Total RNA extraction method and *Prunus* species influence the detection of *Plum pox potyvirus* by real-time RT-PCR

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ABSTRACT

Successful total RNA (totRNA) extraction is a prerequisite for a successful real-time PCR. In the present work we compared one manual and one automated totRNA extraction method for detection of *Plum pox potyvirus* (PPV) in leaves of different *Prunus* sp. using developed real-time RT-PCR assay. Advantages and disadvantages of compared methods are described in the view of sensitivity, reproducibility and in terms of laboratory use. The results suggest that the column based totRNA extraction method is more effective for apricot, plum and peach. In the case of damson the differences in real-time PCR results between both extraction methods were negligible. In case of negative results obtained with automated method, manual column based extraction method should be used additionally.

Key words: 18S rRNA, MGB, PCR inhibition, PPV

IZVLEČEK

RASTLINSKA VRSTA IN METODA IZOLACIJE CELOKUPNE RNA VPLIVATA NA USPEŠNOST DETEKCIJE VIRUSA ŠARKE S PCR V REALNEM ČASU

Uspešnost izolacije RNA je osnova za uspešno izvedbo reakcije PCR v realnem času (qPCR). V svojem delu smo primerjali ročno in avtomatsko metodo izolacije celokupne RNA (totRNA) iz listov različnih rastlinskih vrst iz rodu *Prunus*. Uspešnost izolacije totRNA smo preverjali z uvedeno metodo qPCR za detekcijo virusa šarke (*Plum pox potyvirus* – PPV). Prednosti in slabosti uporabljenih metod smo ugotavljali s primerjavo občutljivosti detekcije, ponovljivosti in intenzivnosti laboratorijskega dela. Rezultati raziskave so pokazali, da je ročna metoda izolacije totRNA učinkovitejša pri marelicah, breskvah in slivah, medtem ko so bile razlike med obema metodama pri ciborah zanemarljive. Pri uporabi preizkušene avtomatske metode izolacije totRNA je priporočljivo, da v primeru negativnih rezultatov le-te še dodatno preverimo z uporabo ročne metode izolacije.

Ključne besede: 18S rRNA, MGB, inhibicija PCR, PPV

1 INTRODUCTION

Sharka caused by the *Plum pox virus* (PPV) is the most devastating viral disease of stone fruits, predominately peaches, nectarines, apricots, plums and prunes. In susceptible varieties it can cause high yield losses and tree decline. The fruit quality is also affected. Estimated cost associated with sharka management worldwide in the last 30 years exceeded 10 000 million euros. Costs of sanitary controls, surveys and eradication programs, but not of indirect trade losses, are included in the estimation (Cambra et al., 2006).

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Sharka was first observed in Slovenia in 1987. In the following years the presence of PPV was confirmed by Double Antibody Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA) in samples originating from orchards, individual trees and propagation material of stone fruits. Due to the high incidence of PPV infection a systematic survey of PPV was initiated in 1998 to prevent and control the spread of sharka. Since 2000 the survey has focused mainly on nurseries, mother trees and stool-beds (Viršček Marn et al., 2004).

Due to the possibility of low virus concentration in plants and its irregular distribution, a great sensitivity and accuracy is required in PPV diagnostics.

Real-time PCR and RT-PCR is extensively used for pathogen detection and quantification; it is a method of choice for gene quantification and it is more and more used also in plant virology. Its use also eliminates or reduces the use of hazardous chemicals (e.g. ethidium bromide) in the laboratories.

Real-time PCR is a method of choice in the view of sensitivity and accuracy but the choice of target tissue and RNA extraction methods can also greatly influence the detectability of viruses in plant material. Plants are known to contain a lot of possible PCR inhibitors such as polysaccharides, polyphenols, proteins and other plant secondary metabolites (Gambino et al., 2008; Demeke and Adams, 1992; Osman and Rowhani, 2006). Their content differs between different plant organs and changes during the growing period. Previous experiences show the importance of validating RNA extraction procedure for different sample matrixes and the ability of the extraction method to provide a suitable nucleic acid free of PCR inhibitors from each sample matrix. Numerous RNA extraction methods have been used in the preparation of total RNA (totRNA) from woody plants with a more or less sufficient quantity of isolated totRNA and reduction of inhibitors, but most of them are time consuming and technically demanding (Gambino et al., 2008; Singh et al., 2002; Maliyakal, 1992).

In the present work we have compared one manual and one automated method for totRNA extraction from leaves of different *Prunus* sp. The quality and quantity of extracted totRNA was evaluated using developed real-time RT-PCR assay for the detection of PPV. Advantages and disadvantages of compared methods are discussed in the view of sensitivity, reproducibility and in terms of laboratory activities.

2 MATERIALS AND METHODS

2.1 Plant material
10 samples of each of 4 different *Prunus* species (*P. armeniaca* (apricot), *P. domestica* ssp. *domestica* (plum), *P. domestica* ssp. *insititia* (damson) and *P. persica* (peach)) were included in the study. Leaves were collected from PPV infected trees previously tested by DAS-ELISA. For each plant species a negative tree was also included.

2.2 Total RNA extraction
TotRNA was extracted from leaf tissue of different *Prunus* species by two extraction methods. Extracts were prepared by homogenizing 100 mg of fresh leaf tissue in 1100 µL of RLT extraction buffer (RNeasy Plant Mini kit (Qiagene, Valencia, USA)). The homogenisation was done using TissueLyser (Retsch, Haan, Germany) shaker for 3 min at 30 Hz in the presence of one stainless steel bead of 5 mm in diameter. Extracts were centrifuged and supernatant was used for further totRNA extraction.

In Method 1 (manual method), 450 µL of each extract were used for totRNA extraction using RNeasy Plant Mini kit (Qiagene, Valencia, USA) according to the manufacturer's instructions. The totRNA elution volume was 50 µL.

In Method 2 (automatic method), the other 450 µL of each extract were used for totRNA extraction using RNeasy Tissue Kit (Qiagene, Valencia, USA) with KingFisher mL instrument (Thermo Electron, Vantaa, Finland), and according to the manufacturer's instructions. The totRNA elution volume was 100 µL.

5 µL of isolated totRNA were treated with DNase I for further analysis with real-time PCR method. The reaction conditions were according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, USA), using 1 U of DNase I. The final volume of the reaction was 50 µL. Aliquots of the resulting RNA were analyzed by electrophoresis on 1 % agarose gel with ethidium bromide staining to check its quantity and integrity (data not shown).

2.3 Reverse transcription
Equalized amounts of DNase treated totRNA (according to agarose gel analysis) was reverse transcribed using a cDNA Archive kit (Applied Biosystems, Foster City, USA) according to manufacturer's instructions with minor modifications. 25 U of RNase inhibitor (Applied Biosystems, Foster City, USA) were added to the final volume of 25 µL. Reactions were incubated at 25°C for 10 min followed by 37°C for two hours.

2.4 Real-time PCR primers and probe design
A high homology 103 bp region of the PPV genome was selected as a target for PCR amplification by aligning 61 sequences published in GenBank database. Primer Express software v2.0 (Applied Biosystems, Foster City, USA) was used to design primers and sequence specific hydrolyzing TaqMan MGB probe (Table 1). The probe was labeled at the 5'-end with 6-carboxyfluorescein (FAM) dye. A GenBank

BLAST search for short nearly exact matches revealed no significant database alignments of either primer or the probe with other than the region of interest in the PPV genome.

Table 1: Primers and probe used for PPV detection

Primer name	Orientation	Sequence (5'→3')
PPV-f	forward	GGA GAC ACA AGT GGA GTA TCC AAT AAA
PPV-r	reverse	AAT GTA CGC TTC AGC CAC GTT A
PPV-probe	probe	FAM-CAC TTT TAG ACA AAT TAT GGC A-MGB

Assay specific for eukaryotic 18S rRNA (PDARS, Applied Biosystems, Foster City, USA) and TaqMan RNaseP Detection Reagents (Applied Biosystems, Foster City, USA) were used for confirmation of successful totRNA extraction and for detection of inhibitors in real-time PCR.

2.5 Real-time PCR (TaqMan) assay
The cDNA of each sample was used in separate real-time PCR reactions for detection of PPV and 18S rRNA.

The 20 μL real-time PCR reactions for PPV were performed in 1X TaqMan Universal Master Mix (Applied Biosystems, Foster City, USA), with 900 nM of each primer and 200 nM probe (Table 1) and 1/10 diluted cDNA as a template.

The 20 μL real-time PCR reactions for 18S rRNA were carried out according to the manufacturer's instructions with 1/10 diluted cDNA as a template.

Real-time PCR reactions were run in duplicates for each undiluted or diluted cDNA on ABI PRISM 7500 (Applied Biosystems, Foster City, USA) using universal cycling conditions (50ºC for 2 min, 95ºC for 10 min, 45 cycles of 95ºC for 15 s and 60ºC for 1 min). Data were acquired and analyzed using the ABI PRISM 7500 Real-Time PCR System Sequence Detection System Software v1.3.

cDNA from a non-infected plant of each *Prunus* species was used as a negative control. A PCR non-template control (NTC) was set up with molecular grade water instead of template.

10-fold serial dilutions of cDNA of each *Prunus* species (samples: apricot 2, damson 10, peach 2, plum 3) were used to obtain standard calibration curve for PPV. For the assessment of inter-run variability a standard calibration curve of three 10-fold serial dilutions of the same sample (apricot 2) was prepared on each of four different real-time PCR plates.

The 18S rRNA assay was used for confirmation of successful totRNA extraction. Additionally, assay for *RNase P* gene was used for detection of inhibitors in real-time PCR step. 10 ng/μL of control human DNA from TaqMan RNaseP Detection Reagents (Applied Biosystems, Foster City, USA) was added to each real-time PCR reaction as external standard of inhibition control.

2.6 Performance of real-time PCR
The ABI Prism 7500 Real-Time PCR System Sequence Detection Software v1.3 generated fluorescence data. For the comparability of the results, identical settings for threshold and baseline were used in all experiments for PPV detection. The baseline was established automatically and the threshold was set at 0.2. The dynamic range of PPV real-time PCR reactions was determined by performing 10-fold serial dilutions of each *Prunus* species template cDNA in water. Standard calibration curves were obtained by plotting cycle threshold (Ct) values against the log of the sample cDNA dilutions.

The real-time PCR efficiency was calculated by the following equation: PCR efficiency [E] = $10^{(1/-S)}$ (Pfaffl, 2001; Rasmussen, 2001).

3 RESULTS

3.1 Total RNA extraction
The quantity of extracted totRNA differed greatly between plant species. The quantity of totRNA from peach samples with Method 1 was high and uniform while the quantity with Method 2 was much lower and there was a big variation between samples (data not shown).

With other three *Prunus* species the used totRNA extraction methods were comparable but the quantity of extracted totRNA was low.

3.2 Real-time PCR (TaqMan) assay
All samples were tested for the presence of eukaryotic 18S rRNA and the results confirmed successful totRNA extraction.

Real-time PCR parameters were determined by amplifying PPV RNA from different *Prunus* species (Table 2). A comparison was made between Method 1 and Method 2. The average coefficients of determination (R^2) were between 0.987 and 0.999 indicating a good correlation between the amount of template and Ct values. Efficiencies and dynamic range of detections of the real-time PCR were assessed (Table

2). No significant differences were observed in efficiency between both isolation methods and between the tested species. Differences were observed in the dynamic range between both methods for plum and peach. The dynamic range with Method 2 was broader for plum but narrower for peach.

The amplification of 10-fold serial dilutions of selected samples of each species (apricot 2, damson 10, peach 2, plum 3) was performed. The Ct values of undiluted samples of peach, apricot and damson ranged between 31 and 39 while the Ct values of samples diluted 1/10 ranged between 18 and 23, what indicates a strong inhibition in undiluted samples using both methods. The strong inhibition of amplification was observed also for plum with Method 2 while the inhibition with Method 1 was weak.

The amplification of *RNase P* gene as external standard of inhibition control (control human DNA) was performed for all tested samples. The results indicate elimination of inhibition in 1/10 diluted samples of all species. Standard deviation (SD) between the different samples was constant and similar to positive control (control human DNA diluted in ddH2O) (Method 1: the average Ct is 27.33 ± 0.14 and Method 2: the average Ct is 27.07 ± 0.13).

Table 2: Comparison of dynamic ranges, efficiencies (E) and coefficients of determination (R^2) between both totRNA extraction methods and plant species

	Method 1			Method 2		
	Dynamic range	E	R^2	Dynamic range	E	R^2
plum	$5 \times 10^{-1} - 10^{-4}$	2.08	0.987	$5 \times 10^{-1} - 10^{-6}$	2.01	0.996
peach	$5 \times 10^{-1} - 10^{-6}$	1.99	0.995	$5 \times 10^{-1} - 10^{-4}$	2.06	0.994
apricot	$5 \times 10^{-1} - 10^{-6}$	2.04	0.999	$5 \times 10^{-1} - 10^{-6}$	1.95	0.995
damson	$5 \times 10^{-1} - 10^{-6}$	1.97	0.999	$5 \times 10^{-1} - 10^{-6}$	2.02	0.997

Most of tested samples were in linear dynamic range of specific species independently of the amount of totRNA used for reverse transcription reactions. All other samples were treated as negative (Fig. 1). Only four apricot samples were found negative with Method 2 and one of them also with Method 1.

According to average Ct of tested samples, the totRNA extraction from plum with Method 1 was much more effective than the extraction with Method 2. The average difference in Ct between Method 1 and Method 2 for all 10 samples was 6.65 ± 3.79. The Method 1 performed better.

For damson we observed lower variability in average Ct compared to plum. The average difference in Ct between Method 1 and Method 2 for all damson samples was -0.34 ± 0.85. Both methods were comparable.

The average difference in Ct between Method 1 and Method 2 for all 10 samples of peach was 2.22 ± 1.48. The Method 1 performed better.

The average difference in Ct between Method 1 and Method 2 for positive apricot samples was 4.04 ± 2.20. The Method 1 performed better.

Figure 1: Real-time PCR cycle threshold (Ct) values for detection of PPV in samples of four different *Prunus* species extracted with two different totRNA extraction methods.

Prunus persica (PEACH)

Prunus domestica ssp. *domestica* (PLUM)
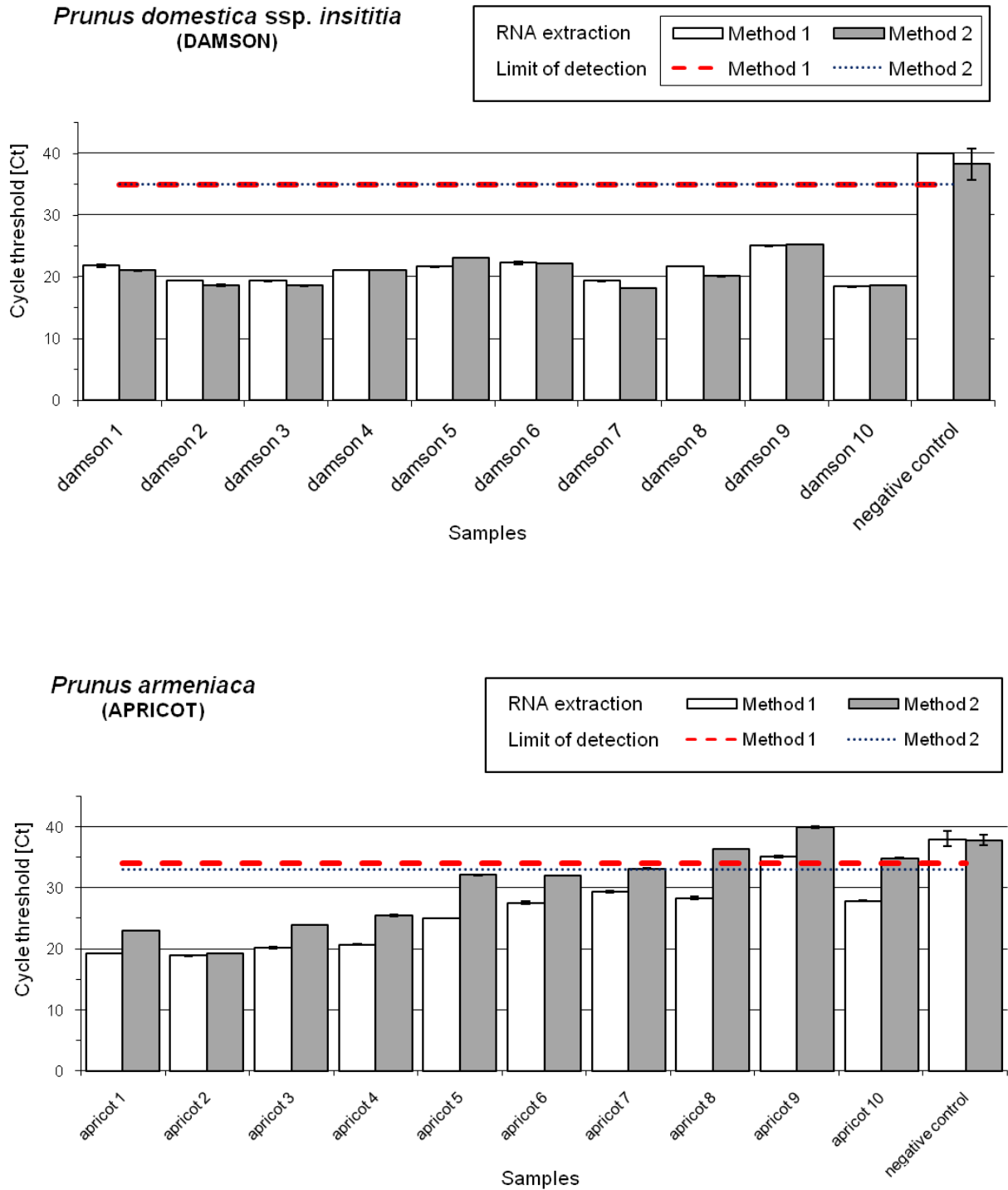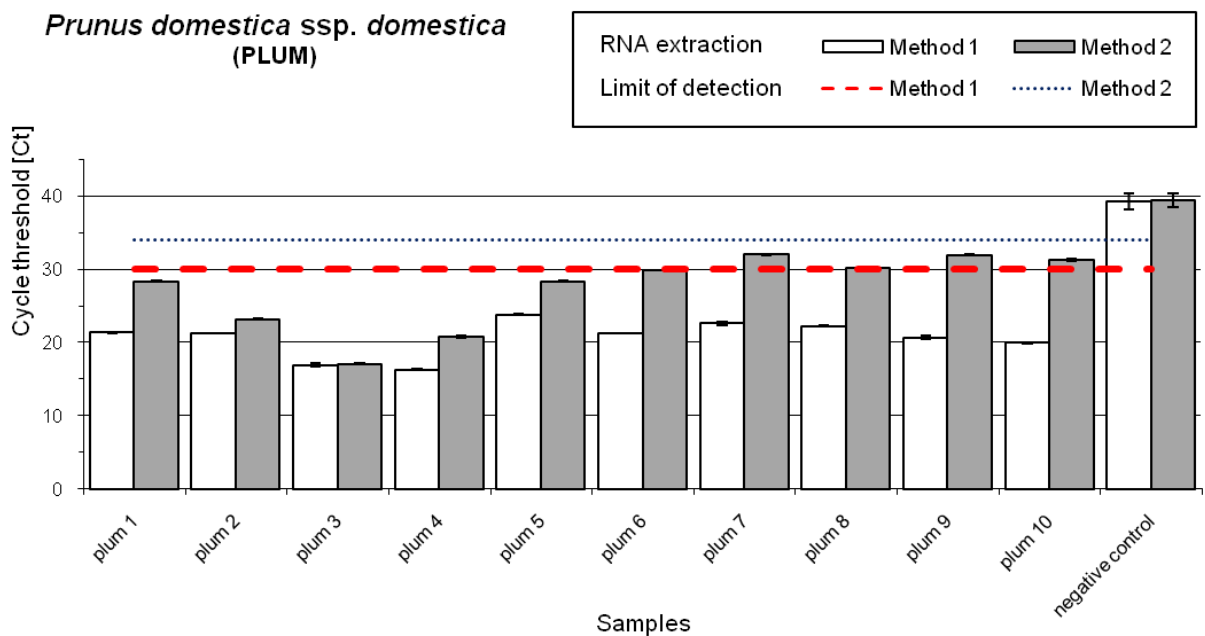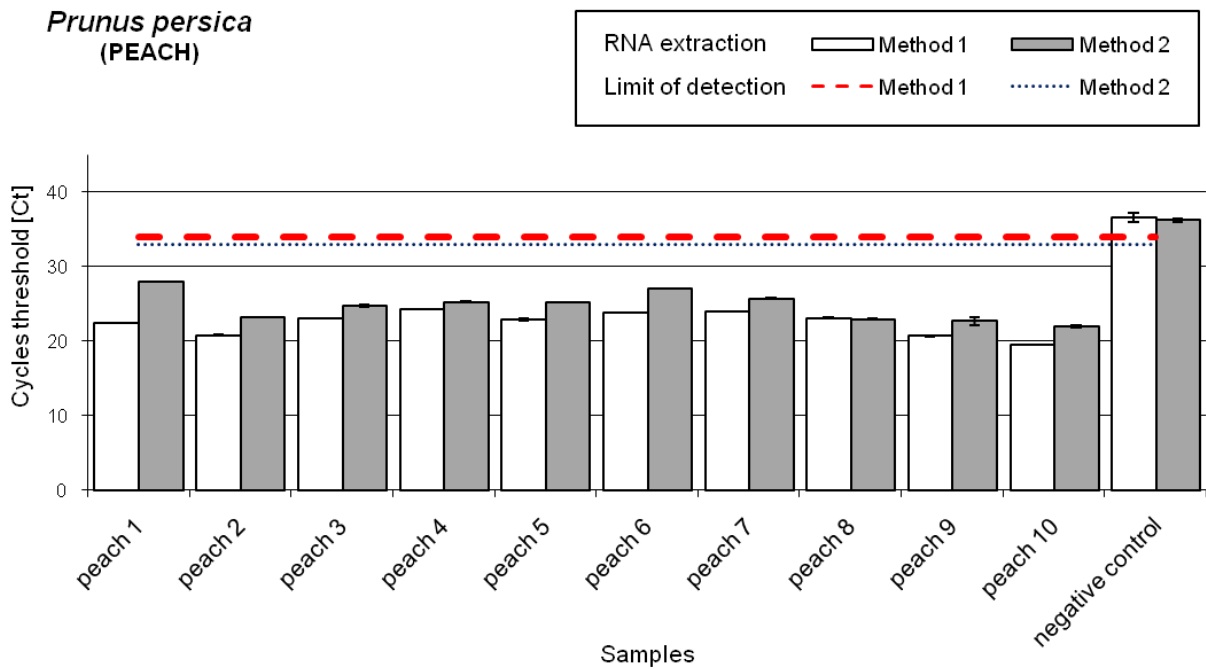
Samples with C_t values of less than marked Ct, indicating the detection limit for each totRNA extraction method and tested *Prunus* species, were considered as positive.

3.3 Inter-run variability

Inter-run variability was assessed by preparing the standard curves with three 10-fold serial dilutions of the same sample in four different plates. The quantification was linear over a range of all three log units. The average slope of all compared standard curves was –

3.355 ± 0.028, the efficiency was 1.986 ± 0.011 and the average R^2 was 0.997 ± 0.003 (data not shown). These results show that the conditions of amplification on all plates were stable and the comparison of the results from different plates was possible.

4 DISCUSSION

PCR based methods, including real-time PCR are widely used for the detection of plant viruses. The choice of nucleic acid extraction procedure can greatly influence the reliability of detection and quantification of target sample. It is important to validate the extraction procedure for different sample matrixes and the ability of the extraction method to provide suitable nucleic acid from each matrix. It is known that different matrixes can contain different substances that could affect the efficiency and reliability of real-time PCR (Cankar et al., 2006). Components that inhibit the amplification of nucleic acids by PCR based methods are present with the target nucleic acids from many sources and inhibitory effects may have important implications for clinical investigations, investigations in food and environmental screening (Wilson, 1997). The mechanism of inhibition is considered to be chelation of the Mg^{2+} cofactor which is important for Taq polymerase activity, or by binding to target DNA/RNA/Taq polymerase or by inhibition of Taq polymerase (Wilson, 1997; Mayr et al., 2005). Inhibitors can generate inaccurate quantitative results and high degree of inhibition can create false-negative results (Nolan et al., 2006).

The significant reduction in the sensitivity and kinetics of real-time PCR caused by inhibitory components frequently found in biological samples is well known (Rådström et al., 2004; Jiang et al., 2005).

Different *Prunus* species may have different concentration of secondary metabolites, similar to other plants like citrus, strawberry and blackberry (Borah et al., 2008; Wei et al., 2008) which can have inhibitory effects. In plant tissue we usually find polysaccharides and polyphenols as components with inhibitory effect on PCR (Demeke and Adams, 1992). Numerous extraction methods have been used in the preparation of totRNA from plant samples with a more or less sufficient quantity of isolated totRNA and reduction of inhibitors (Gambino et al., 2008; Singh et al., 2002; Maliyakal, 1992; Koonjul et al., 1999; Thompson et al., 2003). These approaches are often time-consuming but they are essential for reliable diagnostic work.

In our study one manual and one automated totRNA extraction method were compared in terms of

elimination of potential PCR inhibitors. The column based method for totRNA extraction (e.g. RNeasy Plant Mini Kit) is easily adapted for high throughput processing, and can yield amounts of up to 10 μg of totRNA from small amount of starting material, which is sufficient for real-time PCR applications. Samples isolated by the column based method contain fewer impurities such as phenol compounds which are a particular problem with plant samples and have a significant influence on the efficiency of PCR amplification (Singh and Singh, 1996; Singh, 1998; Myslik and Nassuth, 2001). The use of RNeasy columns manually is time consuming but a higher quantity of totRNA is isolated. An automated alternative for manual extraction with RNeasy columns could be advanced technology, instrument called QIAcube (Qiagen) which we were not able to use during our experiments. Sample preparation on the QIAcube use RNeasy spin columns follows the same steps as the manual procedure and no change in purification chemistry is required.

The KingFisher instrument uses patented technology in which magnetic rod moves particles through purification process. The technology used is based on silica particles which bind totRNA in the presence of chaotropic salt. The use of KingFisher instrument is automated and fast, it saves time and money. The financial disadvantage is the initial investment into the instrument but it can be used for many different applications (for DNA, RNA and protein extraction) (Lassailly et al., 2007; Zhao et al., 2008; Josefsen et al., 2007).

For comparison of manual and automated totRNA extraction we developed a real-time PCR assay that can be used for detection of PPV in different *Prunus* species.

With both totRNA extraction methods real-time PCR assay for PPV detection was shown to be reproducible and linear amplification was achieved over a range of 4 or 5 orders of magnitude. The range depends on the extraction method and the *Prunus* species used.

The results of this study suggest that the column based totRNA extraction method is more effective for apricot, plum and peach. It is a manual method and more time is

needed for processing of the same amount of samples compared to automated method using KingFisher instrument. The differences between both methods were very small when analyzing damson. For the processing high number of samples in large scale detection of PPV the automated method such as KingFisher instrument is more appropriate. It saves time even if the amount of extracted totRNA is not as high as with manual method. According to our results we would recommend to use cDNA diluted 1/10 in real-time PCR of *Prunus* sp. In case of negative results obtained with automated method, manual column based extraction method should be used additionally.

5 ACKNOWLEGEMENTS

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