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Research article

How to store plant tissues in the absence of liquid nitrogen? Ethanol preserves the RNA integrity of *Cannabis sativa* stem tissues

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Abstract: The preservation of intact RNA is a limiting step when gene expression profiling is performed using field-collected plant material. The use of liquid nitrogen ensures the optimal preservation of RNA, however it is not always practical, especially if the plant material has to be sampled in remote locations. Ethanol is known to preserve DNA in plant tissues even after a long storage period and here its suitability to preserve the RNA of textile hemp cortical tissues was tested. Hemp (*Cannabis sativa* L.) is an economically important fibre crop because it supplies cellulosic bast fibres used in different industrial sectors. In this study we demonstrate the suitability of ethanol for RNA preservation by analyzing tissues stored at 4 °C for 1, 2, 4 and 8 days. We show that in all the cases the extracted RNA is intact. We finally analyze hemp stem tissues stored in ethanol for 1 month and demonstrate the preservation of the tissue structure, particularly of bast fibres

Keywords: RNA preservation; ethanol; bast fibres; *Cannabis sativa*; integrity

1. Introduction

The cortex of fibre crops like textile hemp (*Cannabis sativa* L.) harbours the bast fibres, long extraxylary cells which are used both in the textile and biocomposite sectors [1]. Given its importance, this tissue is subjected to molecular investigations which aim at understanding the mechanisms of bast fibre differentiation and development [2,3]. Field-grown plants are interesting to study from the molecular point of view as they provide information in "real-life" conditions. Sample storage and nucleic acid preservation can however be challenging. Several papers in the literature have analyzed

different nucleic acid preservation media for molecular analysis on samples collected in the field [4,5], or on museum specimens [6].

RNA preservation media are available (e.g. RNAlater®), however, for plant specimens, they might not always be optimal, given the presence of the cell wall and waxes, which hinder the efficient diffusion of the medium within the cells and tissues. This is especially the case for plant bast fibres, which possess a thick cellulose-rich cell wall.

A recent study has shown the suitability of ethanol (EtOH) in preserving the tissues and nucleic acids from yeast and plant tissues [7]. In particular, EtOH was suitable not only for the preservation of plant tissues, but also enabled the recovery of high quality RNA, even from recalcitrant plant tissues, like the needles of *Larix decidua* [7]. EtOH easily permeates the tissues and subtracts water, thereby drying the tissues. Tissue dehydration was shown to be essential for the successful preservation of biomolecules from biospecimens for subsequent molecular analyses (even preserved at room temperature) [8,9].

The analysis of plant tissues sampled in the fields helps shedding light on mechanisms regulating the cross-talk between genome and environment: the "field -*omics*" domain is gaining interest as it enables understanding the complexity of field systems by combining -*omics* with practices in crop science [10]. For example, different hemp cultivars (cultivated for fibre or seed production) grown in different locations could be sampled and analyzed using targeted gene expression or even transcriptomics. These studies are important, as they contribute to understand the basis of complex traits (for instance the resistance to specific stresses).

With the aim of providing a more convenient and cheaper alternative to liquid nitrogen for the preservation of plant tissues for molecular analyses (as is the case for specimens sampled in the field), the suitability of EtOH 80% for the storage of textile hemp cortical tissues is here evidenced. EtOH 80% was chosen as it is the medium used to mechanically separate the fibres from the surrounding tissues of the cortex [11]. The results hereafter shown demonstrate that EtOH preserved the morphology of hemp fibres after 1 month and, importantly, it kept RNAs intact even after 8 days at 4 °C. This study can be useful for those scientists interested in studying plant samples collected in the fields using molecular techniques.

2. Material and methods

2.1. Sampling and storage of hemp tissues, RNA extraction and assessment of its integrity.

The hemp plants (cv. Santhica 27, 1.5 months old) were grown as described by Guerriero et al. [11]. The internodes of two plants were pooled per biological replicate, the cortex was quickly peeled off and either immediately plunged in liquid nitrogen (control) or put in 20 mL freshly prepared 80% (v/v) EtOH. For RNA extraction, the cortical tissues were kept at 4 °C for 1-2-4-8 days. Three independent biological replicates were analyzed. The RNA was extracted with the CTAB-C/I+RNeasy procedure described by Guerriero et al. [11]. Purified RNAs were analyzed at the NanoDrop ND-1000 (Thermo Scientific). The integrity of the RNA was measured using a 2100 Bioanalyzer with the RNA Nano 6000 kit (Agilent Technologies), following the manufacturer's instructions. For microscopy observations, whole internodes were kept for 1 month at 4 °C.

2.2. Reverse transcription and PCR

The RNA (1 μg) was retrotranscribed using the ProtoScript II reverse transcriptase (NEB) and random primers, following the manufacturer's instructions. One microliter of cDNA was used for the

PCR reactions with the Q5 Hot Start High-Fidelity DNA Polymerase (NEB) using the primers Actin 5′ qPCRFwd/Actin 3′ qPCRRev and GAPDH 5′ qPCRFwd/GAPDH 3′ qPCRRev described by Guerriero et al. [11]. The PCR parameters were the following: 98 °C for 1 min, 30 cycles at 98 °C for 10 sec, 65 °C for 30 sec, 72 °C for 45 sec, a final extension at 72 °C for 2 min, then a final hold at 12 °C.

2.3. Optical microscopy

Free-hand sections were performed on control and EtOH-preserved hemp internodes. The sections were mounted in 50% glycerol; images were acquired with a Leica DMR microscope.

3. Results and Discussion

3.1. EtOH 80% preserves RNAs in hemp stem tissues

Hemp peeled cortical tissues were stored in EtOH 80% (v/v) at 4 °C for up to 8 days, they were then quickly blot-dried using autoclaved wipers (WypAll) and RNA was extracted using the optimized protocol described by Guerriero et al. [11]. As can be observed in Table 1 and Figure 1, the RNA integrity numbers (RINs) of the extracted samples were all above 8, notably even after storage for 8 days in EtOH. The yield was within the range usually obtained for hemp cortical tissues (>100 ng/µL).

These results show that EtOH is suitable as a preservation medium for the storage of hemp stem tissues for molecular analyses. Targeted, as well as high-throughput analyses like RT-qPCR or Next Generation Sequencing (NGS) require a starting RNA sample of high integrity. The degradation of RNAs can indeed strongly affect the resulting gene expression in a given sample, lead to a bias in the data and, consequently, to a misinterpretation of the biological phenomenon under investigation.

Table 1. RNA concentration and RIN from samples stored in ethanol for 0-1-2-4-8 days. R1-R2-R3 indicate the three replicates.

Sample	Days in EtOH 80%	RNA concentration (ng/µL)	RIN
R1	0	216.91	8.1
R2		274.75	8.2
R3		178.37	8.2
R1	1	236.32	8.6
R2		108.34	8.3
R3		235.16	8.5
R1	2	114.16	9
R2		121.54	8.3
R3		139.96	8.6
R1	4	261.65	8.5
R2		293.15	8.6
R3		213.4	8.4
R1	8	197.65	8.9
R2		107.15	8.1
R3		240.83	8.6

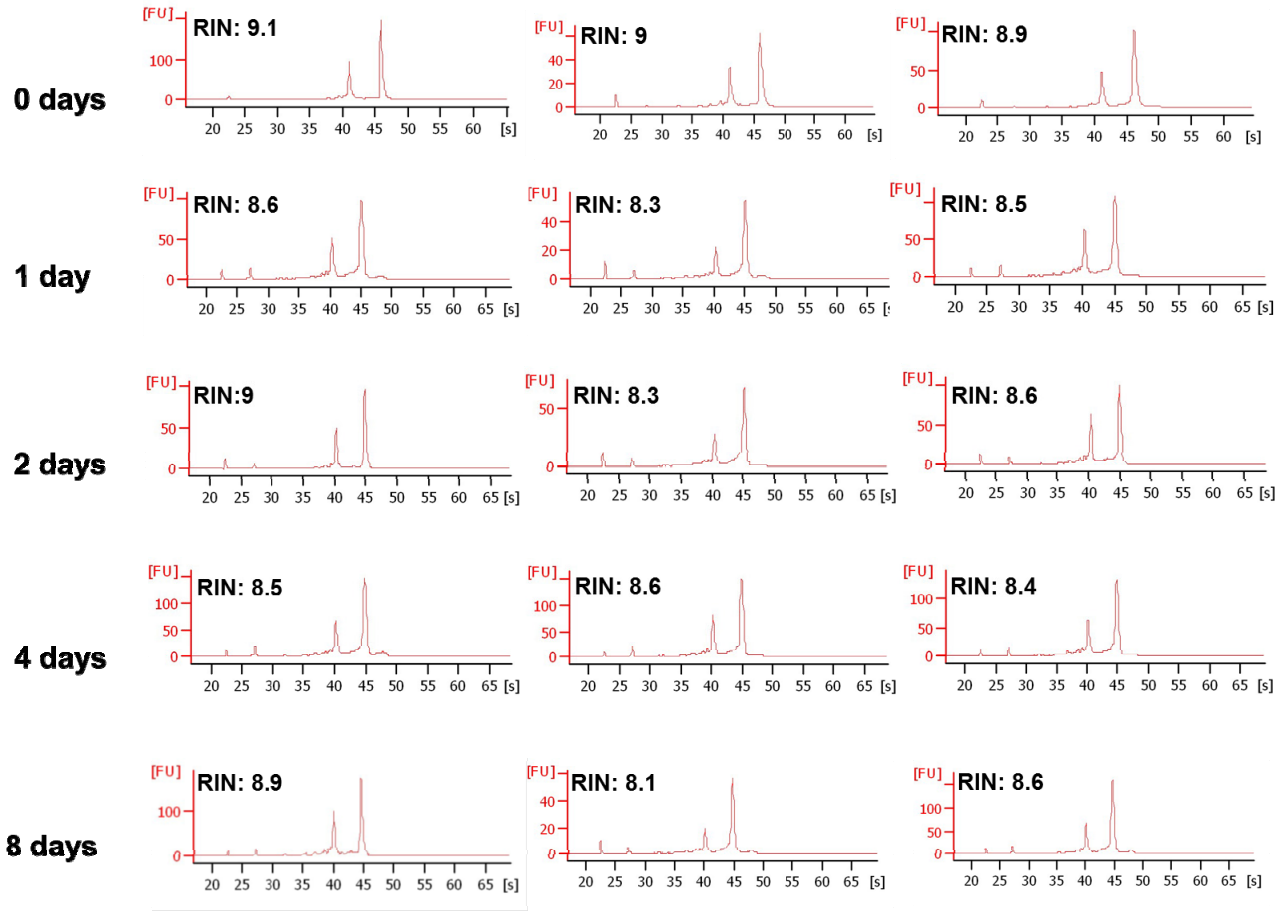

Figure 1. Integrity of the extracted RNAs. Bioanalyzer electropherograms of the RNA samples extracted from hemp cortical tissues plunged immediately in liquid nitrogen (0 days) or stored in ethanol for 1-2-4-8 days. [FU]: arbitrary fluorescent unit; [s]: migration time in seconds.

In order to validate the integrity of the extracted mRNAs, PCRs using primers designed on the 5' and 3' UTR region of the actin and GAPDH genes [11] were performed on the control and 8 days EtOH-preserved samples. The choice of primers localized upstream/downstream the start/stop codon can reveal degraded mRNAs in the EtOH-treated samples (and therefore the absence of an amplification product). As can be seen in Figure 2, all the samples showed the presence of the expected bands (1279 bp for actin and 1324 bp for GAPDH), thereby confirming the integrity of the transcripts.

3.2. EtOH preserves the morphology of hemp stem tissues after 1 month

Hemp produces bast fibres which are characterized by a specific morphology, i.e. a thick gelatinous cell wall composed of crystalline cellulose [12,13]. Cross sections of hemp stems visualized under an optical microscope revealed the presence of bundles of bast fibres with a thick cell wall. Interestingly, the morphology of the bast fibres was preserved in the samples stored in EtOH 80% for 1 month (Figure 3). Indeed, the thick gelatinous cell walls of the control and EtOH-preserved samples appeared very well preserved without any sign of morphological deterioration. The only difference between the control and EtOH-treated sample is the higher tissue transparency of the latter: this is expected, since EtOH is used as a common clearing agent in plant tissue microscopy.

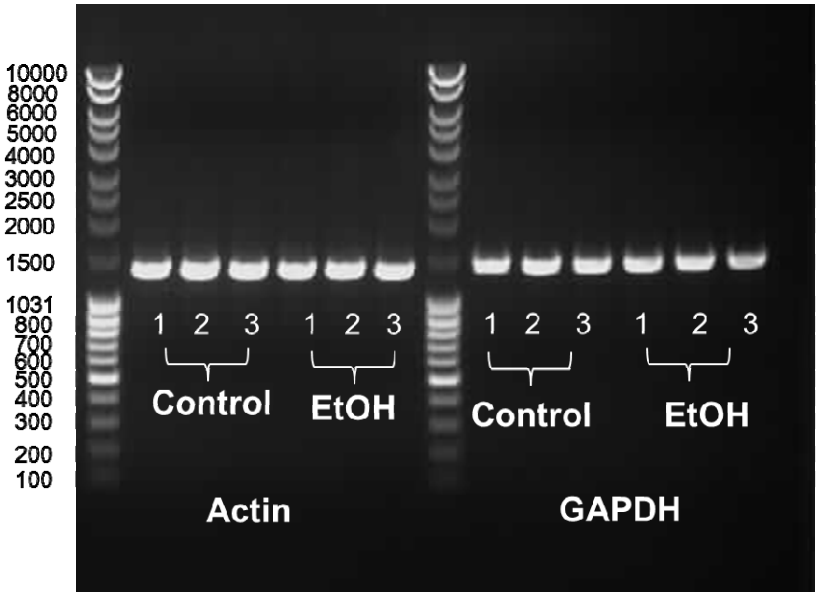

Figure 2. Amplification products of actin and GAPDH from control and EtOH-stored samples (8 days). The ladder is the MassRuler™ DNA Ladder Mix (Thermo Scientific).

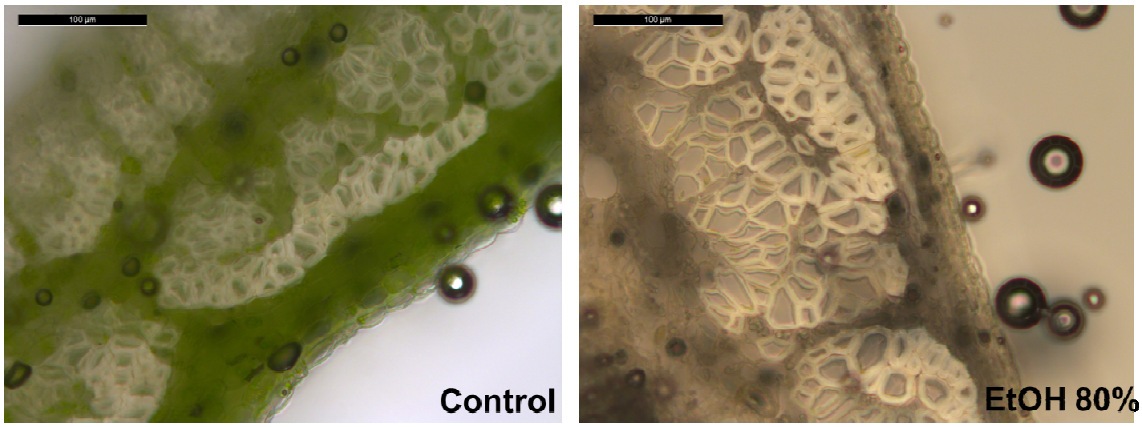

Figure 3. The morphology of hemp bast fibres is preserved after 1 month in ethanol 80%. Representative image of internodes sampled from the bottom of the stem. Scale bars are 100 μm.

4. Conclusion

For field-collected plant material, the preservation of nucleic acids is a challenge: the field location and its distance from the laboratory are important factors to consider and liquid nitrogen might not always be the easiest/most convenient solution (regulations for its transport during field sampling excursions, evaporation even in very large tanks [14]).

Since EtOH was previously shown to be a suitable preservation medium both for DNA [5] and RNA [7], we here sought to test its validity for the preservation of RNA from hemp stem tissues. *C. sativa* is an economically important plant since it is a multipurpose crop with applications e.g. in the textile and biocomposite sectors [1]. It is not only a source of fibres, oil and molecules [15], but also a

practical model to study the molecular processes associated with secondary growth [16]. The results here shown indicate that EtOH 80% preserves the integrity of RNAs in hemp stem tissues for up to 8 days at 4 °C. It can therefore be used to store tissues from field-grown hemp plants for analyses using molecular techniques. Such analyses are helpful to decipher the molecular events linked to the response to actual environmental threats and to unveil specific mechanisms, i.e. those associated with the cell wall response to abiotic stresses, like heavy metals [17]. The approach here described can be tested on other economically-important fibre crops (e.g. flax, jute, ramie, nettle), as well as other members of the Cannabaceae, as for instance hop (*Humulus lupulus* L.) [18].

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Conflicts of interest

The authors declare no conflict of interest.

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