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Short communication

Total RNA quality of lyophilized and cryopreserved dormant grapevine buds

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ABSTRACT

Background: Plant tissues must be preserved in their collection state, especially for genome-wide expression profile studies. Lyophilization is a feasible, affordable tool when fresh tissues cannot be shipped at ultralow temperatures from their origin to the place of analysis. In this study, the total RNA quality of dormant grapevine buds (*Vitis vinifera* L. cv. 'Flame Seedless') of freeze-dried samples stored at room temperature conditions was evaluated and compared to that of cryopreserved (-80°C) grapevine buds.
Results: Good yield and quality of RNA were obtained from freeze-dried dormant buds stored at room temperature for 0, 3 and 6 weeks after they were lyophilized. Further experiments confirmed that the extracted total RNA could be used for actin and β-tubulin PCR gene amplification.
Conclusion: High-quality RNA that is useful for downstream applications was obtained from freeze-dried dormant grapevine bud tissue, similarly to the RNA obtained from cryopreserved dormant grapevine buds.

1. Introduction

The isolation of high-quality RNA and DNA is very important for biological studies, and the ability to obtain this material depends on tissue preservation after collection. Maintaining the integrity of RNA represents an important problem, particularly for the preservation and long-distance shipment of biological samples for international exchange between collaborators or for analysis. Generally, RNA methodologies require fresh tissues, minimum processing, or freezing (liquid nitrogen [LN_2]) at ultralow temperatures. The use of LN_2 and dry ice for long-term tissue preservation has been well established; however, it is not suited for long transportation times or unexpected delays. In order to maintain RNA integrity, samples must be shipped with expedited delivery, resulting in high fees, and in bulky LN_2 or dry ice containers. Additionally, sample transportation in dry ice or LN_2 has to meet several troublesome requirements according to the International Air Transport Association (IATA) [1]. In some countries, samples in dry ice are not approved by transportation companies, especially airlines, because the gases released (CO_2 and N_2) may cause explosion and suffocation and are considered Hazard Class 9 and Class 2, respectively [1,2]. Accordingly, these methods are unsuitable for storage and/or international exchange because of the potential safety, high costs and inconvenience of operation. Over long distances, the sample may thaw, and multiple freeze-thaw cycles and prolonged exposure to increased temperatures must be avoided, as these conditions promote degradation of labile RNA samples [3].

There are new technologies that help preserve RNA at room temperature, such as RNALater® (Ambion, Carlsbad, CA, USA), which helps preserve sample tissues for further RNA extraction, and RNA stable® (Biometrica, San Diego, CA, USA), which keeps isolated RNA in anhydrobiosis at room temperature for weeks [3,4]. However, these methods are costly and need to be in hand at the laboratory at the moment of use. Lyophilization is an alternative appropriate method for processing samples for transportation, as well as for room temperature storage, when an ultralow freezer is a limiting factor [5]. Lyophilization has been widely used for the freeze-drying and storage of various biological samples in the food industry, pharmacy biotechnology and tissue engineering. Despite the advantages that this tool offers, RNA extraction from lyophilized tissues, such as mouse tissues [6], tea leaves [7], tuber and root tissues, such as potato, turnip, sweet potato and radish [5], has not been extensively reported. Saha et al. [8] reported the disadvantage that lyophilized cotton tissue had total RNA low quality. Theoretically, lyophilization should limit or delay cellular component degradation by inactivation of proteolytic enzymes and nucleases [7,8], allowing long-term room temperature storage or long-distance transportation if the seal is maintained. In the present study, we evaluated the effects of the freeze-dried process using dormant grapevine buds on the quality of the obtained RNA that will be used for further cDNA synthesis, amplification and gene expression in transcriptomic contexts.

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2. Materials and methods

2.1. Plant material

Cuttings were collected from ecodormant grapevine plants (*Vitis vinifera* L. cv. 'Flame Seedless'). The plant material came from a commercial vineyard in an agricultural zone in San Miguel de Horcasitas, Sonora, Mexico (29° 20′N, 110° 51′O). Grapevine buds were dissected and immediately frozen in liquid nitrogen and were cryopreserved at -80°C for 6 months. Cryopreserved buds were divided into 4 batches. One batch was kept in an ultralow freezer (-80°C), and 3 batches were lyophilized and packed in Falcon tubes that were closed and sealed with parafilm and stored at room temperature for 0, 3 and 6 weeks. Total RNA extractions of the lyophilized buds were performed after each storage time.

2.2. Total RNA extraction

RNA extraction was performed according to Reid et al. [9], using 8 grapevine buds per sample with 3 replications. The samples were purified using the commercial system Spectrum Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA) starting from binding columns. Genomic DNA traces were eliminated using DNase I (QIAGEN, Valencia, CA, USA). The integrity and quantity of the total RNA were evaluated by the spectrophotometric absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA). The integrity of RNA was determined by electrophoresis on a denaturing (formaldehyde) 1% agarose gel, as well as by using a Bioanalyzer 2100 RNA LabChip (Agilent Technologies, Palo Alto, CA, USA). The obtained RNA was used for synthesis of cDNA.

2.3. Semi-quantitative and real-time RT-PCR

First-strand cDNA was synthesized with 1 μg of total RNA (DNA free) using the SuperScript™ First-strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Semi-quantitative and real-time PCR amplifications were performed for actin and β-tubulin. The primers used for actin were VvACTFw (5′-GCT GAG AGA TTC CGT TGT CC-3′) and VvACTRv (5′-GCC ACC ACC TTG ATC TTC AT-3′) (GenBank accession no. AF369524), and the primers used for β-tubulin were Vvβ8TUBFw (5′-GCA GTG AAC CTG ATC CCA TTT CC-3′) and VvβTUBRv (5′-GCT CAC TCA CCC TCC TGA ACA-3′) (GenBank accession no. AF196485) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To prove the suitability of the cDNA prepared from the RNA from freeze-dried grapevine buds, semi-quantitative PCR was conducted with Go Taq DNA polymerase (Promega, Madison, WI, USA) using the gene-specific primers above. The samples were initially denatured at 95°C for 1 min, followed by 30 cycles of 1 min at 95°C, 1 min at 59°C (β-tubulin) or 63°C (actin) and 2 min at 72°C with a final extension at 72°C for 10 min. The reactions were analyzed by electrophoresis in a 1% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA).

Real-time PCR was performed in triplicate reactions for each sample using iTaq™ SYBR® Green Supermix kit (BIO-RAD, CA, USA) in a 48-well plate with a StepOne™ Real-time PCR system (Applied Biosystems, CA, USA). Reactions were done in 20 μL volume containing 125 nM of each primer, 5 μL cDNA (corresponding to 4 ng) and 10 μL 2× iTaq™ SYBR® Green Supermix reagent. Aliquots from the same cDNA sample were used with both set of primers. Reactions were run using the manufacturer's recommended cycling parameters of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. No-template controls were included for each primer pair.

2.4. Statistical analysis

The data were subjected to analysis of variance using the NCSS software (2006, Kaysville, UT, USA). The effect of cryopreservation and lyophilization on the RNA quality was evaluated by the Tukey–Kramer's comparison test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

The RNA extraction results obtained from freeze-dried and cryopreserved grapevine buds are shown in Table 1. The RNA quality and yield from freeze-dried grapevine buds that were stored at room temperature for 0, 3, and 6 weeks after lyophilization were very similar among all the samples and those obtained from cryopreserved samples. Although the RNA yield from lyophilized buds was somewhat lower than those obtained with cryopreserved buds, no significant differences ($p > 0.05$) were observed. High-quality RNA was obtained in all samples (Fig. 1), as two sharp bands corresponding to 18S and 28S rRNA were obtained from each sample using denaturing 1% agarose gel electrophoresis (Fig. 1a), Bioanalyzer electrophoresis (Fig. 1b) and electropherograms (Fig. 1c). Pearson et al. [10] reported similar results in a study where they compared the RNA quality and yield of frozen and lyophilized brown algae and seagrasses, which did not show significant differences in the RNA quality and yield obtained from frozen and lyophilized tissues [10]. Contrary to this, Saha et al. [8] reported a complete degradation of RNA from freeze-drying cotton tissues compared with RNA from non-freeze-dried tissues. However, in this study, the RNA integrity was also very good in all samples evaluated, as is shown in the values of the RNA integrity number (RIN) in Table 1. The RIN is a standardized method for the interpretation of quality RNA control that takes into account the entire electrophoretic trace, generating an automatic ratio of the 18S and 28S ribosomal RNAs that is based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact RNA [11]. The RIN values of the RNA obtained from freeze-dried grapevine buds immediately after lyophilization was 7.03 ± 0.05, which was very good (week 0, samples 1, 2, and 3). The RNA obtained from samples of freeze-dried buds that were stored for 3 (samples 4–6) and 6 (samples 7–9) weeks at room temperature had RIN values of 6.80 ± 0.00 and 6.46 ± 0.05, respectively, which are also good values. These values were slightly lower than the RIN values for RNA from week 0 but were higher compared to the results obtained for the RNA from the cryopreserved buds (samples 10–12) with a RIN value of 6.60 ± 0.17; it is considered good that the value was still greater than 6. RNA samples with RIN values from 3 to 6 are generally considered degraded [12]. The electropherograms generated by the Bioanalyzer (Fig. 1c) showed a similar peaks pattern in RNA obtained from both tissues (lyophilized and cryopreserved). Schroeder et al. [12] take degraded RNA into consideration when there are small peaks in the area prior to the 18S rRNA peak [12]. However, the presence of these bands in the electropherograms in our samples seems to be more related to other smaller rRNA fragments [12] because the electrophoresis (Fig. 1a and Fig. 1b) shows defined bands instead of degraded bands in these areas. The few degraded RNAs were related to the $A_{260/280}$ ratio values of RNA obtained for each sample (Table 1); the cryopreserved buds showed a higher value ratio of 2.11 ± 0.01, followed by the RNA obtained from freeze-dried buds immediately after lyophilization (time 0) and from the buds stored for 3 weeks, with ratio values of 2.09 ± 0.01, and from the tissue stored for 6 weeks, with a ratio of 2.10 ± 0.01. There was no significant difference ($p > 0.05$) among these values. Additionally, the polyphenol and polysaccharide contamination was low in the RNA obtained from buds, with $A_{260/230}$ ratio values that ranged between 2.34 to 2.39, indicating a low presence of contamination [13]; there was no significant difference ($p > 0.05$) among these values. The low values of polyphenol and polysaccharide contamination in lyophilized buds may be related to the lyophilization process because this may inhibit the

Table 1
Quality of total RNA isolated from lyophilized and cryopreserved dormant grapevine buds. Each date is the average of three independent extractions of each sample and the ± SD.

Sample	RNA (ng/μL)	RIN	Absorbance ratio		C_T ratio
			260/280	260/230	actin/tubulin
Lyophilized					
1–3	231.60 ± 51.35[a]	7.03 ± 0.05[a]	2.09 ± 0.01[a]	2.35 ± 0.03[a]	0.617 ± 0.01[a]
4–6	211.90 ± 35.19[a]	6.80 ± 0.00[ab]	2.09 ± 0.01[a]	2.39 ± 0.03[a]	0.619 ± 0.02[a]
7–9	250.10 ± 19.32[a]	6.46 ± 0.05[c]	2.10 ± 0.01[a]	2.36 ± 0.02[a]	0.625 ± 0.01[a]
Cryopreserved					
10–12	252.00 ± 30.49[a]	6.60 ± 0.17[bc]	2.11 ± 0.01[a]	2.34 ± 0.07[a]	0.627 ± 0.01[a]

Eight grapevine buds were used for each RNA extraction. Samples 1–3 correspond to lyophilized grapevine buds at time zero; samples 4–6 were lyophilized and stored at room temperature for 3 weeks; and samples 7–9 were lyophilized and stored at room temperature for 6 weeks. Samples 10–12 were cryopreserved at -80°C. Different letters mean a significant difference ($p < 0.05$) between treatments.

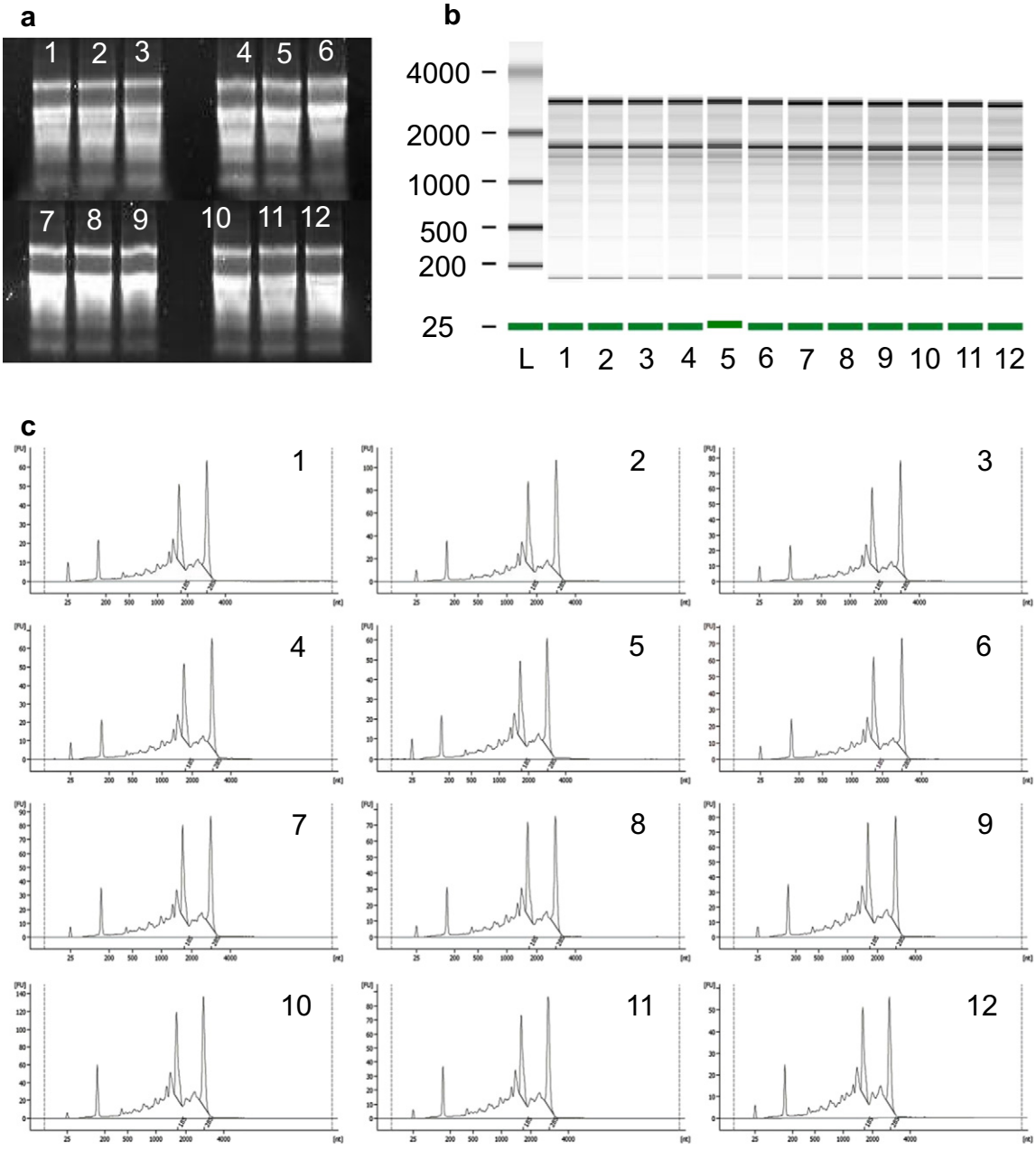

Fig. 1. Quality RNA comparison among samples extracted from dormant grapevine buds. (a) Agarose gel image stained with GelRed™. (b) Bioanalyzer gel image. (c) Electropherograms; note that the scales differ. Samples 1–3, lyophilized buds time zero; 4–6, lyophilized buds stored for 3 weeks at room temperature; 7–9, lyophilized buds stored for 6 weeks at room temperature; 10–12, cryopreserved buds. L: ladder.

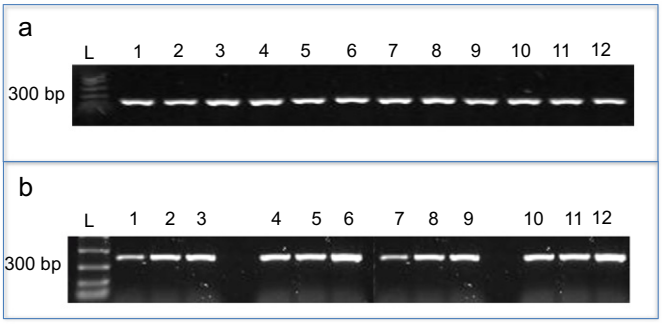

Fig. 2. PCR amplifications performed with the cDNA synthesized from RNA obtained from lyophilized and cryopreserved dormant grapevine buds. (a) Gel bands corresponding to actin (239 bp). (b) Gel bands corresponding to β-tubulin (390 bp). The amplifications were performed using template cDNA synthesized from RNA of lyophilized buds at week zero (lanes 1–3); lyophilized buds stored at room temperature for 3 weeks (lanes 4–6); lyophilized buds stored at room temperature for 6 weeks (lanes 7–9); and buds cryopreserved at -80°C (lanes 10–12). L: ladder (PCR Markers, Promega).

activity of proteolytic enzymes and nucleases, diminishing oxidation and cellular component degradation [7].

According to the RIN values in all the samples evaluated, we report that RNA obtained from these tissues has good integrity [13]. These results demonstrate that the lyophilization process did not affect the quality, yield and integrity of total RNA from grapevine buds.

To assess the suitability of isolated RNA from lyophilized buds for use in further cDNA synthesis, we used reverse transcription coupled to PCR. The cDNA that was synthesized from RNA obtained from the cryopreserved buds and from the lyophilized stored buds at 0, 3, and 6 weeks was used to perform PCR to amplify the housekeeping genes actin (239 bp) (Fig. 2a) and β-tubulin (390 bp) (Fig. 2b). No significant differences were found among the amplified DNA samples using cDNA as the template. Also real-time PCR was performed to assess the amplification efficiency and the C_T ratio was calculated. C_T ratio from lyophilized samples showed no significant differences ($p > 0.05$) from the C_T ratio obtained from cryopreserved samples (Table 1), which may indicate that the transcript abundance ratio of the evaluated genes is similar among samples. Therefore, the RNA stability is not affected by the lyophilization of grapevine buds that are kept for 6 weeks at room temperature prior to RNA extraction. Pearson et al. [10] demonstrated that 3-month-old stored lyophilized sea grasses lead to successful PCR for 16S rRNA, *tsf*, *atpB* and *ycf4* [10]. In this study, the PCR analysis demonstrated that the RNA obtained from lyophilized tissues was well suited for downstream applications, such as gene amplification, which can be further used in studies involving analysis of gene expression.

In conclusion, the freeze-drying process is an adequate method for preserving woody tissues for RNA extraction. In this way, samples can be transported by air or surface mail over short or long distances. It is also good for maintaining RNA quality and integrity for 3 weeks of storage at room temperature. Six weeks after tissue lyophilization, the RNA quality undergoes slight degradation. However, the RNA is still competent for downstream applications such as cDNA and PCR. Freeze-drying is a useful tool for samples with high polyphenol and polysaccharide contents, as it decreases nuclease and protease activities, diminishing the RNA contamination mediated by these compounds.

Conflict of interest

None.

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