# DETECTION OF BEET YELLOWS VIRUS BY RT-PCR AND IMMUNOCAPTURE RT-PCR IN *TETRAGONIA EXPANSA* AND *BETA VULGARIS*

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**Summary. –** Two sensitive methods, RT-PCR with phenol-extracted RNA or Triton X-100-released RNA and immunocapture RT-PCR (IR-RT-PCR) were used for the detection of Beet yellows virus (BYV) in young and old leaves of *Tetragonia expansa* and sugar beet (*Beta vulgaris*) and in sugar beet roots. Four oligonucleotide primer pairs proved suitable for the detection of BYV. The release of BYV RNA with Triton X-100 was shown to be a very effective and easy as compared to isolation of total RNA by phenol extraction with the same or higher sensitivity of subsequent PCR. Using the Triton X-100 release of RNA and IC-RT-PCR the sensitivity of detection was so high that pg amounts of BYV RNA occurring in dilutions up to $10^{-6}$ of saps from young *Tetragonia* and sugar beet leaves could be detected.

**Key words:** Beet yellows virus; immunocapture-RT-PCR; RT-PCR; Triton X-100

## Introduction

BYV and Beet mild yellowing virus (BMYV) are responsible for sugar beet yellows, one of the most important diseases of sugar beet, *Beta vulgaris ssp. vulgaris* (Russell, 1965). BYV is the type member of the genus *Closterovirus*. It has flexuous filamentous particles, 1250–1450 nm long and 12 nm in diameter, containing 5% of single-stranded positive-sense RNA (Carpenter *et al.,* 1977; Candresse and Martelli, 1995). The genome of BYV is composed of 15,480 nt encoding nine putative ORFs (for review see Dolja *et al.,* 1994; Agranovsky, 1996).

The detection of BYV by serological assay, such as double antibody-sandwich ELISA (DAS-ELISA) (Chevalier and Putz, 1982), amplified DAS-ELISA (Stevens *et al.,* 1997; Kundu, 1999) and by some microscopical methods, such as immunogold labeling for electron microscopy (IGL) and immunogold-silver staining for light microscopy (IGS), are well established (Kundu, 1999). Amplification of a targeted nucleic acid-based assay, the RT-PCR has been widely used for RNA virus detection. The coat protein (CP) gene is frequently used for plant virus detection. The CP gene was recently used in the detection closteroviruses, namely BYV (Stevens *et al.,* 1997) and Citrus tristeza virus (CTV) (Mehta *et al.,* 1997). The CP gene together with papain-like leader proteinase (L-Pro), replicase (putative methyltransferase (MT)), helicase (HEL), RNA-dependent RNA polymerase (POL), and heat shock protein 70 (HSP70) are the best conserved genes among BYV strains (Agranovsky, 1996).

In this study we used L-Pro, MT and HSP70 gene for RT-PCR-based detection of BYV. Besides classical phenol extraction of total plant RNA we used also Triton-X-100 for release of RNA from virions of BYV. We also employed the

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**Abbreviations:** AMV = Avian myeloblastosis virus; BSA = bovine serum albumin; BYV = Beet yellows virus; CTV = Citrus tristeza virus; DAS-ELISA = double-antibody sandwich ELISA; HEL = helicase; HSP70 = heat shock protein 70; IC-RT-PCR = immunocapture RT-PCR; L-Pro = papain-like leader proteinase; M-MLV = Moloney murine leukemia virus; MT = methyltransferase; PBS = phosphate-buffered saline; p.i. = post infection; PLRV = Potato leafroll virus; POL = RNA-dependent RNA polymerase; PPV = Plum pox virus; TRV = Tobacco rattle virus

IC-RT-PCR method, in which virus RNA obtained from antibody-captured viral particles is used as a template for cDNA synthesis prior to PCR amplification (Wetzel *et al.,* 1992; Nolasco *et al.,* 1993).

## Materials and Methods

*Plant material and virus isolate.* For initial optimization of PCR methods for the detection of BYV we used leaves of infected *Tetragonia expansa*. The BYV isolate Praha-Ruzyne was maintained in sugar beet plants, variety Domona. *Tetragonia* and sugar beet plants in two true leaves stage were infected using viruliferous *Myzus persicae* Sulzer as described earlier (Kundu and Ryšánek, 1999). The upper leaves of *T. expansa* and *B. vulgaris* showing symptoms three weeks post infection (p.i.) were used for routine testing. Later, both central and external leaves and roots (central part) of greenhouse-grown sugar beet (6–12 months p.i.) and bottom leaves of *Tetragonia* (3 months p.i.) were tested as well. Only fresh tissues were subjected to analysis.

*Phenol extraction of total RNA* from 1 g of BYV-infected plant material was performed according to the procedure described by Kundu (2003). The plant material dilutions in the range of 1 $x10^{-1}$–2 $x10^{-6}$ were prepared and subjected to the extraction either by the procedure employing Tris buffer (Kundu, 2003; Robinson, 1992) or glycine buffer (Robertson *et al.*, 1991). For comparison, also a method based the on use of the RNeasy Kit (Qiagen Inc.) was employed (100 mg of plant material was used). Extracts from healthy plants prepared by the same methods were always included as negative controls.

*Release of BYV RNA with Triton X-100.* A BYV-infected plant material was grinded in liquid nitrogen, homogenized in sterile water (1:10), centrifuged at 10,000 rpm for 10 mins and the obtained supernatant was serially diluted as described above. Triton X-100 was added to each dilution to final concentration of 1%. To include a special control, in some experiments, this step was omitted. The mixture was incubated at 60°C, 65°C or 70°C for 10 mins with occasional mixing.

*Immunocapture of virus particles.* Virus particles were captured on a solid-phase pre-coated with virus-specific antibodies. The first steps were performed as in DAS-ELISA as described by Clark and Adams (1977) either in microtiter plates or in microtubes, coated with antibodies against BYV (Loewe) diluted 1:200 in a carbonate buffer. After washing with PBS the wells or microtubes were filled with 200 µl aliquots of plant extracts in PBS-Tween with 2 g/l BSA and 20 g/l PVP diluted as described above. After incubation either overnight at 4°C or for 1 hr at 37°C and washing with PBS, the captured virus particles were treated with 1% Triton X-100 in DEPC-treated water. To each well or microtube 100 µl of 1% Triton-X-100 was added and incubated at 70°C for 10 mins with occasional mixing (Wetzel *et al.,* 1992). For comparison, also 1x SSC (Koenig *et al.,* 1995) or AMV RT buffer was emploed in this step in some experiments. RNA was then transferred to a microtube and used as a template for cDNA synthesis.

*Primers.* Four pairs of oligonucleotide primers were designed based on the BYV sequences published by Agranovsky *et al.* (1994) and optimized by the software the Oligo program.

The sense primers:

BYV-1A (5'-TTCATTACTTTTGGGACCTTGCG-3', nt 1008–1030)

BYV-2A (5'-CTATTCGGACCACCCTGCG-3', nt 2132–2150)

BYV-3A (5'-TAAGCCGCACTACAAGACAGAG-3', nt 9871–9892)

BYV-4A (5'-CTTATGGTGGGTGGGTCTTCT-3', nt 10601–10621)

The anti-sense primers:

BYV-1B (5'-CAGCGTGGGCGAGGTAACAT-3', nt 1631–1650)

BYV-2B (5'-AGGAGAATACGCTGAACGAGTGAT-3', nt 2760–2783)

BYV-3B (5'-ATTTTGTAGTTCACGGGCAGTT-3', nt 10359–10380)

BYV-4B (5'-AATCGTAAGCAGCGTTTCCA-3', nt 11101–11120

The primer pairs designed from the 5'-proximal part of ORF 1a containing a domain of L-Pro (BYV-1A/1B) and MT (BYV-2A/2B) showed a 99.2 % sequence homology between two main BYV isolates, BYV-U (Ukraine) (NC_001598) and BYV-C (California) (AF0056575). The other two primer pairs (BYV-3A/3B and BYV-4A/4B) designed from ORF3, HSP70 protein gene, showed a 95.8 % sequence homology between the isolates BYV-U, BYV-C and BYV-G (Germany) (X73457). All samples testing in this study was done with the primer pair BYV-2A/2B.

*Reverse transcription.* Synthesis of first strand cDNA was performed as follows: 1 µl of RNA extract (regardless of the extraction method used) was diluted to 10 µl with DEPC-treated water and 10 pmoles of the anti-sense primer was added. The mixture was incubated at 70°C for 5 mins, chilled on ice and complemented with all remaining components of RT reaction mixture: 8.25 µl of RNase-free water, 5 U/0.5 µl of AMV reverse transcriptase, 5 µl of the buffer for AMV reverse transcriptase, 20 U/0.5 µl of RNasin and 0.2 mmol/l dNTP, (all Promega). Some experiments were also done without heat incubation before completion the RT reaction mixture. The reaction mixtures was incubated at 42°C or 48°C for 1 hr, boiled for 5 mins and chilled on ice. For comparison, the Enhanced AMV reverse transcriptase (Sigma) with transcription at 60°C and M-MLV (Promega) with transcription at 37°C were also tested.

*cDNA synthesis.* The PCR reaction mixture (24 µl) containing 2.5 µl of *Tfl* DNA polymerase buffer, 2.5 U/0.5 µl of *Tfl* DNA polymerase, 10 pmoles of each sense and antisense primers, 0.2 mmol/l dNTPs, 1.25 mmol/l $MgSO_4$ (Promega) and sterile deionized water was prepared. Alternatively, 2 U/1 µl of Dynazyme (Finnzymes) with appropriate buffer was used instead of *Tfl* DNA polymerase. One µl of cDNA was used in each reaction. In all cases the PCR mixture was overlaid with 25 µl of mineral oil. PCR was carried out in a MJ Research PTC 150 Thermocycler using the following conditions: initial incubation at 72°C for 5 mins, 40 cycles of 94°C/1 min, 60°C/30 sesc and 72°C/1 min, with a final 4 mins elongation step at 72°C.

*Analysis of PCR products.* Aliquots of PCR mixtures were mixed with ethidium bromide (0.5 µg/ ml), analyzed by 1% agarose gel electrophoresis in 1x TBE buffer (90 mmol/l Tris-borate and 2 mmol/l EDTA) and visualized under UV light. The size of PCR products was determined using DNA size markers (MBI Fermentas).

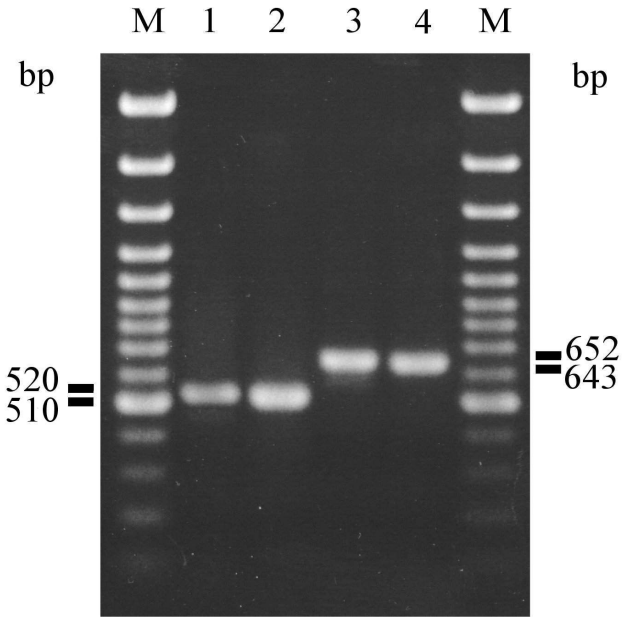


**Fig. 1**

**RT-PCR with RNA extracted with the Qiagen kit from young *T. expansa* leaves**

Primer pairs BYV-4A/AB (expected length of the PCR product 520 bp, lane 1), BYV-3A/3B (510 bp, lane 2), BYV-2A/2B (652 bp, lane 3), BYV-1A/1B (643 bp, lane 4). DNA size marker, 100 bp ladder (lanes M),

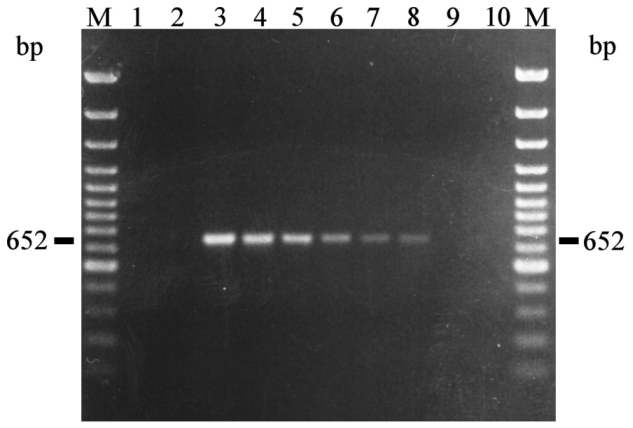


**Fig. 2**

**RT-PCR with RNA released from upper leaves of *T. expansa* with Triton X-100**

Crude sap dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, 1 x $10^{-5}$, 2.5 x $10^{-5}$, 5 x $10^{-5}$, $10^{-6}$ and 2 x $10^{-6}$ (lanes 1–9), negative control (lane 10). DNA size marker, 100 bp ladder (lanes M).

## Results and Discussion

The objective of this study was to optimize conditions for the detection of BYV by RT-PCR and to develop a simple method for sample preparation. All four primer pairs tested amplified the expected RT-PCR product (Fig. 1). The genes selected for primer designing, namely L-Pro, MT, HSP70 and CP are highly conserved among BYV strains (Agranovsky, 1996). A high sequence homology was observed among BYV isolates in the genome part that was amplified by RT-PCR and includes C-terminal part of L-Pro, N-terminal part of MT and HSP70 (see Materials and Methods). BYV L-Pro, MT and HEL proteins have membrane-binding function and are probably associated with induction of typical cytopathic effect and formation of so called BYV-like vesicle (Zinovkin *et al.,* 2003). Summing up these data, the primers described above appear to be reliable for a large scale RT-PCR-based detection of BYV.

The methods of total RNA extraction tested were not found to affect significantly the results of PCR. This finding is similar to those of Stevens *et al.* (1997), though it seemed that precipitation of RNA with LiCl generated slightly stronger bands of PCR products in gels (data not shown). Extraction of RNA using the RNeasy Qiagen Kit required similar efforts as classical phenol extraction. However, the exclusion of hazardous chemical is an unbeatable advantage of the former method. Similar results were obtained irrespective of either the temperature at which reverse transcription was carried out (42 or 48°C with AMV reverse transcriptase, 37°C with M-MLV reverse transcriptase), or the polymerase used (*Tfl* DNA polymerase or Dynazyme). However, transcription at 60°C with the Enhanced AMV reverse transcriptase was unsuccessful. The dilution end-point of RT-PCR with total RNA from young leaves of both *Beta vulgaris* and *Tetragonia expansa* was usually between $10^{-5}$ and $10^{-6}$ (data not shown).

The release of RNA from BYV particles in crude homogenate with Triton X-100 was found to be an easy and effective method of sample preparation with final results equal or better than those obtained with extraction of total RNA. Dilution end-points for various plant materials were 1:$10^{-6}$ for *T. expansa* upper leaves and sugar beet central leaves, 1:5 x $10^{-4}$ for *T. expansa* bottom and sugar beet external leaves, and 1:2.5 x $10^{-5}$ for sugar beet roots (Figs 2 and 3). The best results were obtained with direct addition of the RT reaction mixture and Triton X-100 to crude plant extracts and subsequent RT reaction. Both the incubation at higher temperatures (60°C, 65°C and 70°C) and omitting Triton X-100 reduced slightly the efficiency of PCR amplification (data not shown). Heating plant extracts with Triton X-100 for 5 or 10 mins at 60°C, 65°C or 70°C before RT had an adverse effect on product formation and the sensitivity decreased two- to five-fold (Nolasco *et al*., 1993). However, Kundu (2003) used an incubation step of 60°C for 5 mins prior to the RNA release form crude saps containing fruit tree viruses. On the other hand, Thomson

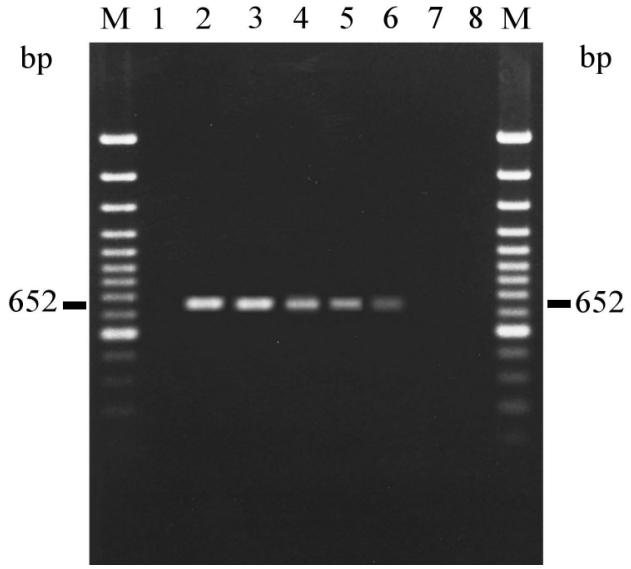


**Fig. 3**

**RT-PCR with RNA released from roots of *B. vulgaris* with Triton X-100**

Crude sap dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, 1 x $10^{-5}$, 2.5 x $10^{-5}$, and 5 x $10^{-5}$ (lanes 1–7), negative control (lane 8). DNA size marker, 100 bp ladder (lanes M).

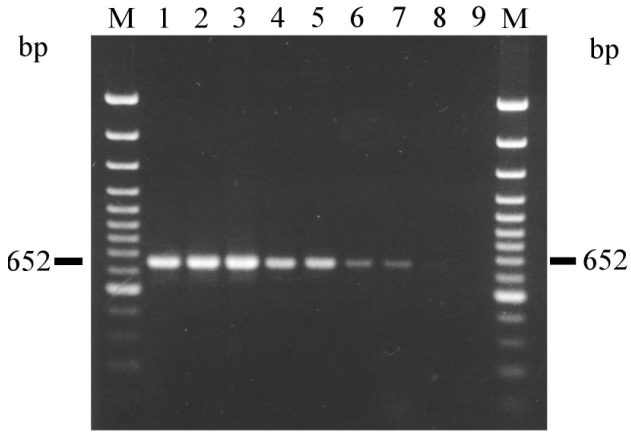


**Fig. 4**

**IC-RT-PCR with upper leaves of *T. expansa***

The IC step lasted overnight. Crude sap dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, 1 x $10^{-5}$, 2.5 x $10^{-5}$, 5 x $10^{-5}$, 1 x $10^{-6}$ and 2 x $10^{-6}$ (lanes 1–9), negative control (lane 10). DNA size marker, 100 bp ladder (lanes M).

and Dietzgen (1995) used successfully a heating at 95°C for 10 mins to release virus RNA from small pieces of intact plant tissue. Heating BYV particles to 42°C or 48°C appeared sufficient for at least partial disintegration of viral particles

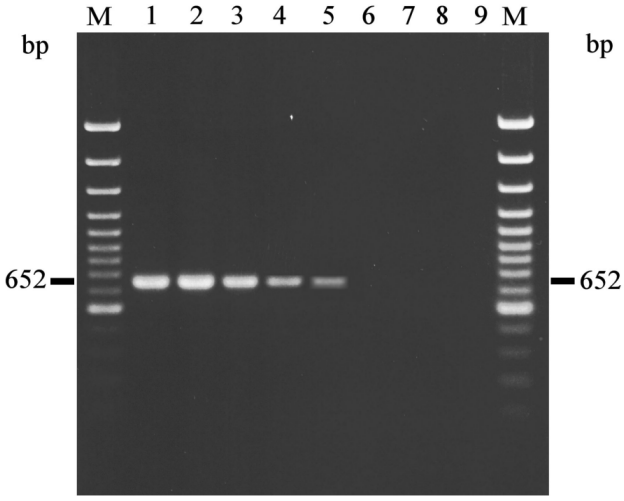


**Fig. 5**

**IC-RT-PCR with upper leaves of *T. expansa***

The IC step lasted 1 hr. Crude sap dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, 1 x $10^{-5}$, 2.5 x $10^{-5}$, 5 x $10^{-5}$, 1 x $10^{-6}$ and 2 x $10^{-6}$ (lanes 1–9), negative control (lane 10). DNA size marker, 100 bp ladder (lanes M).

and making the RNA accessible to RT. Initially, RNA denaturation by heating or by methylmercury hydroxide during did not appear to be necessary for crude extract preparation (Wetzel *et al.,* 1991). This step was later omitted without any decrease of sensitivity of the assay. RT-PCR with RNA released with Triton X-100 usually failed at low dilutions of plant extracts (1:10 and 1:100), probably due to the presence of inhibitors of either RT or PCR. Adverse effects of these putative inhibitors could be overcome by clarification step after homogenization and by diluting the crude sap to at least 100 times or more (Kundu, 2003). In this way, however, the ability of this method to detect the virus at very low concentrations would be reduced. Despite these inhibitors, the release of virus RNA with Triton X-100 represents an alternative way to simple and quick total RNA extraction not required highly hazardous chemicals. Moreover, it is less expensive and enables the same or better sensitivity in subsequent RT-PCR.

The IC RT-PCR was also performed with a dilution series of a plant sap (Fig. 4). This assay had the same maximum dilution endpoint (1:$10^{-6}$) as the Triton X-100 method. The use of 1% Triton X-100, SSC or AMV RT buffer for releasing BYV RNA yielded comparable results. The simplest protocol turned out to be the best, namely addition of complete RT reaction mixture and Triton X-100 into wells/tubes without any previous treatment and running the RT reaction at 42°C. Reducing the IC step to 1 hr at 37°C reduced 100–1,000 times the sensitivity of the assay (Fig. 5). The dilution endpoints for various plant materials were the same as for

direct Triton X-100 release of RNA from plant extracts. Using IC-RT-PCR, Wetzel *et al.* (1992) obtained 250 times higher sensitivity of Plum pox virus (PPV) detection (in terms of pg of virus/ml) as compared to direct RT-PCR. However, in our hands IC-RT-PCR did not increase the sensitivity of BYV detection as compared to RT-PCR. Indeed, decreasing the incubation time for the IC step led to a substantial decrease in sensitivity. Therefore, IC-RT-PCR can only be recommended for use if the presence of inhibitory compounds in the materials tested is expected (Kruse *et al.,* 1994) and the extracted RNA cannot be properly diluted (see above). Wetzel *et al.* (1992) have treated pre-heated immunocaptured PPV particles with 1% Triton X-100 at 65°C. Initially, we applied this step successfully to our IC-RT-PCR. Later, Nolasco *et al.* (1993) have suggested that this step could be deleterious to RNA. Later, we omitted this step from our IC-RT-PCR without loss of sensitivity.

The results presented above showed that RT-PCR and IC-RT-PCR are highly sensitive tools for BYV detection. Fifteen to 30 mg of virus particles were purified from 100 g of BYV-infected leaves of *T. expansa* (Rogov *et al*., 1993). Considering the fact that BYV contains 5% of RNA, the estimated sensitivity of our RT-PCR-based methods is of the order of pg of BYV RNA (~7.5–15 pg). Our results correspond to those of many other authors (e.g. Wetzel *et al.,* 1992; Hadidi *et al.,* 1993; Van der Wilk *et al.,* 1994) working with PPV, Potato leafroll virus (PLRV) and Tobacco rattle virus (TRV), respectively.

Summing up, the described RT-PCR and IC-RT-PCR exploiting a simple procedure of release of BYV RNA from plant materials with Triton X-100 have 100–1000 times higher sensitivity than ELISA (Kundu, 1999) and represent tools more convenient for routine testing BYV in sugar beet crop.

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