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A rapid and inexpensive RNA-extraction method for high-throughput virus detection in grapevine

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Summary

The extraction of RNA from grapevine tissue is a crucial step for virus diagnostics via multiplex reverse transcription-polymerase chain reaction (mRT-PCR). Conventional methods are either time-consuming or expensive when convenient extraction kits are used. Here we present an easy, but reliable extraction method that fulfills the requirements of epidemiological research (high sample throughput with maximum accuracy). A further advantage of the protocol beside the low costs is the absence of harmful chemicals like phenol or chloroform and the possibility to manage 'high-throughput' extractions and analyses.

K e y w o r d s : *Vitis vinifera*; virus diagnostic; modified silica-extraction.

Introduction

Grapevine is a cultivated plant of major worldwide importance. Nearly 70 different viruses are reported for this plant species (Martelli 2017). Almost all of them have a worldwide distribution. There is no other cultivated plant that shows more different virus diseases than grapes (Martelli 2017). The economically most relevant viruses belong to the genera Ampelovirus, Nepovirus and Vitivirus (Martelli 2014). Viruses of the genera Ampelovirus, e.g. Grapevine leafroll-associated virus (GLRaV), and Vitivirus, e.g Grapevine virus A (GVA) are transmitted by phloem-feeding scale insects (Homoptera Coccina) (Raski *et al.* 1983, Hommay *et al.* 2008, Tsai *et al.* 2008, Martelli 2014, Herrbach *et al.* 2016). Viruses of the genus Nepovirus, e.g. Grapevine fanleaf virus (GFLV), are transmitted by ectoparasitic nematodes (Sitanath and Raski 1968, Hewitt *et al.* 1958).

Since grapevine viruses are graft transmissible, excluding infected mother plants from propagation in the frame of certification schemes is crucial for the production of high quality planting material (Alley and Golino 2000, Almeida *et al.* 2013). A major phytosanitary problem related to viruses of the genera Ampelovirus and Vitivirus is the field transmission by scale insects that disseminate the pathogens within and between vineyards (Herrbach *et al.* 2016). The major drawback for the visual diagnosis of virus infection is that symptoms expressed by virus infected grapes are highly variable (Naidu *et al.* 2014, Sudarshana *et al.* 2015, Walsh and Pietersen 2013). Biotic and abiotic interactions affect the symptoms expressed by infected grapevines (Almeida *et al.* 2013). The specific virus and the grapevine variety also affect symptomatology (Basso *et al.* 2010). Therefore, visual inspection is neither sufficiently reliable for epidemiological studies nor for certification (Cabaleiro and Segura 1997). Therefore, regular testing of mother plants is a prerequisite for the production of healthy propagation material. Virus analyses are carried out by serological techniques like DAS- ELISA (Double Antibody Sandwich- Enzyme- Linked Immunosorbent Assay) or via nucleic acid-based techniques like PCR (polymerase chain reaction), LAMP (loop-mediated isothermal amplification) and NGS (next generation sequencing) (Martelli 1993, Walsh and Pieterson 2013, Boonham *et al.* 2014). The most commonly used methods for routine detection are PCR and DAS-ELISA (Adams *et al.* 2009, Boonham *et al.* 2014). Virus diagnosis in the frame of certification as well as epidemiological studies of leafroll disease and its spread within and between vineyards require a high throughput of samples as well as specific and reliable detection of the associated viruses. It is therefore essential that virus detection can be carried out reliably but also quickly and inexpensively.

The aim of this work was to improve available methods for RNA-extraction from grapevine. Here we present a RNA-extraction method that provides templates of sufficient quality for virus detection via multiplex reverse transcription-polymerase chain reaction (mRT- PCR). The improved extraction protocol was compared to a commercial RNA-extraction kit in regard to sensitivity, reliability, the labor and costs.

Material and Methods

P l a n t m a t e r i a l : Phloem scrapings from mature canes have proven to be the optimal tissue for virus detection (Fiore *et al.* 2009). In winter (November-January 2016-2018), wooden samples of different cultivars of *V. vinifera* were collected in the German winegrowing regions Nahe,

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Rheinhessen, Württemberg, Pfalz and Baden. The bark of the canes was peeled off with a scalpel and 50-100 mg of phloem tissue was scraped off and cut in 2 mm pieces on frozen metal plates (approximately -20 °C) to avoid RNA degradation during processing. These phloem tissue samples were transferred into 2 mL collection-tubes (Axon, Kaiserslautern, Germany), loaded with two steel beads (Ø 3 mm) and were shock frozen in liquid nitrogen. The samples were stored thereafter at -20 °C up to several months until extraction.

To check the possibility of using pooled samples, phloem scrapings of single canes were combined and an aliquot of 50-100 mg of the mixed sample was processed as described above.

Silica-reduced-RNA-extraction (ScR-RNA-extraction): To each tube with frozen sample tissue a volume of 300 µL of lysis- buffer (200 mM Tris- HCl pH 7.5, 25 mM Na_2-EDTA pH 8, 250 mM sodium chloride, 2.5 % PVP-40, 0.5 % SDS, modified from Lemke *et al.* 2011) was added at room temperature (RT). The tissue was disrupted in a TissueLyser (Schwingmühle TissueLyser2, Qiagen GmbH, Hilden, Germany) for 3 min at 30 Hz, adaptors turned round, followed by 3 min at 25 Hz. Then, after fixing the caps with a plate and clamp, the mixture was incubated in a water bath at 68 °C for 10 min with intermittent shaking two times per hand. Then the samples were centrifuged for 2 min at 3400 g. To each sample 150 µL of precipitation- buffer (1.25 M potassium acetate, pH 6.5, modified from Lemke *et al.* 2011) and 150 µL binding-buffer (2 M guanidinhydrochloride, Lemke et al, 2011) were added and shaken several times. This mixture was stored at -20 °C for 10 min and centrifuged for 5 min at 3400 g. A volume of 70 µL of the supernatant was transferred in a 0.2 mL tube (8-stripes, Axon, Kaiserslautern, Germany) with 35 µL ethanol propounded. According to the method of Rott and Jelkmann (2001), 70 µL 6 M sodium iodide and 10 µL resuspended silica (60 g silica (Sigma S5631) per 60 ml distilled water, pH 2,0 with HCl) were added to the samples and incubated for 10 min at room temperature with intermittent shaking by hand. The mixture was centrifuged for 1 min at 3400 g. The supernatant was discarded and the pellet was resuspended in 170 µL washing- buffer (10 mM Tris- HCL pH 7.5, 50 mM NaCl, 0.5 mM Na_2EDTA pH 8, 50 % EtOH, Rott and Jelkmann 2001) and centrifuged for 1 min at 3400 g. The washing step was repeated once. Then the pellet was dried at 70 °C for about 2 min in a thermomixer (peqLab, VWR International GmbH, Darmstadt, Germany) and resuspended in 50 µL RNAse free water by shaking for 5 min at 70 °C with 1000 u·min^{-1}. The mixture was centrifuged for 3 min at 5000 g. A volume of 30 µL of the supernatant was transferred into a new 0.2 mL tube, ready for further analyses or storage at -20 °C (-80° for long time storage). All centrifugation steps were carried out at RT with the centrifuge Sigma 6-16K (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The yield and purity of RNA were determined with a Nanodrop spectrophotometer (Nanodrop 2000c, Thermo scientific).

RNA-extraction with the RNeasy Plant Mini Kit: To compare the RNA extraction protocol with the results using a commercial kit, RNA was extracted from the same samples with our method and the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The phloem fragments prepared for extraction were processed according to the manufacturer's specifications. Yield and purity were determined as described above.

Multiplex reverse transcription- polymerase chain reaction (mRT-PCR): First-strand cDNA synthesis was performed using 2.5 µL of extracted RNA, 2 µL sterile water and 1.5 µL of primermix (reverse Primers of HSP70, P24, POLR1, CP and 18s, see

Table 1

Primers for multiplex reverse transcription-polymerase chain reaction (mRT-PCR) used in this study

Target	Primer	Length (bases)	Sequence	Amplified DNA size (bp)	Conc. of primers for reverse transcription (µM)	Conc. of primers for multiplex-PCR (µM)	Reference
Vitis 18S	18S rRNA-for	20	5'-CGCATCATTCAAATTTCTGC-3'	844	0.1	0.2	Gambino and Gribaudo 2006
	18S rRNA-rev	20	5'-TTCAGCCTTGCGACCATACT-3'			0.2	Gambino and Gribaudo 2006
GLRaV-1	HSP70-for	21	5'-GTTGGTGAATTCTCCGTTCGT-3'	382	25	35	Beuve et al. 2013
	HSP70-rev	22	5'-ACTTCGCTTGAACGAGTTATAC-3'			35	Beuve et al. 2013
GLRaV-2	P19-for	25	5'-ATGGAGTATTGTTTGAAGCAGGTAC-3'	120	25	10	Beuve et al. 2007
	P24-rev	25	5'-AGAATGTCTTCAGCTTCATAAGGAG-3'			10	Beuve et al. 2007
GLRaV-3	POLF1-for	22	5'-ACGTAACGGGGCAGAATATAGT-3'	282	25	23	Beuve et al. 2013
	POLR1-rev	24	5'-TATCAACACCAAGTGTCAAGAGTA-3'			23	Beuve et al. 2013
GVA	CP-for	21	5'-GGCTACGACCGAAATATGTAC-3'	524	25	35	Beuve et al. 2013
	CP-rev	21	5'-AGAAACGATGGGTCATCCATC-3'			35	Beuve et al. 2013

Tab.1, Beuve *et al.* 2007 and 2013, Gambino and Gribaudo 2006). This mixture was incubated for 10 min at 70 °C followed by incubation on ice for 5 min. Afterwards the preparation was completed with a mix of 2 µL of 5x reaction buffer (Genaxxon bioscience GmbH, Ulm, Germany), 1 µL of 10 mM dNTPs (Genaxxon bioscience GmbH, Ulm, Germany), 0.2 µL (200 $U \cdot \mu L^{-1}$) M-MuLV reverse transcriptase (Genaxxon bioscience GmbH, Ulm, Germany) and filled up with 0.8 µL sterile water to a final volume of 10 µL. The whole mixture was placed in a thermal cycler (Applied Biosystems, 2720 Thermal Cycler, Darmstadt, Germany) at 25 °C for 10 min, 42 °C for 1 h and 70 °C for 10 min (all steps are according to Genaxxon protocol for first strand cDNA synthesis). The received cDNA was either immediately used for mPCR or stored at -20 °C.

The multiplex-PCR reaction mix (final volume 10 µL) for the detection of four viruses (GLRaV-1, -2, -3, GVA) and a part of the grapevine 18SrRNA-gene as an internal control contained 1.5 µL of cDNA solution, 5 µL of 2x KAPA2G fast multiplex mix (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 2.5 µL of PCR-grade water, and 1 µL of primermix with different concentrations of specific primers (Tab. 1). Cycling conditions consisted of an initial denaturation step at 95 °C for 3 min, 30 cycles (95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s) and a final step at 72 °C for 3 min. Reaction products were analyzed by electrophoresis in 2 % agarose gel in 1x tris-acetate-EDTA-Puffer, stained with (1.5 $\mu L \cdot 100\ mL^{-1}$) DNA stain clear G (Serva Electrophoresis GmbH, Heidelberg, Germany) and visualized using UV light.

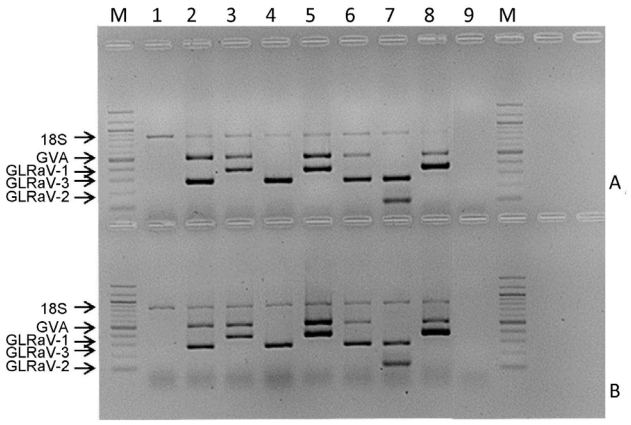

Fig. 1: Comparison of mRT-PCR results with RNA-templates extracted either with a commercial RNA-extraction kit (**A**) or the ScR- method (**B**) for detection of Grapevine virus A, Grapevine leafroll-associated virus -1, -2, -3, and the plant 18SrRNA-gene in grapevine varieties. Lane 1: 'Aligote' healthy control, lane 2: 'Alvarinho' infected with Grapevine virus A and Grapevine leafroll-associated virus-3 (GVA, 524 bp, GLRaV-3, 282 bp), lane 3: 'Silvaner' infected with Grapevine virus A and Grapevine leafroll-associated virus-1 (GVA, 524 bp, GLRaV-1, 382 bp), lane 4: 'Tempranillo' infected with Grapevine leafroll-associated virus-3 (GLRaV-3, 282 bp), lane 5: 'Auguster' yellow infected with Grapevine virus A and Grapevine leafroll-associated virus-1 (GVA, 524 bp, GLRaV-1, 382 bp), lane 6: 'Bachet Noir' infected with Grapevine virus A and Grapevine leafroll-associated virus-3 (GVA, 524 bp, GLRaV-3, 282 bp), lane 7: 'Redora' infected with Grapevine leafroll-associated virus-3 and -2 (GLRaV-3, 282 bp, GLRaV-2, 120 bp), lane 8: 'Dornfelder' infected with Grapevine virus A and Grapevine leafroll-associated virus-1 (GVA, 524 bp, GLRaV-1, 382 bp), lane 9: no template control M: DNA size marker (100 bp, NEB).

Results

The results of RNA-extraction with the ScR- method presented here were compared to those achieved with a commercial kit. The mean RNA concentration measured for the ScR-extracted samples was slightly higher than the value for the commercial kit (Tab. 2) but Nanodrop measurement does not differentiate between RNA and DNA. There is no DNA-digestion-step in the ScR-protocol and therefore the concentration of RNA in the ScR extract may actually be lower than the one of the Qiagen RNA extract. The 260/280 nm absorbance ratio indicated sufficient purity of the extracted RNA with both protocols, while the 260/230 nm ratios indicated the presence of contaminants especially polysaccharides and polyphenols in both extracts, but more in the ScR- extract (Schultz *et al.* 1994). However, these impurities did not affect mRT-PCR since the detection of four viruses led to concordant results with RNA samples extracted with each of the two protocols (Fig. 1).

To compare the quality of RNA extracted with either of the two methods, the extracts of infected samples were serially diluted in healthy grapevine extract before incubation and gained RNA was used as template for mRT-PCR. The ScR- extraction method showed a reliable accuracy up to a dilution of 1:10, while ambiguous results were achieved with higher dilutions (Fig. 2a). In contrast, PCR with template RNA extracted with the commercial kit showed clear results up to a dilution of 1:400 (Fig. 2b). To reduce the number of mRT-PCR reactions, we considered the possibility to prepare mixed samples. Phloem scrapings of a GLRaV-1 infected cane were combined with material of up to nine

Table 2

Yield and purity (mean ± SD) of total RNA extracted from dormant canes with a commercial RNA-extraction kit and the ScR- method, respectively eight canes were extracted individually with both protocols. A: absorbance

Method	RNA yield ($ng \cdot \mu L^{-1}$)	A_{260}:A_{230}	A_{260}:A_{280}	RT-PCR[1] n pos / n reactions
Commercial kit	17.0±12.6	0.5±0.3	1.8±0.2	8 / 8
ScR- method	19.8±4.3	0.1±0	2.2±0.4	8 / 8

[1] RT-PCR detection of Grapevine leafroll-associated virus -1, -2, -3, and the plant 18SrRNA-gene.

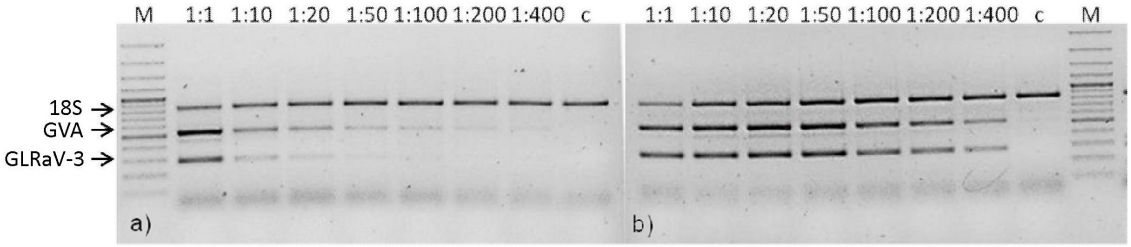

Fig. 2: Results of mRT-PCR using serial dilutions of RNA extracts. ScR-extract (**a**) in comparison a commercial RNA-extraction kit extract (**b**). c: plant extract with no virus infection. M: DNA size marker (100 bp- Ladder, NEB). Grape variety: 'Aligote'.

non-infected canes. One infected cane in a batch of ten canes led to a positive PCR result (Fig. 3). Corresponding results were achieved with batches of up to ten canes with one cane infected by GLRaV-2, GLRaV-3 or GVA (data not shown). Samples with multiple infections led to the same result (data not shown). The costs of an extraction using the new ScR-method presented here was estimated at 0.50 € per sample, while an extraction using the commercial kit costs 6.60 €. The working time for the ScR- method is about two hours per sample series, while up to 96 samples can be extracted at the same time. In contrast, the extraction with the commercial kit takes only one hour and up to 24 samples can be extracted simultaneously.

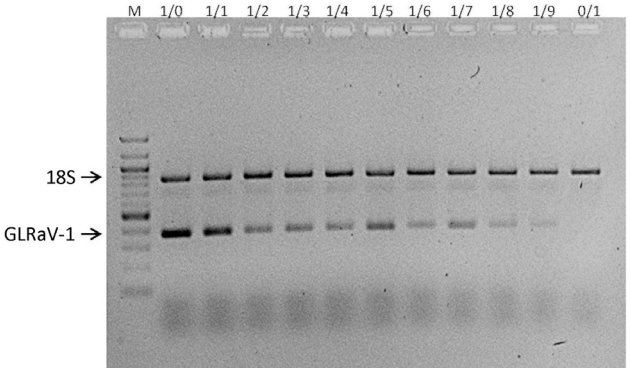

Fig. 3: Results of mRT-PCR using RNA extracted from mixed samples with different proportions of GLRaV-1 infected and non-infected canes. M: DNA size marker (100 bp-Ladder, NEB). Grape variety: 'Riesling'.

Discussion

There is a need for high throughput, though reliable virus diagnosis of grapevine samples regardless of any visible symptoms, both for certification purposes (Almeida *et al.* 2013) and for epidemiological studies (Cabaleiro and Segura 2006). A rapid and reliable RNA extraction method is a prerequisite to achieve conclusive mRT-PCR results.

Here we present a reliable, fast and inexpensive RNA- extraction protocol. It was tested on more than 3000 grapevine samples in a study of the regional incidence of GLRaV-viruses and the spread of leafroll disease in vineyards, where experimental plots were tested vine by vine every year. We achieved consistently RNA extracts of sufficient quality to perform virus diagnostics by mRT-PCR. Although the Nanodrop measurements indicated comparable nucleic acid contents for the two extraction methods, the comparison of RT-PCR results of serial dilution series indicated a considerably higher RNA quantity in extracts achieved with the commercial kit. This is most likely due to DNA residues in the extracts of the ScR extraction method, as the Nanodrop measurements do not differentiate between DNA and RNA.

The extraction protocol enables one person to extract 96 samples of phloem scrapings within two hours. In comparison, RNA-extraction using a commercial kit allows the extraction within one hour, but only 24 samples can be extracted simultaneously. With a high throughput of samples the ScR RNA-extraction protocol is less time consuming than a commercial kit and much more cost effective. Other RNA- extraction methods require between three and six hours for five or six samples, or they are as quick as the protocol presented here but more expensive (Gambino *et al.* 2008). Our results show, that both methods applied in this study provide a good yield of RNA and sufficient quality for mRT-PCR. In this study the RNA extracted with the presented ScR protocol was successfully used as template in the mRT-PCR protocols of Beuve *et al.* (2007, 2013). However, it proved to be a suitable template with the PCR-protocol of Gambino and Gribaudo (2006), too (data not shown).

With the presented ScR-RNA extraction protocol a single infected sample could be detected in batches of up to ten canes. This allows reducing the high number of samples, which often accumulate in epidemiological investigations. However, if a pooled sample is tested positive, all canes have to be re-tested individually to determine the infection status of each plant. In order to obtain a practical benefit from the batch testing, the size of pooled samples needs to be adjusted to the expected field infection rate. The optimal batch size as a function of the field infestation rate can be calculated by transforming the equation provided by Bhattacharyya *et al.* (1979). With a field infection rate of 0-10 % a batch size of 5 samples is the most economic, while batches should be reduced to 3-4 samples for field infection rates between 10 and 30 %. If the expected infestation exceeds 30 % the samples should be tested individually.

The presented new RNA extraction protocol provides a fast, simple and reliable tool for epidemiological studies on grapevine viruses. Combined with an appropriate mRT-PCR protocol for testing of batch samples it allows a high throughput testing of entire vineyards or propagation plots.

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