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One prep to catch them all: "2 in 1", an efficient method for the simultaneous extraction of DNA and RNA from Grapevine tissues

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ABSTRACT

Recent advances in our understanding of plant physiology and adaptation to the environment are tightly related to the development of 'omics' technologies such as metabolomics, transcriptomics, genomics and epigenomics that allow a more comprehensive view of the plant functioning. In this context, the ability to extract DNA and RNA from small amounts of plant material can be a limiting factor, worse in the case of non-model plants for which efficient nucleic extraction procedures are lacking. In the case of grapevine, extraction of high-quality DNA is typically limited by the high polyphenolic and polysaccharide contents of the different tissues. Here, we propose an adaptation of the method of Reid *et al.* (2006) that allows the simultaneous and efficient extraction of DNA and RNA from grapevine vegetative and berry tissues from *in vitro* grown grapevine plants and cells and from other plants. The protocol allows the extraction of high-quality RNA and DNA for standard molecular biology methods as well as for Next Generation Sequencing (NGS). It also works with a limited amount of plant material, such as young developing buds, and provides the means to analyse "omics" data from a single plant sample.

KEYWORDS: *Vitis*, nucleic acids, DNA, RNA, extraction protocol, grapevine, tomato, rapeseed

INTRODUCTION

Grapevine, one of the most important perennial crops globally, is consumed as table grapes and dried raisins, although it is primarily used for winemaking. Thirty-three billion bottles of wine have been consumed worldwide in 2019 (https://www.bourrasse.com/en/key-facts-about-wines-global-status/). International wine export represents a turnover of more than 31 billion dollars per year (Oiv-2019-statistical-report-on-world-vitiviniculture.pdf). However, grapevine culture is subjected to several threats worldwide, such as pathogen attacks that impair annual grapevine development, for example, through vascular conductivity disruption (Bortolami *et al*., 2019) and fungi attacks (Pertot *et al*., 2017). In addition, abiotic stresses and, more globally, climate changes can drastically alter grapevine yield and thus wine production (Guilpart *et al*., 2017; Gambetta *et al*., 2020; Venios *et al*., 2020).

Recent advances in the understanding of plant development and adaptation to the environment are tightly related to the implementation of "omics" technologies such as metabolomics, transcriptomics, genomics and epigenomics, which allow a more comprehensive view of the physiology of plants (Fabres *et al*., 2017) including grapevine (Fortes and Gallusci, 2017). Indeed, Multi-Omics data integration is highly relevant to the study of environmental impacts on grapevine growth and on-vine quality (Fabres *et al*., 2017). Accordingly, the number of grapevine genomics, transcriptomics and epigenomics publications has been rising during the last decade (Battilana *et al*., 2017; Cochetel *et al*., 2017; Magris *et al*., 2019; Ruperti *et al*., 2019).

Molecular studies rely on extracting high-quality DNA and/or RNA in sufficient amounts, eventually from small amounts of tissues. However, as for many woody perennials, DNA extraction in grapevines is challenging because of high levels of polysaccharides and other types of secondary metabolites such as polyphenols that may accumulate in both vegetative (Loupit *et al*., 2020; Martín-Tornero *et al*., 2020) and reproductive (Gabaston *et al*., 2020) organs. The simultaneous presence of these various metabolites is a challenge for extracting both DNA and RNA (Varma *et al*., 2007) as it is for the extraction and analysis of active enzymes for isoform analyses (Pierpoint, 2003). Several protocols have been developed and used over the years for grapevine genomic DNA extraction (Marsal *et al*., 2013), allowing the extraction of good quality DNA, but sharing a major drawback as they require a significant amount of plant material (over 200 mg FW) to extract a minimum of 0.5 µg of genomic DNA (summarised in Supplementary Table S1). This becomes a limitation when multi-omics analyses, requiring the extractions of both metabolites, RNA and DNA, have to be performed from the same sample. Various commercially available kits, such as the Qiagen DNAeasy Plant Mini Kit (Qiagen, id: 69204), allowing the purification of DNA in sufficient amounts from limited quantities of plant material, are available; in addition, in most cases, they lack consistency on non-model plants, thus limiting their efficient use for Next Generation Sequencing (NGS) technology. As for DNA, RNA extraction from grapevine tissues or other perennials can be challenging and requires adaptation of existing protocols to get sufficient yields (Gambino *et al.*, 2008; Reid *et al*., 2006).

In addition, the quality and purity of DNA and RNA are critical for their use. Southern, Northern, Polymerase Chain Reaction (PCR) and NGS require nucleic acids of high quality, with limited proteins, salts, or carbohydrate contaminations. Assessment of nucleic acid purity usually relies on the ratio between the absorbance of nucleic acid (A260 nm), proteins (A280 nm) and polysaccharides, polyphenols and EDTA (A230 nm) (Sambrook *et al*., 1989). Classically, the A260/280 (A280) and A260/230 (A230) ratios are both expected to range between 1.8 and 2 to assess the purity of DNA. However, in many cases, these values are not reported for grapevine nucleic acid extractions (Supplementary Table S1), and only the nucleic acid abundance is estimated by measuring absorbance at 260 nm (Reid *et al*., 2006; Varma *et al*., 2007). This is most likely because of qualitative variations due to the tissue used for nucleic acid extractions. However, this makes it difficult to evaluate the purity of the extracts. Approaches based on the absorbance properties of nucleic acid extracts have limitations nevertheless because they do not allow discriminating degraded from non-degraded DNA or RNA (Simbolo *et al*., 2013). Fluorometric methods using DNA or RNA intercalating agents provide an alternative, which allows a more reliable evaluation of RNA quality and quantity and non-degraded/double-stranded DNA (Simbolo *et al*., 2013). The 'RNA Integrity' score (RIN score), which is based on the 28S to 18S rRNA ratio analysed by capillary electrophoresis and should be above 6.5, is now used as a robust and reproducible method to assess the quality of RNA extracts (Schroeder *et al*., 2006). In the present work, we propose an adaptation of the grapevine RNA extraction protocol from Reid *et al*. (2006) that allows the simultaneous extraction from a single plant sample of DNA and RNA of sufficient quality and quantity for many applications, starting from limited amounts of plant material (from 10 to 220 mg FW). The method was initially tested on young grapevine leaves and subsequently on grapevine cell suspensions, mature leaves, stems, roots, grape berries, whole plants cultivated *in vitro* and extended to other plants such as tomato and rapeseed.

In all cases, DNA and RNA suitable for classical molecular studies such as PCR and RT-PCR or NGS, including RNA seq and Whole Genome Bisulphite Sequencing (WGBS), were obtained.

MATERIALS AND METHODS

1. Plant material

Young and mature grapevine leaves (*Vitis vinifera*, Cabernet-Sauvignon, CS) were harvested at different developmental stages from plants grown in greenhouse

or field conditions. Grape cell suspensions from Cabernet-Sauvignon Berries (CS) were provided by Atanassova and co-workers (Atanassova *et al*., 2003) from culture cells initiated as described in (Krisa *et al*., 1999). The CS cells were collected from different *in vitro* suspensions cultivated and sampled, as presented in Berger (2021).

Plantlets of 1103 Paulsen (1103P) and Riparia Gloire de Montpellier (RGM) were propagated *in vitro* on McCown Woody Plant Medium (Duchefa) supplemented with 3 % sucrose, 0.7 % agar and 0.27 µM 1-naphthalene acetic acid, in a growth chamber at 25 °C/20 °C (day/night). Plants were grown with a photoperiod of 16 h light/8 h dark with a light intensity of 145 µmol photons m–2 s–1. *In vitro* plants were collected after two (for 1103P) and four (for RGM) months of growth.

Leaf, stem, and root from 1103P and RGM samples were collected from plants grown in a phytotron on a substrate composed of a mix of zeolite, perlite and sand (1/3/3) and irrigated with a full nutrient solution for 8 weeks, after 1 month of acclimation on perlite and irrigated with tap water. Before the acclimation step, the plants were propagated *in vitro* as described above for 1 month only.

The CS and Pinot noir (*V. vinifera*, PN) berries were collected 6 weeks after flowering, and skin and pulp were dissociated and stored separately. CS buds were collected at " Pointe verte" or "C Stage" (according to the Institut Français de la Vigne et du Vin, vignevin.com) from 3 different plant cuttings. Tomato (*Solanum lycopersicum* cv VWA106) and Rapeseed (*Brassica napus*) leaves were collected from plants grown in greenhouse conditions and divided into 4 technical replicates after grinding.

All plant materials were frozen in liquid nitrogen upon harvest and stored at –80 °C until processed. Sample manipulation and grinding were made in liquid nitrogen. Quality and quantitative analysis of DNA and RNA

2. Qualitative and quantitative analysis of DNA and RNA

The Invitrogen « Qubit dsDNA BR assay » kit and Qubit 3.0 (Qubit dsDNA BR Assay Kit, Q32853) fluorometer were used to quantify ds-DNA according to the manufacturer instructions (Simbolo *et al*., 2013). RNA and DNA yield were measured at 260 nm with a spectrophotometer (Nanodrop 2000c Thermo Scientific™), and DNA and RNA purity was determined by calculating the A260/280 (referred to as A280 ratio) and A260/230 (referred to as A230 ratio) ratios to assess for protein and polysaccharide contaminations, respectively.

According to manufacturer instructions, RIN for RNA extracted from CS berry (skin and pulp) and RGM tissues were determined using the Agilent 2100 expert Bioanalyzer. The RIN values for RNA extracted from CS leaves samples were provided by the BGI sequencing platform quality control (https://bgi.com) using an Agilent 2100 BioAnalyzer. When indicated, contaminating DNA was removed from RNA samples using the DNA free TM DNA removal kit from Invitrogen (AM1906) according to manufacturer instructions.

DNA and RNA were analysed by gel electrophoresis in 0.8 % and 1.5 % agarose gel, respectively, stained with Gelgreen (Nucleic Acid gel stain, 10,000X from Biotium. SKU 37-41004/41005) and visualised using a Biorad Gel Doc Ez Imager and the Image Lab software (version 5.2) gel documentation system.

3. PCR amplification

All PCR amplifications of the grapevine Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were performed using the PCR Universal master mix (Thermofisher) according to manufacturer instructions with samples diluted at 20 ng/µL. The PCR conditions were the following: initial denaturation Step (95 °C, 3 min), 35 cycles each composed of a denaturation Step (95 °C, 30 sec), a hybridisation Step (60 °C, 30 sec) and an elongation Step (72 °C, 1 min). A final elongation Step (72 °C, 5 min) was performed at the end of the PCR reaction. Amplicons of 230 bp were loaded on 1 % agarose gels and migrated for 25 minutes (Primers: GAPDH3_F:5'-GCTGGAATTGCTCTGAATGA-3'; GAPDH3_R: 5'-GCTGAAAATTCTGGGTCCAA-3').

4. Statistical analysis

Statistical analyses were performed using the R software (version 1.4.1103). The DNA and RNA yields presented in Figure 5 were compared using Student's t-test after performing a one-way ANOVA. ANOVA assumptions, such as sample normality (Shapiro test with a pval > 0.05) and variance uniformity (Bartlett test with a pval > 0.05) were controlled for both DNA and RNA yields data prior to statistical analysis (DNA: Shapiro test pval = 0.3592, Bartlett test pval = 0.9436; RNA: Shapiro test pval = 0.8995, Bartlett test pval = 0.2896). Tukey's honestly significant difference test (HSD) was used as a comparison test when the samples were significantly different after the analysis of variance (DNA Anova pval = 1.32e-07; RNA Anova pval = 4.86e-05).

RESULTS

1. Protocol

A two-day protocol was adapted from the procedure of Reid *et al*. (2006) to isolate RNA and DNA using a single extraction procedure (Figures 1 and 2). All materials necessary for the extraction and composition of buffers are listed in Supplementary Information 1. During day 1 (Figure 1, Step 1 to 7), cell nucleic acids are extracted. A differential purification of RNA (Step 8 to 11) and DNA (Step 12 to 17) is performed during day 2 (Figure 2). The steps indicated below correspond to those shown in Figures 1 and 2.

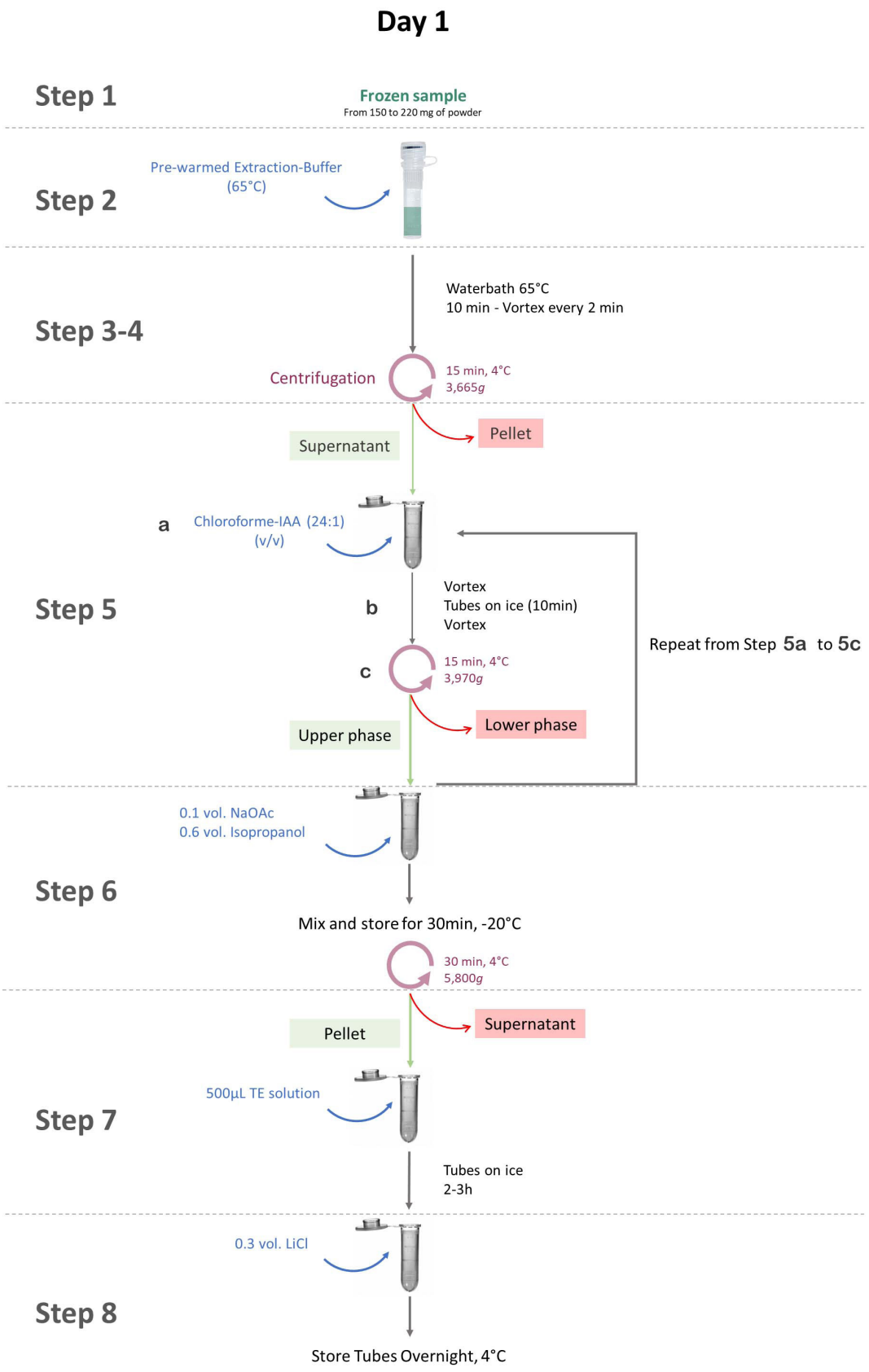


FIGURE 1. DNA/RNA extraction procedure. Day 1: The procedure is detailed in the results (protocol section, day1) and the steps refer to those indicated in the text of the protocol section. NaOAc: 5M Sodium Acetate, pH5.6; TE: 10 mM/1 mM Tris EDTA, pH8.0; LiCl: 10M Lithium Chloride; EtOH: ethanol.

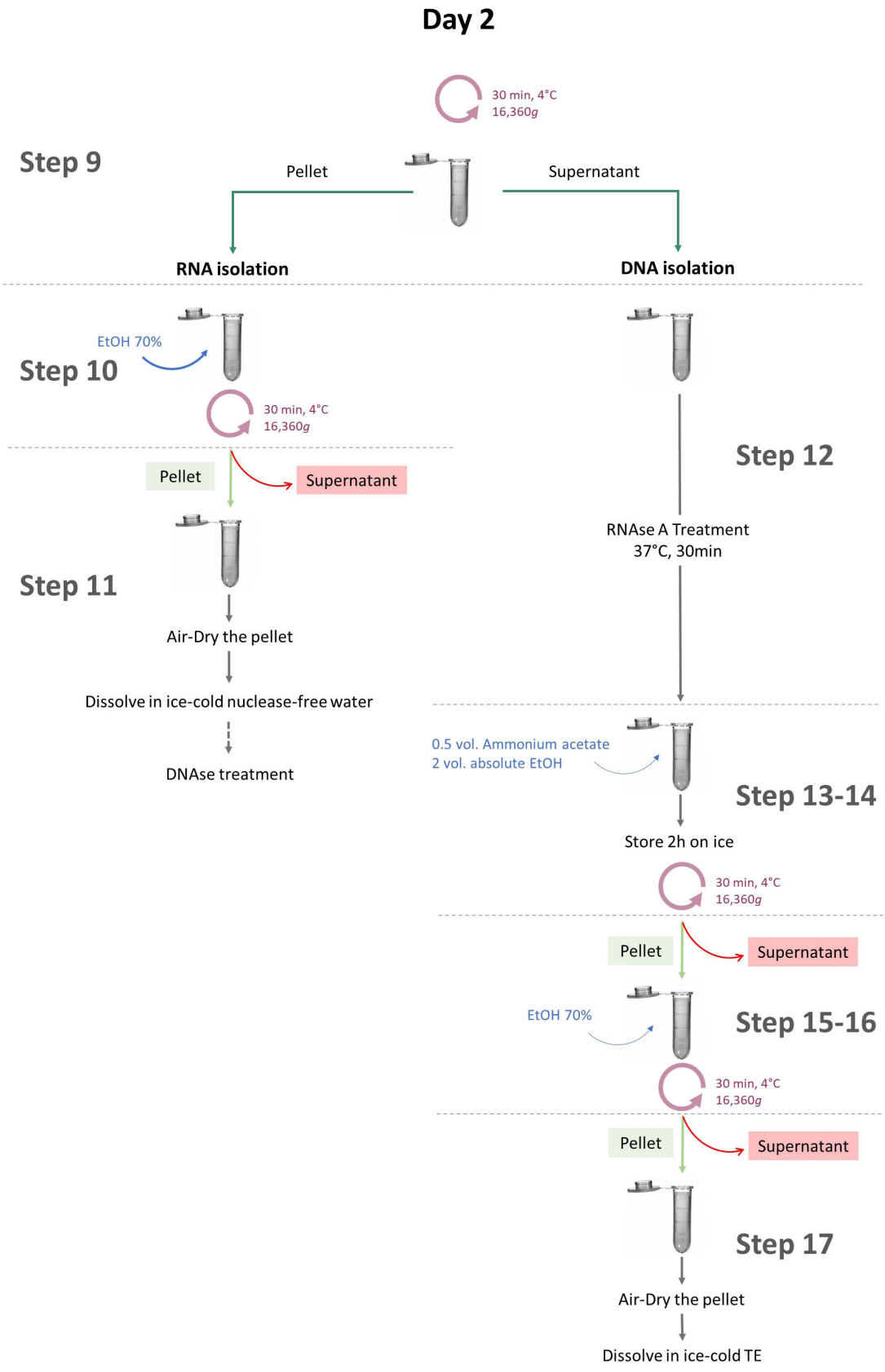


FIGURE 2. DNA/RNA extraction procedure. Day 2. The procedure is detailed in the results (protocol section, day 2) and the steps refer to those indicated in the text of the protocol section. EtOH 70 %: 70 % ethanol (V/V) in nuclease free water. Ammonium Acetate 7.5M, pH7.7; TE: 10 mM/1 mM Tris/EDTA (Ethylene-Diamine-Tetra-Acetic Acid), pH8.0; LiCl: 10M Lithium Chloride.

Day 1, (Figure 1):

Step 1 Weight between 160 and 200 mg of ground frozen tissues in a 2 mL screw microtube (pre-cooled in liquid nitrogen), and store in liquid nitrogen until use.

Step 2 Open the tube and allow liquid nitrogen to evaporate (~30 sec) before the addition of 1.2 ml pre-warmed (65 °C) Extraction Buffer (EB, Supplementary information 1) and mix thoroughly to obtain a homogeneous solution.

Steps 3 and 4 Incubate for 10 min at 65 °C with vigorous mixing (vortex) every two minutes. Centrifuge samples for 15 min (4 °C; 3,665 *g*).

Step 5 Washing step: transfer the supernatant (from 0.9 to 1.2 ml) to a fresh 2 mL Eppendorf tube before extracting with an equal volume of chloroform: isoamyl alcohol (24:1). Vortex and let on ice for 10 minutes. Centrifuge for 15 min maximum (4 °C; 3970 *g*). Repeat this Step twice. Transfer the aqueous phase to a new 2 mL Eppendorf tube.

Step 6 Precipitate nucleic acids by adding 0.3 volume of 5M Sodium Acetate (NaOAc) (pH5.2) and 0.6 volume of isopropanol and incubate at – 20 °C for 30 min. Recover precipitate by centrifugation at 5,800 *g* (30 min, 4 °C).

Step 7 Suspend the nucleic acid pellet in a 500 µL ice-cold Tris-EDTA (Tris-Ethylene-Diamine-Tetra-Acetic Acid) solution. Tubes are then kept on ice for 2 to 3h with occasional gentle shaking to improve nucleic acid resuspension.

Step 8 Add 0.3 volume of 10M Lithium Chloride (LiCl) and incubate overnight (ON) at 4 °C to precipitate total RNA.

Nota Bene: Pellet resuspension is critical (1) for optimal DNA recovery, (2) to limit the DNA contamination of RNA samples due to incomplete DNA solubilisation. To limit DNA contamination of RNA samples, the resuspension time can be extended overnight. Alternatively, the aqueous phase can be transferred to a new Eppendorf tube before LiCl precipitation of RNA.

Day 2, (Figure 2):

RNA purification:

Step 9 Pellet the RNA by centrifugation at 16,360 *g* (30 min, 4 °C) and transfer the supernatant to a new 2 mL tube. The supernatant will be used for DNA purification (Step 11 to 15, see below *DNA purification* section).

Step 10 Wash the RNA pellets with 500 µl of ice-cold 70 % Ethanol/Diethylpyrocarbonate (DEPC) treated water to eliminate residual salts and centrifuge at 4 °C for 20 min at 16,360 *g*.

Step 11 Air-dry the RNA pellets before resuspending in 50 µL ice-cold DEPC-treated water. Let tubes on ice to allow RNA solubilisation for up to 2 h and store at –80 °C before quantity and quality check.

NB: It may be necessary to include a DNA nuclease (DNAse) digestion Step if some DNA contamination is observed. However, extending the solubilisation time and changing tubes for Lithium chloride precipitation results in very low levels of DNA contamination. When necessary, DNA contamination was removed from RNA samples using a DNA-*free*™ DNA removal kit from Invitrogen (AM1906) according to manufacturer instructions (Figure 2, Step 11).

DNA purification:

Step 12 Eliminate remaining RNA by RNase A (Thermo Scientific) treatment at a final concentration of 7.7 µg. mL-1 (0.5 µL of 10 mg/mL RNAse A) at 37 °C for 30 min in a dry-heat-bath.

Step 13 Precipitate DNA by addition of 0.5 volume 7.5 M Ammonium acetate (pH 7.7) and 2 volumes of Ethanol 70 % in nuclease-free water precooled at –20 °C and incubate 2 hours on ice.

Step 14 Centrifuge 30 min at 16,360 g, 4 °C. to pellet DNA

Step 15 Carefully eliminate the supernatant by pipetting.

Step 16 Wash the pellet with 500 µL of ice-cold 70 % ethanol (prepared with nuclease-free water) and centrifuge (16,360 *g*, 30 min, 4 °C).

Step 17 Air dry the pellets at room temperature and resuspend in 50 to 60 µL of ice-cold TE buffer. Incubate ON at 4 °C.

During development, plant organs and tissues specialise and accumulate various metabolites that may interfere with the extraction of nucleic acids (Varma *et al*., 2007). To evaluate the efficiency and robustness of the RNA/DNA extraction protocol described above, various grapevine vegetative organs (leaf, stem, roots) from different genotypes were used. Berry tissues (pulp, skin), cell suspensions, and leaves from other plants (tomato, rapeseed) were also tested. Extractions were performed using tissues of the grapevine genotypes RGM, CS and 1103P. For berries, we used the Cabernet Sauvignon (CS) and Pinot Noir (PN) genotypes (Table 1). Biological and technical replicates were performed on all samples to evaluate the robustness of the protocol (Supplementary Table S2).

2. Nucleic acid extraction from different cultivars and vegetative tissues of grapevine

2.1. Grapevine leaves

DNA and RNA were extracted from 204 ± 8 mg of grounded grapevine mature leaves (cv. RGM) as described above. For DNA, the A280 and A230 ratios were 1.8 ± 0.01 and 1.6 ± 0.05, respectively, and the average yield was 53.1 ± 1.7 µg.gFW-1 (Table 1, Supplementary Figure S1). Although the A230 ratio was below the recommended value (1.8), DNA samples were successfully used for standard PCR amplification (Figure 3A).

The RNA extracted during this procedure had A280 and A230 ratios, both ranging from 2.0 to 2.4, indicating low contamination levels, with an average yield of 221.1 ± 18.7 µg.gFW-1 (Table 1). A weak DNA contamination was detected, the remaining DNA was easily removed by standard DNA nuclease (DNAse) treatment (Figure 2, protocol Step 11, Supplementary Figure S2). The RNA RIN score

TABLE 1. Details of the Extraction performed on different grapevine tissues. DNA and RNA were extracted from the berry and vegetative tissues of Cabernet-Sauvignon (CS) and Riparia Gloire de Montpellier (RGM) grapevine cultivars and resuspended, respectively, in 60 µL (DNA) or 50 µL (RNA) of TE. Each extraction was performed two times. The Yield, quality ratios (A230; A260), sample weight and final nucleic acid concentrations are indicated. F3: Fruit set + 3 weeks; F6: Fruit set + 6 weeks; F11: Fruit set + 11 weeks; FW: Fresh Weight.

Tissue	Cultivar	[DNA] µg.gFW-1	DNA A280	DNA A230	[RNA] µg.gFW-1	RNA A280	RNA A230	RIN score	FW (mg)	[DNA] ng/µL	[RNA] ng/µL
Leaf	RGM	53.1 ± 1.7	1.8 ± 0.01	1.6 ± 0.05	221.1 ± 18.7	2.1 ± 0.01	2.4 ± 0.06	7.15 ± 0.10	204 ± 8	180.5 ± 12.7	903.0 ± 110.9
Stem	RGM	30.9 ± 3.6	1.8 ± 0.00	1.4 ± 0.05	165.2 ± 19.4	2.1 ± 0.02	2.3 ± 0.06	8.35 ± 0.69	206 ± 2	106.3 ± 13.2	680.7 ± 86.5
Root	RGM	22.2 ± 7.2	1.7 ± 0.01	1.3 ± 0.10	91.7 ± 3.9	2.1 ± 0.02	2.3 ± 0.06	8.85 ± 0.10	211 ± 3	77.9 ± 24.0	386.1 ± 11.0
In vitro plant	RGM	46.0 ± 1.2	1.8 ± 0.00	1.6 ± 0.03	121.1 ± 52.3	2.0 ± 0.06	2.2 ± 0.10	6.80 ± 0.00	185 ± 32	141.5 ± 28.4	455.6 ± 271.2
Berry skin (F3)	CS	3.3 ± 0.9	2.0 ± 0.01	2.2 ± 0.03	38.7 ± 5.5	2.1 ± 0.02	2.3 ± 0.09	8.3 ± 0.1	242 ± 8	13.1 ± 3.3	187.4 ± 32.5
Berry pulp (F3)	CS	1.4 ± 0.4	2.0 ± 0.10	2.2 ± 0.02	36.2 ± 1.9	2.1 ± 0.01	2.4 ± 0.00	8.3 ± 0.0	246 ± 9	5.9 ± 1.7	177.4 ± 2.7
Berry skin (F6)	CS	2.7 ± 0.2	1.7 ± 0.03	0.9 ± 0.05	22.7 ± 3.8	2.0 ± 0.07	2.4 ± 0.00	8.90 ± 0.00	229 ± 7	10.2 ± 0.5	104.0 ± 13.7
Berry pulp (F6)	CS	1.7 ± 0.2	1.8 ± 0.00	1.2 ± 0.02	22.3 ± 3.5	2.1 ± 0.00	2.3 ± 0.01	9.05 ± 0.10	231 ± 15	6.7 ± 1.4	103.0 ± 22.5
Berry skin (F11)	CS	2.5 ± 0.3	1.9 ± 0.00	1.4 ± 0.12	38.8 ± 3.6	2.1 ± 0.01	2.2 ± 0.01	7.0 ± 0.2	247 ± 2	10.3 ± 1.0	191.8 ± 16.1
Berry pulp (F11)	CS	0.4 ± 0.2	2.3 ± 0.03	2.5 ± 0.05	5.9 ± 4.3	2.1 ± 0.03	2.4 ± 0.14	8.4 ± 0.0	232 ± 4	1.6 ± 0.8	27.58 ± 20.3

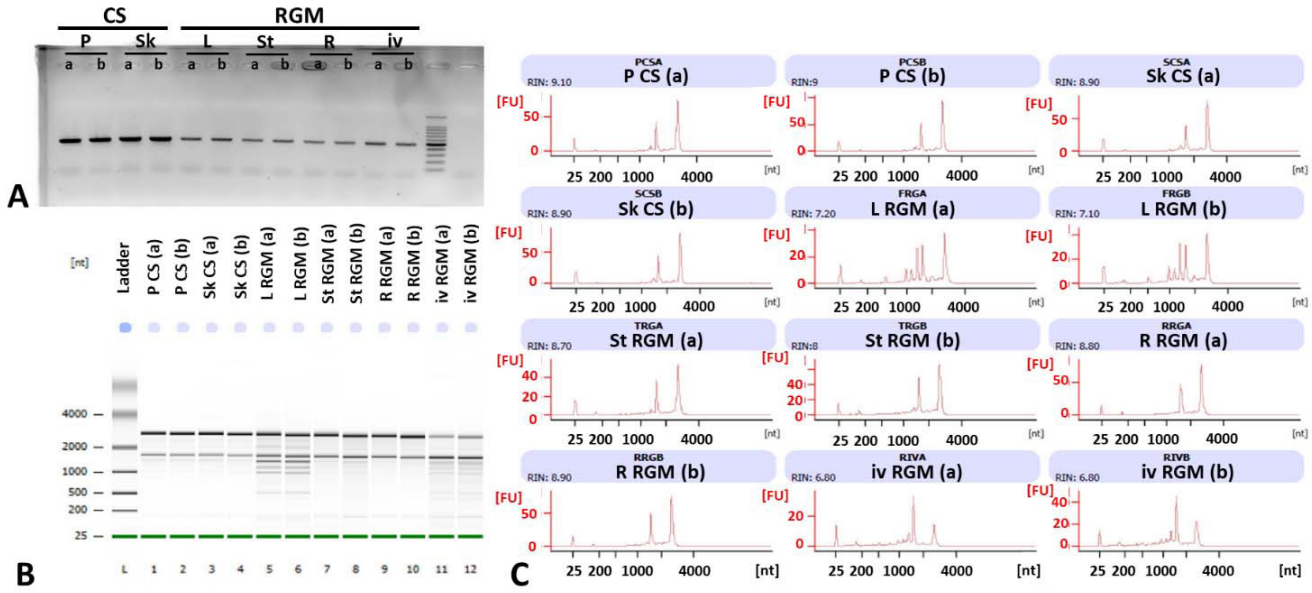


FIGURE 3. Evaluation of the quality of the DNA and RNA extracts. (A) Gel electrophoresis of PCR products performed on DNA extracted from vegetative and berry tissues of RGM and CS plants; The 1kb ladder (Promega) is used as a size standard. (B and C) Agilent quality check of RNAs extracted from technical duplicates (a, b) performed on CS berry tissues (F6 Pulp, F6 Skin), RGM vegetative organs (Leaf, Stem, Root) and *in vitro* grown plant (iv). P: Pulp dissected from F6 Berries; Sk: Skin dissected from F6 berries; L: Leaf; St: Stem; R: Root; iv: *in vitro* plant.

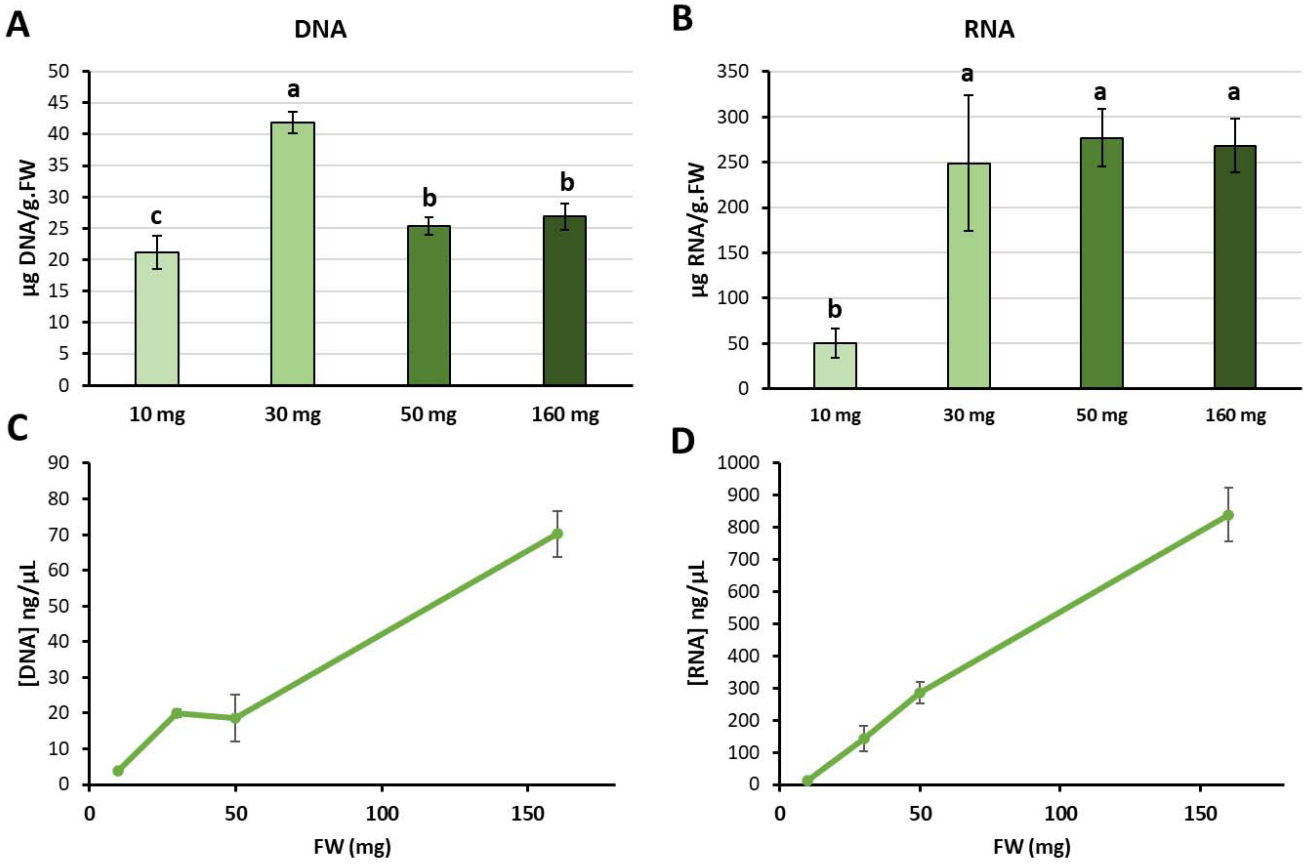


FIGURE 4. DNA and RNA yield and concentration were obtained from different amounts of leaf samples. Graphical representation of DNA (A,C) and RNA (B,D). The nucleic acid yield and final concentration (x-axis) obtained from 10 to 160 mg (y-axis) of grapevine leaf samples are indicated. Errors bars represent the confidence intervals (CI).

was, on average, 7.15 ± 0.10, which is above the 6.5 value recommended by the manufacturer (Figure 3C, Table 1). To test whether the genotype of the plant could influence the quality or yield of the DNA and/or RNA extractions, the protocol was used with mature leaves of various grapevine cultivars and rootstocks including CS, RGM and 1103P, starting from ~160 mg (FW) of grounded leaf tissue (Supplementary Table S2). As for RGM leaves, the DNA and RNA extracted from CS mature leaves were of high quality with A280 and A230 ratios above 1.9 and for RNAs a RIN score of 7.8 ± 0.2. Calculated yields were 17.4 ± 2.8 µg.gFW-1 for DNA and 184.3 ± 67.2 µg.gFW-1 for RNA (Supplementary Table S2, Supplementary Table S3, Supplementary Table S4). Nucleic acids extracted from CS leaves were successfully used for RNAseq and DNA Bisulfite sequencing (Berger, 2021; Supplementary Table S3, Supplementary Table S4). Genomic DNA was also extracted from RGM and 1103P mature leaves with yields of 25.1 ± 3.8 µg.gFW-1 and 23.7 ± 5.3 µg.gFW-1, respectively. For all extractions, quality was assessed by spectrophotometry. The A280 ratio was 1.8 in all cases, whereas the A230 ratio was lower, 1.63 ± 0.08 and 1.49 ± 0.15 for RGM and 1103P DNA, respectively, suggesting higher contamination levels with sugars. However, all samples were successfully used for standard PCR amplification analysis (Supplementary Figure S3). RNA extracted from RGM and 1103P leaves also had A230 and A280 ratios above 2.0, demonstrating the purity of the RNA extracts, with yields estimated at 268.2 ± 29.5 and 320.3 ± 61.7 µg.gFW-1 for RGM and 1103P (Supplementary Table S2).

We further analysed whether the developmental stages of leaves could impact the DNA/RNA extraction procedure. The DNA and RNA were extracted from 228 ± 7 mg FW of 'Young developing' CS leaves corresponding to the fourth leaf from the apex. The DNA and RNA extracts had A280 and A230 ratios above 1.9, and RNAs RIN score was 7.7 ± 0.1 (Supplementary Table S2, Supplementary Table S3, Supplementary Table S4).

Extraction yields were 35.6 ± 8.7 µg.gFW-1 for DNA and 225.5 ± 39.2 µg.gFW-1 for RNA (Supplementary Table S2). In addition, both DNA and RNA extracted from these tissues qualified for Whole Genome Bisulfite Sequencing (WGBS) and RNAseq analysis (Supplementary Table S3, Supplementary Table S4).

Altogether, these results indicate that both DNA and RNA can be extracted from leaves at different developmental stages and different grapevine genotypes and used for standard PCR and NGS sequencing (WGBS and RNA seq).

2.2. The efficiency of the DNA/RNA extractions varies with different amounts of starting material

As the quantity of plant samples can be a limiting factor in some experiments, the efficiency of the "2 in 1" protocol was evaluated with decreasing quantities of starting plant material using the same volume of EB. The "2 in 1" protocol was first tested with 10, 30, 50 and 160 mg FW of RGM leaf material. Starting from 10, 50 and 160 mg of grounded tissues resulted in comparable DNA yields of 21.1 ± 2.7, 25.3 ± 1.4, 26.9 ± 2.1 µg.gFW-1, respectively, which represents half of the yield obtained when 30 mg of powder were used (41.9 ± 1.7 µg.gFW-1, Figure 4A, Supplementary Table S2). These results are consistent with the idea that the ratio tissue powder/volume of buffer EB is an important parameter of the DNA extraction procedure. The efficiency of RNA extraction was also strongly affected by the amount of starting material as shown by the 5-fold reduction in yield observed when the extraction was performed with 10 mg of powder (50.45 ± 14.1 µg.gFW-1) as compared to extractions with 30 to 160 mg of starting material (Figure 4B, Supplementary Table S2).

We also examined the quality of the RNA and DNA that were extracted. In contrast to RNA that presented A230 and A280 ratios above 2.0 regardless of the amount of starting material, those of DNA showed contrasted behaviours. The A280 ratio remained high in all situations, ranging between 1.69 (50 mg) and 1.81 ± 0.37 (10 mg) with low amounts of starting powder and increased to 1.9 ± 0.02 with 160 mg of fresh leaf tissue (Supplementary Table S2). In contrast, the A230 ratio was strongly affected by the amount of plant material used with an average value of 1 ± 0 when 50 mg of fresh powder or less were used. Hence, the "2 in 1" extraction protocol of DNA and RNA is efficient enough even with low amounts of plant tissue. However, the A280 and A230 ratios indicate higher contamination levels of DNA with proteins and sugars when low amounts of starting powders are used.

We also evaluated the "2 in 1" protocol using a low quantity of powder from buds, an organ containing tissues enriched in dividing cells compared to fully expanded leaves. Three extractions were performed using 20, 55 and 62 mg of FW. Both RNA and DNA were successfully extracted regardless of the quantity of starting material. The yield in DNA and RNA was much higher than the one of mature leaves and any other tissues tested in this study (Figure 5, Supplementary Table S5), most likely reflecting the higher number of actively dividing cells. RNA yields were in average of 661.99 ± 29.51 µg.gFW-1 with both A280 and A230 ratio above 2.0, while DNA yields were estimated at 184.64 ± 70.89 µg.gFW-1 with an A280 ratio of 1.7 ± 0.02 and an A230 ratio of 2.0 ± 0.04 (Figure 5, Supplementary Table S5).

2.3. DNA and RNA extraction from other vegetative tissues

We also investigated the extraction of DNA/RNA using root and stem tissues from different cultivars. DNA and RNA were extracted from 211 ± 3 mg. FW of RGM root powder. Concerning RNA, both A260 and A230 ratios were above 2.0, with a RIN score of 8.85 ± 0.10 and an average yield of 91.7 ± 3.9 µg.gFW-1 (Table 1, Figure 3). DNA was also efficiently extracted with an average yield of 22.2 ± 7.2 µg.gFW-1.

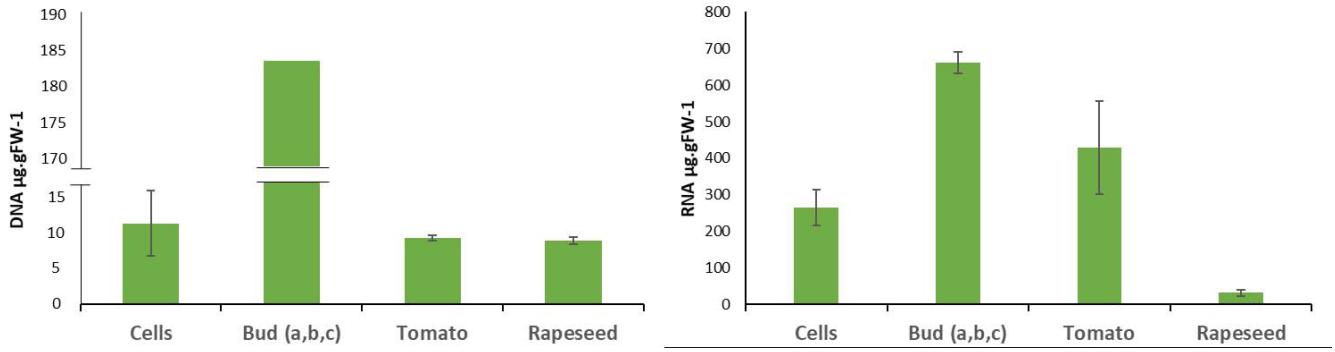


FIGURE 5. Graphical representation of DNA and RNA yields obtained from grapevine (cells, buds) and other plants leave samples (tomato, rapeseed). Values are indicated in Supplementary Table S5. Error bars represent confidence interval (CI). Buds DNA yield CI value (70.89 µg.gFW-1) is out of scale for the bar chart and is indicated in Supplementary Table S5.

The A280 ratio was 1.7 ± 0.01 and the A230 was 1.3 ± 0.10, suggesting that DNA was contaminated probably with pectin or other compounds absorbing at 230 nm. However, this did not interfere with standard PCR amplification (Figure 3A).

Extraction was also performed using roots of 1103P as a starting material (Supplementary Table S2). Similar to RGM, RNA extracted from 1103P roots were characterised by A280 and A230 ratios above 1.9 and yield of 116.0 ± 10.1 µg.gFW-1 (Supplementary Table S2). DNA was extracted with an average yield of 13.4 ± 1.1 µg.gFW-1 and quality scores remain low with an A280 ratio of 1.61 ± 0.05 and an A230 ratio of 1.17 ± 0.03 (Supplementary Table S2) but suitable for PCR amplification (Supplementary Figure S3). Hence, DNA and RNA extracted from roots were comparable in terms of quantity and quality, regardless of the cultivar.

The extraction of DNA and RNA was also performed from 206 ± 2 mg FW of RGM stems. As for other tissues, RNA extracts showed A280 and A230 ratios above 2.0, a RIN score of 8.35 (± 0.69), and a yield of 165.2 ± 19.4 µg.gFW-1 (Table 1). The average DNA yield was 30.9 ± 3.6 µg.gFW-1 and the A280 and A230 ratios were 1.8 ± 0.0, and 1.4 ± 0.05, respectively (Table 1). Even with a relatively low A230 ratio, samples were successfully used as a matrix for standard PCR amplification (Figure 3A).

To compare different grapevine cultivars, DNA and RNA were concomitantly extracted from 1103P stem and RGM. The two cultivars did not show significant differences in yield or the A230 and A280 ratios. The RNAs obtained from the 1103P stem were of high purity with both ratios above 2.0 and a yield of 173.1 ± 30.8 µg.gFW-1 (Supplementary Table S2). DNA yields obtained were measured at 12.9 ± 5.3 µg.gFW-1, and the A280 and A230 ratios reached 1.78 ± 0.07 and 1.35 ± 0.09, respectively (Supplementary Table S2).

Stem DNA and RNA can be efficiently extracted using the DNA/RNA extraction protocol, regardless of the grapevine cultivar. RNA was obtained in sufficient amounts for RNA seq analyses with high-purity and quality scores. Even though the A230 ratio remains low (< 1.8), extracted DNA could be efficiently used for standard PCR analysis (Supplementary Figure S3).

2.4. DNA/RNA extraction from *in vitro* grown plants and cells

Nucleic acids were also extracted from 185 ± 32 mg FW of *in vitro* grown RGM grapevine plants. Concerning DNA, an average yield of 46.0 ± 1.2 µg.gFW-1 was obtained with A280 and A230 ratios of 1.8 ± 0.00 and 1.6 ± 0.03, respectively. The genomic DNA was successfully used for standard PCR amplification (Figure 3A). The extraction of RNA provides an average yield of 121.1 ± 52.3 µg.gFW-1 with A280 and A230 ratios both above 2.0 and a RIN score of 6.80 ± 0.00. This demonstrates that RNAs were of good quality with little contamination levels (Table 1).

The DNA/RNA extraction protocol was also tested on *in vitro* grown plants of the additional genotypes 1103P. Both DNA and RNA were successfully extracted without significant differences between genotypes (Supplementary Table S2).

When extractions were performed using 200 ± 14 mg. FW of CS suspension cells, the DNA yield was 11.2 ± 4.5 µg.gFW-1, with A280 and A230 ratios of 1.97 ± 0.01 and 2.34 ± 0.07, respectively (Figure 5, Supplementary Table S5). Concerning RNA, the yield was 265.7 ± 48.7 µg.gFW-1, with A280 and A230 ratios both above 2.0 (Figure 5, Supplementary Table S5). The low DNA yield can be explained by the high intracellular water content of grapevine cells that was measured at 95.6 ± 0.06 %, compared to 70–85 % and 50–55 % of water content usually reported in grapevine leaves and buds, respectively (Popescu and Popescu, 2014). However, both DNA and RNA were extracted in sufficient quality and quantity to be used for subsequent WGBS and RNAseq analysis, respectively (Berger, 2021).

3. Nucleic acid extraction berry tissues at different developmental stages and from different cultivars in grapevine

The "2 in 1" extraction protocol was also tested on grapevine berry tissues. On average, 229 ± 7 mg or 231 ± 15 mg of skin and pulp dissected from CS berries harvested 3 (F3), 6 (F6) and 11 (F11) weeks after fruit set were used for DNA and RNA extractions (Figure 3, Table 1, Supplementary Figures S4, S5 and S6). Although successful, the extraction of genomic DNA from either of the tissue resulted in very low yields irrespective of the berry stage (ranging from 0.4 ± 0.2 to 1.7 ± 0.2 µg.gFW-1 for the pulp and from 2.5 ± 0.3 to 3.3 ± 0.9 µg.gFW-1 for the skin, Table 1). Of note, the yield was lower in the pulp of old berries (F11) than the younger ones (F3, F7). In addition, the A230 ratio was below the recommended values –0.9 ± 0.05 for skin, and 1.2 ± 0.02 for pulp at the F3 stage, and 1.4 ± 0.12 in the skin only for F11 berries- suggesting significant contamination of the genomic DNA with 230 nm absorbing compounds such as phenolic compounds, pectin, or other complex sugars in these samples. In contrast, the A280 ratio varied between 1.8 ± 0.00 and 2.3 ± 0.03 in the pulp, and between 1.7 ± 0.03 and 2.0 ± 0.01 in the skin of F3, F7 and F11, with no evidence of degradation as controlled after electrophoresis on agarose gel (Supplementary Figure S1, Supplementary Figure S4). All DNA extracted from berry tissues have been successfully used in standard PCR amplification (Figure 3A, Supplementary Figure S5).

For RNA extraction, even though the yield was low (22.7 ± 3.8 and 22.3 ± 3.5 µg.gFW-1 for the skin and the pulp, respectively), the A230 and A280 ratios were above 2.0 for both tissues showing little contamination levels (Table 1). Furthermore, the RNA integrity score was 8.90 ± 0.00 for skin and 9.05 ± 0.10, both indicative of RNAs of good quality (Table 1, Figure 3C, and Supplementary Figure S6).

To evaluate the robustness of the protocol on grape berry samples, additional extractions were performed on F6 CS and PN berry tissues, which resulted in DNA and RNA yield and quality similar to those described above (Supplementary Table S2).

4. DNA and RNA extraction works efficiently with other plant species

We have further measured the efficiency of the protocol developed from grapevine tissues on other plants using leaves from tomato and rapeseed, starting with similar quantities of leaf powders, 150 ± 4 and 148 ± 3 mg, respectively (Supplementary Table S5). Genomic DNA and RNA were successfully extracted from both species and showed A230 and A280 ratios above 1.8 in all situations. DNA yields obtained were comparable between tomato and rapeseed, 9.2 ± 0.4 and 8.8 ± 0.5 µg.gFW-1, respectively (Figure 5, Supplementary Table S5). However, RNA yields were significantly higher when extracted from tomato compared to rapeseed with 427 ± 127.2 µg.gFW-1 in tomato and 31.0 ± 7.5 µg.gFW-1 in rapeseed (Figure 5, Supplementary Table S5).

Thus, the successful extraction of DNA and RNA with the protocol developed in this study can be used with other plant species with similar efficiencies as those observed in grapevine.

DISCUSSION

We have successfully performed the sequential extraction of DNA and RNA with a single extraction procedure using nine different grapevine tissues and leaves from tomato and rapeseed. The protocol was extended from an existing RNA extraction protocol developed specifically for grapevine berry tissues (Reid *et al*., 2006). We aimed to adapt this protocol to extract RNA and DNA sequentially by adding a few steps after LiCl RNA precipitation (Steps 8 to 11) using the supernatant that contains the genomic DNA. The supernatant recovered after the RNA precipitation was then used to recover DNA. Although the protocol was successfully used for all tissues tested, the yield and quality of nucleic acids show some variations between tissues, organs, and developmental stages (Table 1, Supplementary Table S2). In all cases, although there was a 30-fold difference in yield between tissues and species, RNA was obtained in sufficient quantities (≥ 1 µg) in low cases, even when low amounts of starting material were used. The A280 and A230 ratios were always equal to or above 2.0, consistent with low protein and 230 nm absorbing compound contamination levels. Additionally, the RNA integrity scores, when measured, were above 6.5 for all vegetative and berry tissues (Table 1), thus showing that this protocol is appropriate to obtain grapevine RNA eligible for most applications, including RNA seq as performed on RNA obtained from CS leaves and cells (Berger, 2021).

As for RNAs, the yield of the DNA extractions also showed significant variations (over 50-fold) depending on the tissue used. Variations in extraction efficiency between technical replicates could be partly due to the protocol that includes a resuspension Step of all nucleic acids before RNA precipitation (Step 7). For practical reasons, this Step was limited to 3 h, which may be limiting in some situations to fully resuspend the genomic DNA, especially when DNA is abundant, thereby generating differences in recovery rate. Hence, extending this Step from 3 hours to overnight may result in a better recovery of DNA. Furthermore, this may also affect the A230 ratio by increasing the amount of DNA recovered without major impacts on other compounds absorbing at 230 nm, such as pectin. However, variations in yield may also be due to the tissue itself. The lowest DNA extraction efficiencies were observed in berry tissues and, to a lower extent, in cell suspensions (Table 1, Figure 5, Supplementary Table S2 and Supplementary Table S5). As these tissues are water-rich cells (García de Cortázar-Atauri *et al*., 2009), this may explain the limited amount of DNA recovered. Interestingly, whereas the RNA yield from fruit tissues was also the lowest, 10-fold higher than the DNA yield though, the efficiency of the RNA extraction from cells in culture was 20 times higher than for DNA and was in the range of other tissues.

Hence, other parameters than the cell water content might influence the final recovery of DNA. This is also illustrated by analysing the DNA and RNA extraction efficiencies from low amounts of plant materials. A dramatic effect was observed when buds were used instead of leaves for DNA extraction with low amounts of tissues. The DNA yield from buds was 7 to 10 times higher than the one of leaves (Table 1, Figure 5, Supplementary Tables S2 and S5). Buds are young tissues containing highly dividing cells, which may explain the difference in yield with leaves and other tissues mainly composed of fully differentiated cells. Of note, a better yield was observed with 30 mg of FW leaves as compared to 10, 50 and 160 mg, which may reflect a better powder/ EB ratio. Thus, optimising this ratio according to the tissues and the amount of powder used could be a way to improve the yield of the DNA extraction further. It should be emphasised that even when the yield was low, more than 2 µg of DNA was obtained in all cases, an amount sufficient for most applications, including genome and methylome sequencing with NGS technologies.

As mentioned above, the purity of RNAs, as estimated from the A280 and A230 ratios, was in general sufficient, which was not the case for DNA. The DNA extracted from fruits provides a striking example of this situation, combining a low yield and A230 ratio, suggesting that the DNA was contaminated with polysaccharides (pectin). Indeed, sugar contamination interferes with subsequent enzymatic reactions such as polymerase activity (PCR) or enzymatic digestion (Varma *et al*., 2007; Lodhi *et al*., 1994). However, DNA extracted from CS and PN berries was successfully used for standard PCR amplification despite low A230 ratios (Figure 3A, Supplementary Figure S3), suggesting that the overall quality of DNA was sufficient for routine applications. In a more general way, the purity of the DNA extracts was variable between tissues and genotypes (Table 1, Figure 5, Supplementary Table S2, Supplementary Table S5).Whereas all the seven grapevine tissues tested, namely cell suspension, young and mature leaf stems, roots, buds and fruit tissues, showed DNA with no or little protein contamination (A280 $\geq$ 1.7), in most cases, the A230 was variable and below 1.5, indicating significant contamination levels with compounds absorbing at 230 nm. Only CS mature and young leaf and CS cell suspension DNA extracts had an A230 ratio above 1.8. Whether variations in contamination rates reflect metabolic differences between grapevine cultivars cannot be formally excluded (Ershadi *et al.*, 2016). In support of this hypothesis, the A230 ratios of DNA extracts obtained from RGM and 1103P tissues were different, regardless of the tissue used for the extraction.

Finally, the "2 in 1" protocol can be used for other plant species, as illustrated by our first attempts on tomato and rapeseed leaves. Indeed, variations between plants were observed although the same amount of starting material was used, suggesting that the protocol should be adapted to the specificity of the plant tissues under study.

CONCLUSION

Because of the high amounts of polysaccharides and secondary metabolites, such as polyphenols, that may accumulate in different tissues, extracting nucleic acids from grapevine tissues has always been challenging (Akkurt, 2012; Marsal *et al*., 2013). Here, we present an efficient protocol that allows the simultaneous extraction of DNA and RNA from a single sample using a reduced amount of material (~160 to 200 mg). Extracted DNA and RNA are suitable not only for most routine applications but also for NGS technologies. Furthermore, we provide evidence that this "2 in 1" protocol can be used for other plant species, even though some minor adaptations may be necessary to obtain optimal results depending on the tissues studied.

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