X GSB (750 mmol/L Tris-HCl pH 9.0, 200 mmol/L $(NH_4)_2SO_4$, 0.1 g/L Tween 20), 6 μL of 25 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTP's, 1 μL of each 10 μmol/L specific perimers 1 μL Taq DNA polymerase (100 U/μL), 2 μL of cDNA, and 33 μL sterile PCR water to a final volume of 50 μL. The amplification were carried out for 35 cycles with the following cycling parameters: denaturation at 94 ℃ for 35 sec, annealing at 52 ℃, 58 ℃, 62 ℃ for 40 sec, extension at 72 ℃ for 40 sec preceded by an initial incubation at 94 ℃ for 2 min, and a final extension at 72 ℃ for 5 min. in a Stratagene Robocycler Gradient 40 (Stratagene, La Jolla, CA). Samples were then analyzed at once or stored at 4 ℃ until analyzed by electrophoresis. For detection of PNRSV, PCR condition was the same as described, except using PNRSV specific primer pair, and 5 μL $MgCl_2$.

1.5 Analysis of PCR amplified products

Aliquots (10 μL) of PCR amplified DNA were analyzed by electrophoresis through 1.2% agarose gels for 45 min in 1X TAE buffer at 80 V. Separated DNA fragments were visualized following staining with ethidium bromide (0.5 μg/mL in the gel) using a UV transilluminator and photographed. Sizes of fragment were determined by comparison with DNA Ladder mix (Gene Ruler™).

2 Results

2.1 Comparison of nucleic acid extraction using modified silica capture and Qiagen's RNeasy kits

Samples preparation before RT-PCR amplification was found crucial for the detection of PDV and PNRSV from infected cherry growing in field. The RNA extraction protocol described in this paper has facilitated the routine detection of PDV and PNRSV in their woody hosts using RT-PCR. The method was based on the silica capture and was superior to other methods. Compared with the commercially available RNA extraction kits (RNeasy, Qiagen, Inc., Chatsworth, CA), both of them got expected good quality RNA (Fig. 1) and RT-PCR results (Fig. 2: lane 1 and lane 3), but the method described in this paper is much cheaper and simpler.

Using the explored optimum protocol, it only took less than 1.5 h to finish RNA extraction, and totally about 6-7 h to complete virus detection from virus extraction to analysis of the PCR amplified products. The cycle of virus detection was shortened highly. This method is effective and effi-

cient for virus detection by RT-PCR.

Fig. 1 Electrophoresis analysis of total RNA
Lane: 1. Modified silica capture method, cherry leaf sample; 2. Modified silica capture method, cherry phloem samples; 3. Qiagen's RNeasy kits method, cherry leaf samples; 4. Qiagen's RNeasy kits method, cherry phloem sample

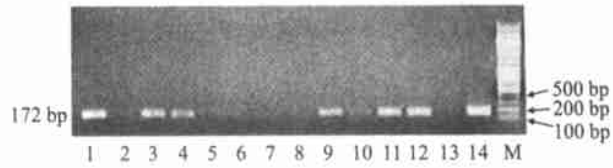

Fig. 2 Agarose gel analysis of PDV RT-PCR products amplified from silica captured total nucleic acids of cherry, peach and plum infected tissue
Lane: 1. PDV infected cherry, sample from leaves; 2. uninfected cherry control; 3. PDV infected cherry leaf RNA extracted by RNeasy kit; 4. sample from infected cherry bark; 5—8. samples from uninfected peach and plum tissue; 9—10. sample from PDV infected cherry buds; 11. PDV infected peach; 12. PDV infected plum; 13. water control; 14. PDV infected control M: DNA Ladder mix

2.2 Samples from different tissue and different plant species

Samples from leaves, buds, and bark of infected cherry, extracted by silica capture, could be detected by PCR for PDV and PNRSV positive (Fig. 2, lane: 1, 4, and 9; Fig. 3, lane: 2, 4 and 5) and isolates from peach and plum (samples from leaves) could also be detected for PDV (Fig 2, lane: 11, 12) These results show that the improved RNA extraction protocol is suitable to not only cherry, but also plum and peach.

2.3 Optimum RT-PCR condition for PDV and PNRSV

During the course of optimizing the RT-PCR protocols for each virus, it was noted that RT (superscript Ⅱ) concentration used during the reverse transcription reaction had a profound effect on the quantity of specifically amplified cDNA that was produced. It was observed that 10～30 U of superscript Ⅱ were required to produce detectable level of amplified DNA following PCR, and less than 5 U is not suitable. Due to the reverse transcriptase was quite expensive, effective and efficient RT concentration we used was 10 U per reaction mixture. For PCR condition, the optimum annealing temperature for PDV was 58 ℃, for PNRSV was 62 ℃. The denaturation and extension temperature were same.

Total RNA extracts and RT (cDNA) samples could be stored for relatively long time under proper condition (at - 20 ℃, several months; at - 80 ℃, for years).

2.4 Results of RT-PCR detection for PDV and PNRSV

Figure 2 and Figure 3 shows agarose gel electrophoretic analysis of PDV and PNRSV isolates from infected tissue of cherry amplified by RT-PCR. The size of the major amplified product for PDV in all case was near 200 bp, about 172 bp (Fig. 2) and for PNRSV, was between 400- 500 bp, about 450 bp (Fig. 3) for PNRSV. These products were not detected in uninfected tissue and water control. All expected DNA fragment were got by RT-PCR.

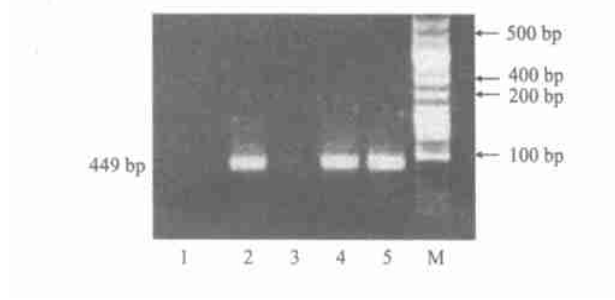

Fig. 3 Agarose gel analysis of PNRSV RT-PCR products amplified from silica captured total nucleic acids of cherry
Lane: 1. uninfected cherry control; 2. sample from PNRSV infected cherry leaves; 3. water control; 4- 5. sample from infected cherry buds and bark. M: DNA Ladder mix

3 Discussion

One of the difficulties with the routine use of RT-PCR for the detection of plant RNA viruses is obtaining sufficient and pure RNA starting materi-

al, cost effectively and efficiently[2, 14]. Tree tissues or other plant tissues which contain high levels of phenolic or polysaccharide compounds are particularly difficult from which to purify[1, 10]. Purification by immunocapture of virus particles using specific antibodies for subsequent use in RT-PCR has also been described[15]. The method of using the commercially available RNeasy extraction columns from Qiagen[16], which is also based on silica capture, fulfils many of the above criteria, its only shortcoming being the relative expense of the method. For this reason, alternative methods were explored. The modified silica capture method used in this paper has all of the advantages of the QIAGEN's RNeasy method with a significant cost saving, making it a valuable additional tool for a great quantity and routine RT-PCR detection from virus infected woody plants. Shortening the procedure while maintaining reliability reduces costs. Costs are further reduced when more than one kind of virus is tested for simultaneously. High-quality RNA suitable for use in RT-PCR could be obtained from cherry, peach and plum by modified silica capture, allowing for detection of PDV, PNRSV LChV-1, LChV-2 and PPV (the results not shown for LChV-1, LChV-2 and PPV in this paper). The ability to use budwood (bark or buds) as a reliable source of viral RNA allowed the routine detection of these viruses in infected plants throughout the year rather than restricting it in the spring or summer months, when leaf or flower blossom tissue was readily available.

To reduce costs and complexity, attempts were made to develop a procedure for the simultaneous detection of several viruses. Since total nucleic acid being extracted, all target cDNA could be got by once reverse transcription (RT), so from one cDNA sample, all virus in question could be detected by PCR. This characteristic make the routine detection and virus survey practical for field woody plants, it is superior to immunocapture purification, which only purify the antibody specific virus. The more complicated procedures improve reliability but take longer time, hence they are more expensive[17, 18] PNRSV was detected early in sweet cherry plantlet *in vitro* with RT-PCR[19], but the virus extraction protocol the author used was difficulty to get enough purified RNA virus from field tissue to detect by RT-PCR.

Using improved RNA extraction protocol based on silica capture described in this paper, we also got expected results to detect PDV, PNRSV and PPV in plum and peach trees, but for pome fruit trees such as apple and pear trees have not being tried.

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改良 RNA 提取法及樱桃 PDV 和 PNRSV 的 RT-PCR 检测

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摘 要: 介绍了一种从木本植物组织中获得高质量 RNA 的快速、简单和高效的核酸提取方法。该方法是基于核酸的二氧化硅捕获, 避免了使用苯酚、氯仿等有机溶剂。利用该方法从樱桃组织中提取的总 RNA 用 RT-PCR 技术检测 PDV, PNRSV 均获得成功。从感病植株的一年生枝的叶片、韧皮部及芽组织中扩增出了预期的目的片段, 即 172 和 449 bp, 而健康组织中无此扩增带。该法提取的总 RNA 用于 RT-PCR 技术检测, 其敏感性至少与商业出售的 Qiagen RNeasy 提取试剂盒相当, 但简单经济。

关键词: 总 RNA 提取; 二氧化硅; 樱桃; PDV; PNRSV; RT-PCR 检测