

# Combining an original method for preserving RNA expression *in situ* with an effective RNA extraction method makes it possible to study gene expression in any banana fruit tissue

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## Combining an original method for preserving RNA expression *in situ* with an effective RNA extraction method makes it possible to study gene expression in any banana fruit tissue.

**Abstract — Introduction.** RNA isolation is a prerequisite to studying gene expression in banana and to understanding changes occurring in response to the environment. Standard extraction methods do not efficiently extract RNA from plants such as banana, with high levels of phenolics, carbohydrates, or other compounds that bind to and/or coprecipitate with RNA. **Materials and methods.** Five to seven RNA extraction methods were compared. Four crown-tissue storage methods were also compared. cDNA-AFLP was used to ensure that the obtained RNA was of sufficient quality for molecular applications and that RNA expression was unaltered by *in situ* storage. **Results and discussion.** The modified hot-borate method proved to be the best RNA extraction method, allowing high yields of good quality, undegraded RNA from the crown, fruit peel and pulp at all stages of ripening. The RNA obtained by this method was of sufficient quality for molecular applications such as cDNA-AFLP that give highly reproducible results. Freeze-drying of fresh tissues and tissue conservation in hot-borate buffer, two original storage methods, appear appropriate for preserving RNA *in situ* without ultra-low temperature. The RNA obtained was of high quality, undegraded, and useful for all downstream applications. The genome expression profile obtained by cDNA-AFLP analysis was unaltered by these methods for storing collected tissues. **Conclusion.** By applying all the suggested procedures in this work, it is possible to store and study gene expression in any banana fruit tissue, whatever the maturity stage, without affecting the RNA expression level.

Belgium / *Musa* sp. / bananas / freeze-drying / RNA / storage / extraction

## La combinaison d'une méthode originale permettant de maintenir l'expression de l'ARN *in situ* et d'une méthode efficace pour extraire l'ARN permet d'étudier l'expression de gènes dans n'importe quel tissu de banane.

**Résumé — Introduction.** L'extraction de l'ARN est un préalable pour étudier l'expression des gènes dans la banane et pour comprendre les changements intervenant en réponse à l'environnement. Les méthodes standard d'extraction de l'ARN ne sont pas efficaces avec des plantes comme le bananier, qui présentent des niveaux élevés de composés phénoliques, d'hydrates de carbone, ou autres composés qui se lient à et/ou coprécipitent l'ARN. **Matériel et méthodes.** Cinq à sept méthodes d'extraction de l'ARN ont été comparées. Quatre méthodes de stockage de tissus de couronne ont été également comparées. La technique de cDNA-AFLP a été utilisée pour s'assurer que l'ARN extrait était de qualité suffisante pour des applications moléculaires et que l'expression de l'ARN était inchangée après un stockage *in situ*. **Résultats et discussion.** La méthode du borate chaud modifiée s'est avérée être la meilleure méthode d'extraction de l'ARN, permettant des rendements élevés de bonne qualité, et donnant de l'ARN non dégradé à partir des tissus de la couronne, de la peau des fruits et de la pulpe à toutes les étapes de la maturation. L'ARN obtenu par cette méthode a été de qualité suffisante pour les applications moléculaires telles que le cDNA-AFLP qui donne des résultats fortement reproductibles. La lyophilisation des tissus frais et la conservation de tissu dans la solution tampon de borate chaud, deux méthodes originales de stockage, semblent appropriées à la conservation de l'ARN *in situ* en absence d'ultra basse température. L'ARN obtenu a été de qualité, non dégradé, et utilisable pour toutes les applications. Le profil d'expression du génome obtenu par la technique de cDNA-AFLP est resté inchangé par ces méthodes de stockage des tissus collectés. **Conclusion.** En appliquant toutes les procédures suggérées dans ce travail, il est possible de stocker et d'étudier l'expression de gènes dans n'importe quel tissu de banane, quel que soit le stade de maturité, sans affecter le niveau d'expression de l'ARN.

Belgique / *Musa* sp. / banane / lyophilisation / ARN / stockage / extraction

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## 1. Introduction

Banana is cultivated in more than 120 countries and holds the second place in world fruit production, just behind orange and in front of grape. Exported dessert bananas belong to the Cavendish subgroup. Gene expression studies are essential to understanding the physiological processes of this plant species [1, 2]. Expression profiling, however, requires adequate sample conservation and high-quality RNA isolation for cDNA library construction and molecular analysis. The total RNA and mRNA obtained must notably be free of protein, genomic DNA and secondary metabolites. RNA extraction is thus a crucial step, but it can be problematic because of the relative instability of RNA, largely due to RNA degradation by ribonucleases [3]. Conservation and isolation of RNA from plant material, particularly from fruit tissues, presents special challenges [4, 5]. First, most plant materials contain relatively high levels of RNase activity, mostly located in the vacuoles [6]. They also contain various plant metabolites, such as polysaccharides and polyphenols. They bind to and/or co-precipitate with RNA, affecting the yield and quality of RNA [7] and rendering it unsuitable for cDNA synthesis. Thus, qualitative and quantitative differences in polysaccharide and polyphenol content among different plant tissues significantly influence the efficiency of nucleic acid extraction and purification procedures [3, 8]. Many procedures [4, 5, 8–14] have been developed to cope with endogenous polysaccharides, phenolics and RNases during RNA isolation from various plants, but the efficiency of an extraction method depends largely on the kind of plant or tissue.

Another potential problem with RNA is its rapid degradation or alteration during tissue storage. In some cases, plant tissues must be preserved as collected, especially when a genome-wide expression profile is to be studied. The most commonly used method to store tissues for preparation of RNA is to remove fresh tissues and to maintain them at a strictly ultra-low temperature [(–50 to –70) °C] [15]. However, other efficient storage methods without ultra-low temperature

could be of interest but have not been extensively documented. Freeze-drying is proposed in some cases to conserve animal tissues [16–20], human tissues [21, 22] and bacteria [23] in order to further RNA extraction. However, in plant tissues, freeze-drying has not been used extensively to preserve RNA [4, 24] and contradictory results have been reported [25, 26].

The first aim of our study was to evaluate various techniques of RNA extraction from fresh banana tissues for quantity, quality and integrity, but also its adequacy for downstream molecular analyses such as cDNA-Amplified Fragment Length Polymorphism analysis (cDNA-AFLP). This RNA fingerprinting technique derives from AFLP (Amplified Fragment Length Polymorphism), a method described by Vos *et al.* [27] for genomic DNA fingerprinting, which was applied for the first time like cDNA-AFLP to plants by Bachem *et al.* [28].

Classical RNA isolation techniques were compared with methods specifically elaborated for banana or other plants rich in polyphenols and polysaccharides.

As collected materials sometimes have to be stored prior to RNA extraction, the second aim of our study was to assess different methods for preserving RNA *in situ*. This means both avoiding RNA degradation between sample collection and RNA extraction and ensuring that nothing happens, during storage, to alter the expression profile obtained. Checking that this latter criterion is met requires obtaining pre- and post-storage differential expression profiles by cDNA-AFLP.

## 2. Materials and methods

### 2.1. Plant materials

Ripe yellow bananas of the Cavendish subgroup were bought on the market. Preclimacteric green bananas of the Cavendish subgroup were obtained from a commercial source in Belgium (Van Damme, Brussels) just before the ethylene treatment that triggers ripening.

## 2.2. RNA extraction methods

RNA extraction methods were tested on various fruit tissues from green and ripe bananas.

Samples of peel, pulp and crown tissue were sliced, immediately frozen in liquid nitrogen, and ground to a fine powder with a pre-cooled mortar and pestle before extraction. Five extractions were performed for each tested method. All materials and reagents were treated so as to be RNase-free. Glassware was oven-baked at 240 °C overnight. Sterile disposable plastic wares were used when it was possible and non-disposable plastic wares were rinsed with 0.1 M NaOH, 1 mM EDTA, followed by RNase-free water. Solutions (water and other solutions) were treated with 0.1% DEPC (diethyl pyrocarbonate), incubated overnight at 37 °C, then autoclaved to eliminate residual DEPC.

Five RNA extraction methods were applied to peel and pulp from green bananas. Methods based on commercial products were carried out according to the supplied instructions. Methods taken from the literature were carried out according to the authors' protocols. These methods were:

1. The commercial ready-to-use RNA isolation product TRIZOL<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA, USA), according to the protocol proposed for extraction from plant tissues: this is an improved version of the single-step RNA isolation method developed by Chomczynski and Sacchi [29]. During sample homogenization or lysis, TRIZOL Reagent maintains RNA integrity while disrupting cells and dissolving cell components.
2. The 'SV Total RNA Isolation System' developed by Promega (Madison, WI, USA): this is a quick and simple technique for preparing purified, intact total RNA. The system combines the disruptive and protective properties of guanidine thiocyanate and  $\beta$ -mercaptoethanol to inactivate the ribonucleases present in cell extract. The system also incorporates a DNase treatment step, and purification is achieved without any phenol/chloroform extraction.
3. The method elaborated by Liu *et al.* [30] to isolate total RNA from banana fruit tissues: RNA was extracted at room tempera-

ture with a high-ionic-strength buffer. Proteins, genomic DNA and secondary metabolites were then removed from the extract by precipitation with pre-cooled potassium acetate and repeated phenol/chloroform/isoamyl alcohol extractions. RNA was recovered by ethanol precipitation without LiCl. This procedure can be completed in less than 4 h.

4. The hot-borate method [31], adapted from that described for cotton [32]: RNA extraction was performed in a basic hot-borate/proteinase K buffer. A RNA-enriched pellet is precipitated from the supernatant with lithium chloride and dissolved in DEPC water. Two phenol/chloroform/isoamyl alcohol (25/24/1) extractions and one chloroform/isoamyl alcohol (24/1) extraction are incorporated into the RNA purification protocol to remove contaminant proteins. Total RNA is precipitated with ethanol.

5. The simple procedure developed by Asif *et al.* [33] for RNA isolation from banana tissue: these authors have modified the CTAB/NaCl method [11] by removing PVP from the extraction buffer and including a simple polysaccharide precipitation step. A RNA-enriched pellet is precipitated from the supernatant with lithium chloride and dissolved in DEPC water before phenol/chloroform extraction. Total RNA is precipitated with ethanol.

On peel and pulp from ripe bananas, in addition to these five protocols, two additional ones among those proposed by Gehrig *et al.* [7] were tested. Their techniques based on GITC (guanidium isothiocyanate) (for technique 6) and GHCL (guanidium hydrochloride) (for technique 7) buffers were selected. Both methods use a high-molecular-weight polyethylene glycol (HMW-PEG). The hot-borate method [31] was also tested on crown tissues. All extractions were performed on a small scale in a 1.5- or 2-mL tube, by using the protocols proportionally.

## 2.3. Evaluation of RNA quality, quantity and integrity

After extraction, RNA was recovered by centrifugation for 20 min at 16 000 *g* and 4 °C.

The pellet was washed once in 1 mL of 70% cold ethanol, vortexed, and centrifuged for 5 min at 16 000 g and 4 °C. The pellet was air-dried at 37 °C, and then resuspended in RNase-free water. The purified RNA was quantified by measuring the absorbance at 260 nm ( $A_{260}$ ) with a Nanodrop (ND-1000 spectrophotometer, Isogen Life Sciences, Maarsse, Netherlands). An  $A_{260}$  of 1 is equivalent to 40 ng RNA· $\mu\text{L}^{-1}$ . The absorbance at 280 nm was also measured, and sample purity was estimated by the [ $A_{260} / A_{280}$ ] absorbance ratio. RNA integrity was evaluated by the sharpness of the bands corresponding to 28S and 18S ribosomal RNA (rRNA) visualized by ethidium bromide staining on a 1% agarose gel.

#### 2.4. Evaluation of the RNA adequacy for downstream molecular analyses

RNA extracts were treated for 30 min at 37 °C with 20 units of RNase-free DNase (Roche, Basel, Switzerland) in the presence of 40 units of RNase inhibitor (Roche Diagnostics, Mannheim, Germany). The cDNA-AFLP technique involves three steps: (1) restriction of cDNA and ligation of oligonucleotide adapters; (2) selective amplification of sets of restriction fragments using PCR primers bearing selective nucleotides at the 3' end; (3) gel analysis of the amplified fragments. Double-stranded cDNA was synthesized from total RNA according to the instructions for use of the Superscript Double-Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). The double-stranded cDNA was digested with EcoRI and

MseI, ligated with EcoRI and MseI adapters, and pre-amplified with the Eco (5'-GACT-GCGTACCAATTC-3') and Mse (5'-GAT-GAGTCCTGAGTAA-3') primers, following the instructions of the AFLP Analysis System kit for microorganisms (Invitrogen, Carlsbad, CA, USA). After twenty-fold dilutions of the PCR fragments, specific amplifications were carried out with combinations of an Eco primer and a Mse primer containing two additional bases at their 3' ends. The Eco primers were labeled with  $\gamma^{33}\text{P}$  dATP. Amplification products were separated by electrophoresis at 50 W on a vertical denaturing polyacrylamide gel (6%) containing 7 M urea. Gels were dried on a Whatman paper before screening with a phosphorimager (BioRad, Richmond, CA, USA). To evaluate the reproducibility of the cDNA-AFLP, a single banana crown was ground in liquid nitrogen to a fine powder and divided into two pools. RNA was extracted according to the hot-borate method [31]. After DNase treatment, two different pre-amplifications were performed per sample and two different selective amplifications were performed per pre-amplified sample (*figure 1*). Two replicates were performed.

#### 2.5. *In situ* RNA preservation methods

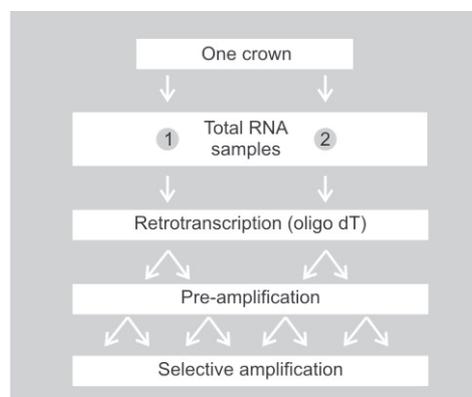
Four tissue storage methods were compared on crown tissue material.

The first two methods involved placing the tissues on RNeasy (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Here, crown tissue was first either sliced into tiny lamellas before storage in RNeasy at 4 °C or ground in liquid nitrogen before storage in RNeasy at 4 °C.

The third conservation method (buffer storage) consisted of storing crown tissues (ground to a fine powder in liquid nitrogen) in the hot-borate buffer [31] (200 mM BORAX, pH 9.0; 30 mM EGTA; 1% SDS; 10 mM DTT; 2% PVP 40; 2% DIECA; 0.5% Igepal) to which 0.5 mg proteinase K·mL<sup>-1</sup> buffer was added. This was stored at room temperature.

The last method consisted of freeze-drying the crown sample at -80 °C for 24 h (Telstar, Cryodos, Barcelona, Spain) and storing

**Figure 1.** Protocol applied to test the reproducibility of the cDNA-AFLP analyses based on RNA extracted from banana fruit tissues.



**Table I.**

[A<sub>260</sub> / A<sub>280</sub>] ratio values according to different tissue and RNA extraction methods. The best methods give ratios between 1.7 and 2.2.

| Methods                                  | Peel    |         | Pulp      |         | Crown     |
|--|---------|---------|-----------|---------|-----------|
|  | Green   | Mature  | Green     | Mature  |           |
| Trizol <sup>®</sup> Reagent (Invitrogen) | 0.9–1.1 | 0.9–1.2 | 0.1–3.4   | 1.0–1.3 | –         |
| SV Total RNA Isolation System (Promega)  | 0.2–0.8 | 0.4–1.0 | 0.06–0.20 | 0.6–0.9 | –         |
| Liu <i>et al.</i> [30]                   | 1.7     | 1.7–2.0 | 1.7–1.8   | 1.8–2.1 | –         |
| Mbéguié-A-Mbéguié <i>et al.</i> [31]     | 2.0–2.2 | 2.0–2.2 | 2.0–2.2   | 2.0–2.1 | 2.00–2.14 |
| Asif <i>et al.</i> [33]                  | 1.2–1.4 | 1.2–2.3 | 1.1–1.5   | 0.8–1.5 | –         |
| Gehrig <i>et al.</i> (GITC) [7]          | –       | 1.7–2.0 | –         | 1.0–2.3 | –         |
| Gehrig <i>et al.</i> (GHCL) [7]          | –       | 1.7–2.0 | –         | 1.7–2.0 | –         |

GHCL: guanidium hydrochloride; GITC: guanidium hydrochloride.

it at room temperature before RNA extraction (freeze-drying storage). The freeze-dried crown samples were stored with silica gel to avoid an aqueous environment.

In each case, RNA extraction was performed 21 d later by the hot-borate method [31]. The extraction efficiency was assessed by analysis of RNA quality, quantity and integrity as described in section 2.2.

The adequacy of the RNA, obtained after a buffer or a freeze-drying storage, for downstream molecular application was evaluated by cDNA-AFLP as described in section 2.4. Furthermore, this technique allows evaluating the effect of the storage conditions on gene expression by comparing pre- and post-storage differential expression profiles. To do this, three samples were collected from a single crown. One of these served as control and RNA extraction was performed immediately; both of the others were stored either by freeze-drying or in hot-borate storage buffer [31] as described above prior to RNA extraction and cDNA-AFLP. This experiment was repeated twice.

### 3. Results and discussion

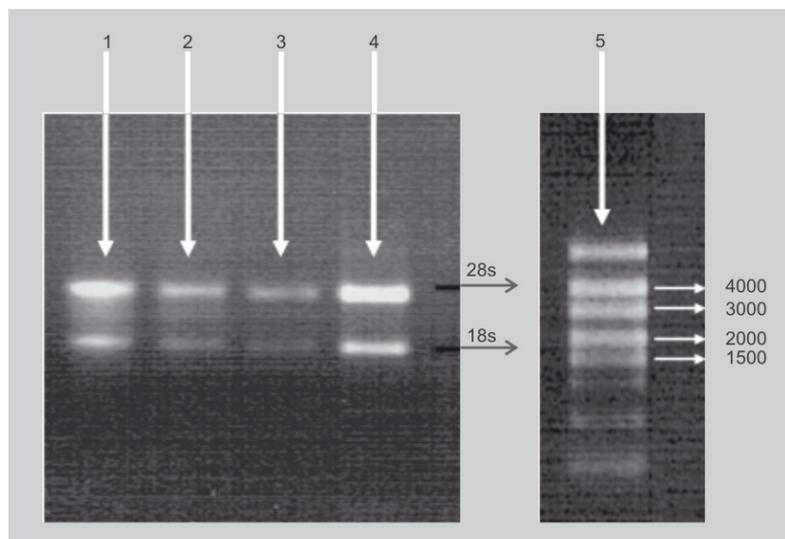
#### 3.1. Compared performances of the RNA extraction methods

Among the RNA extraction methods tested, the hot-borate method [31] emerges as par-

ticularly adequate for banana pulp and peel, whatever the maturity stage, and for the crown, which is particularly fibrous. Total RNA isolated by this method was of high purity and integrity and of sufficient yield, making it suitable for molecular analysis. This was confirmed in several ways.

First, spectrophotometric analysis revealed an [A<sub>260</sub> / A<sub>280</sub>] ratio ranging consistently from 2.0 to 2.2, indicating that the RNA isolated by this approach was largely free of contaminating proteins. This was true whatever the tissue type and maturity stage (*table D*). Pure RNA has an [A<sub>260</sub> / A<sub>280</sub>] ratio of 2.0, but, for most applications, a lower or higher ratio probably will not affect the results [3]. As a result of variations between individual starting materials and in performing the procedure, the expected range of the [A<sub>260</sub> / A<sub>280</sub>] ratio for RNA is 1.7–2.2. Three other methods gave this ratio: Liu *et al.* [30] and both methods described by Gehrig *et al.* [7]. Both commercial kits failed to extract RNA from banana fruit tissues. The TRIZOL reagent was developed for animal tissues, especially those rich in RNase, but it seems unsuitable for recalcitrant plant tissues such as banana. This is in agreement with the results obtained by other authors [12]. The method based on CTAB [33] was also unsuccessful. The same conclusion was reached with different recalcitrant plant species [13].

Secondly, a comparison of RNA yields based on spectrophotometric determinations showed a significantly lower yield (as



**Figure 2.** Band patterns obtained by ethidium bromide staining after electrophoresis, through a 1% agarose gel, of total RNA extracted from green banana peel (1 and 3) or pulp (2 and 4). The extraction protocols used were that of Liu *et al.*, [30] (1 and 2) and the hot-borate method [31] (3 and 4). (5) is a RNA ladder and the positions of the 28s and 18s rRNAs are shown. Similar results were obtained for both protocols with peel and pulp from ripe banana. No rRNAs were observed with any other method tested in this work (results not shown).

much as 15 times lower) for the method of Liu *et al.* [30] than for the hot-borate method [31] (*table II*). The yield obtained in this study by this latter method is comparable with those reported for other methods designed for high-phenol fruit tissues [4, 7]. The yield obtained in this study by the hot-borate [31] protocol depended heavily on the tissue type and, to a lesser extent, on the maturity stage. By decreasing yield (*table II*), the different tissues can be ranked as follows: green pulp (67.8–79.5  $\mu\text{g}\cdot\text{g}^{-1}$  FW) > mature pulp (61.8–65.9  $\mu\text{g}\cdot\text{g}^{-1}$  FW) > green

peel (45.2–60.2  $\mu\text{g}\cdot\text{g}^{-1}$  FW) > mature peel (43.4–50.5  $\mu\text{g}\cdot\text{g}^{-1}$  FW) > crown (10.2–17.4  $\mu\text{g}\cdot\text{g}^{-1}$  FW).

Thirdly, denaturing agarose gel electrophoresis yielded clear rRNA bands with a 28s rRNA band equal to or more abundant than the 18s rRNA band (*figure 2*), indicating that little or no degradation had occurred during extraction with the hot-borate [31] and Liu *et al.*'s [30] methods. In high-quality RNA, the 28s band should be approximately twice as intense as the 18s band [3]. In degraded RNA samples, the 18s band will be enhanced, since the 28s ribosomal RNA is typically degraded to an 18s-like species. No rRNA was observed with any other technique, and notably not with either of those described by Gehrig *et al.* [7]. The latter methods did give, however, a good  $[A_{260} / A_{280}]$  ratio (*table I*) and thus a high estimated yield (*table II*). An explanation might be that  $A_{260}$  could be falsely boosted by contaminants such as salt, organic solvent or protein, or by one or more of the many chemicals used in nucleic acid purification which absorb at 260 nm [34]. Thus, the  $[A_{260} / A_{280}]$  ratio can accurately describe nucleic acid purity, but it can also be misleading [35]. For this reason, it is essential to have both the  $[A_{260} / A_{280}]$  ratio and an agarose gel result to evaluate a RNA extraction method.

**Table II.**

Yield values ( $\mu\text{g RNA}\cdot\text{g}^{-1}$  fresh material), based on  $A_{260}$ , according to different tissue and RNA extraction methods. (x) Means that the yield could not be calculated because the  $[A_{260} / A_{280}]$  ratio was not between 1.7 and 2.2.

| Methods                                 | Peel      |             | Pulp      |             | Crown     |
|---|-----------|-------------|-----------|-------------|-----------|
|   | Green     | Mature      | Green     | Mature      |           |
| Trizol® Reagent (Invitrogen)            | (x)       | (x)         | (x)       | (x)         | –         |
| SV Total RNA Isolation System (Promega) | (x)       | (x)         | (x)       | (x)         | –         |
| Liu <i>et al.</i> [30]                  | 3.9–5.7   | 7.6–8.9     | 3.6–9.9   | 50.3–56.8   | –         |
| Mbégué-A-Mbégué <i>et al.</i> [31]      | 45.2–60.2 | 43.4–50.5   | 67.8–79.5 | 61.8–65.9   | 10.2–17.4 |
| Asif <i>et al.</i> [33]                 | (x)       | (x)         | (x)       | (x)         | –         |
| Gehrig <i>et al.</i> (GITC) [7]         | –         | 72.0–75.8   | –         | (x)         | –         |
| Gehrig <i>et al.</i> (GHCL) [7]         | –         | 102.4–109.7 | –         | 117.5–125.2 | –         |

GHCL: guanidium hydrochloride; GITC: guanidium hydrochloride.

**Table III.**

$[A_{260} / A_{280}]$  ratio and yield values (based on  $A_{260}$ ) according to four RNA *in situ* preservation methods. Extractions were performed by the hot-borate method [31].

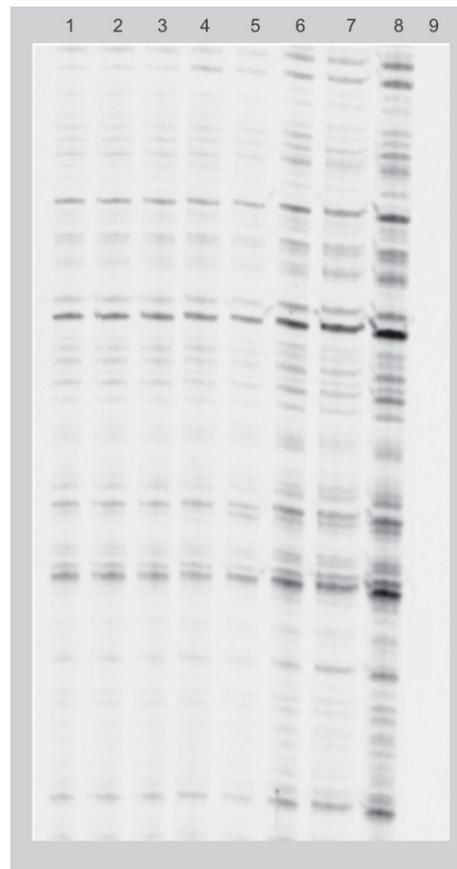
| Methods  | $[A_{260} / A_{280}]$ ratio | Yield ( $\mu\text{g RNA}\cdot\text{g}^{-1}$ fresh material) | 28s:18s RNA bands |
|--|-----------------------------|---|-------------------|
| Crown sliced into tiny lamellas and stored in RNAlater at 4 °C for 21 d  | 1.9–2.0                     | 3.2–5.7   | Yes               |
| Crown ground in liquid nitrogen and stored in RNAlater at 4 °C for 21 d  | 1.8                         | 1.5–3.7   | Yes               |
| Crown ground in liquid nitrogen and stored in the hot-borate buffer [31] + proteinase K at room temperature for 21 d | 1.9                         | 10.2–14.0   | Yes               |
| Crown freeze-dried and stored for 21 d at room temperature before extraction   | 2.2                         | 12.0–16.2   | Yes               |

Finally, the adequacy of the hot-borate method [31] for extracting total RNA from banana tissues in order to generate cDNA for downstream molecular analyses was successfully performed by cDNA-AFLP after DNase treatment (figures 3, 4).

For these reasons, although the hot-borate method [31] is labor-intensive and time-consuming, it seems to be the most suitable for extracting RNA from banana tissues in order to carry out genome-wide expression analyses. This RNA extraction technique is applicable to large samples of up to 25 g fresh material [36], but also to small samples of 0.24 g of material in a 2-mL tube, as in this work. It could be more convenient to extract RNA from a small sample, because many samples can be extracted at the same time, and the yield obtained is still sufficient for subsequent molecular applications.

### 3.2. *In situ* RNA preservation methods

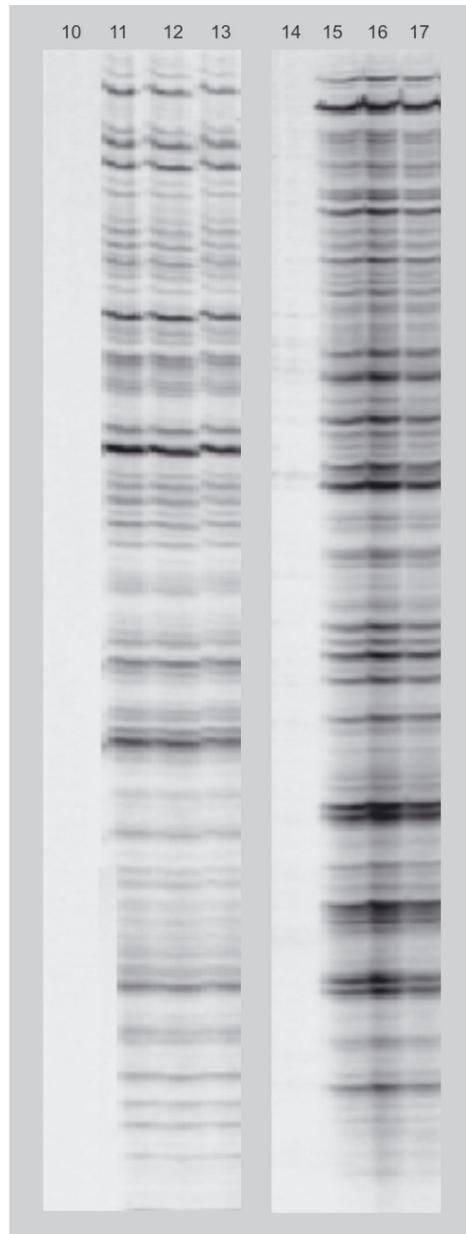
On the basis of quality, quantity and integrity, it appears that all four methods tested can preserve RNA (table III). The choice of a method must thus be based on the experimental constraints and the equipment available. Yet, although all four techniques appeared to preserve RNA quality and integrity, their yields were different. RNAlater is

**Figure 3.**

Reproducibility of cDNA-AFLP based on total RNA. cDNA-AFLP was applied to various pools of total RNA from a single crown according to figure 1. (1) to (8) represent the various cDNA-AFLP pools and (9) is a blank.

an aqueous tissue storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue samples. According to

**Figure 4.** Reproducibility of the cDNA-AFLP gene expression profile whatever the *in situ* preservation method. (10–13) = replicate 1; (14–17) = replicate 2. (10) and (14) are blanks; (11) and (15) are profiles obtained after immediate RNA extraction from fresh material; (12) and (16) are profiles obtained with RNA from freeze-dried tissues; (13) and (17) are profiles obtained with RNA from tissue samples stored in the hot-borate buffer [31].



Ambion (Austin, TX, USA), it eliminates the need to process tissue samples immediately or to freeze them in liquid nitrogen for later processing. Tissue pieces can be harvested and submerged in RNeasy for storage without jeopardizing the quality or quantity of RNA obtained by subsequent RNA isolation. In this study, RNeasy has the advantage of being a simple and fast technique, but its yield is two to five times lower than those

obtained by tissue storage in extraction buffer or freeze-drying. For the latter two methods, the yields are similar. These two techniques present the advantages of storage at room temperature and a similar RNA extraction quality and yield to those obtained after immediate extraction of RNA from fresh crowns (*tables II, III*). Freeze-drying has not been used extensively in plant tissues [4, 24]. Contradictory results, ranging from good RNA preservation in leaf tea [25] to complete degradation in cotton [26], have been reported. In these previous studies, only the RNA quantity and quality on agarose gel were analyzed [24]. In some cases, authors have tested the expression conservation of a single or two particular genes [19–21, 23, 25]. In this original study, the entire pre- and post-storage genome-wide expression profile were compared, in order to evaluate the eventual global modification of RNA expression that could happen during the freeze-drying process or storage. The results obtained by cDNA-AFLP clearly indicate that the RNA obtained by both buffer storage and freeze-drying was of sufficient quality for molecular application and that cDNA-AFLP pre- and post-storage differential expression profiles were identical (*figure 4*). Thus, these two storage methods do not affect either the RNA quantity and quality, or the genome expression. For this study, cDNA-AFLP was selected because it is a non-biased technique based on PCR amplification, offering the advantage of almost no false positives [37]. It provides a straightforward way to check band identity and homogeneity [37] and it is more sensitive than hybridization-based techniques and highly specific. It was also confirmed in this study that cDNA-AFLP is a highly reproducible method for genome-wide expression profile analysis (*figure 3*). In both replicates, the steps comprising DNase treatment, amplification and selective amplification led to the same AFLP profile. Reproducibility of cDNA-AFLP has already been reported by many authors [28, 38–40]. Khun [37] even evaluates the reproducibility of cDNA-AFLP at 100%.

Both storage techniques are thus perfectly suitable for cDNA-AFLP applied to the study of differential gene expression in different cell populations. However,

freeze-drying, which preserves enzymatic activity, offers further advantages highlighted in this study; they can be summarized: (1) the extraction yield, quality and integrity equal those obtained by fresh tissue RNA extraction or by conventional storage techniques; (2) the freeze-dried RNA can be stored even at room temperature, without any alteration of the expression profile, for further RNA extraction; (3) freeze-drying is an easy way to obtain a fine dry powder without using liquid nitrogen, which is not always available or economically feasible in all banana-producing countries; (4) freeze-dried tissues can be easily transported from one laboratory to another and even between countries; (5) a final advantage of freeze-drying is the large amount of RNA that can be obtained in a single extraction carried out in a 2-mL tube. Such a tube can hold only about 0.25 g fresh sample material, but if the material is freeze-dried, a mere 0.2 g is equivalent to about 2.4 g fresh weight. Assuming the same yield from freeze-dried material as from fresh material, the amount of RNA that can be obtained per tube is ten to twelve times higher when the material is freeze-dried. In applications requiring a lot of RNA, it could be very convenient to meet this requirement while working on a small scale with a 2-mL tube. These advantages make freeze-drying an attractive alternative to conventional storage.

Moreover, the two efficient storage methods proposed in this study to conserve fruit banana RNA *in situ* without altering the quality, quantity or expression profile could probably be applied to other plant tissues in particular fruits which are particularly recalcitrant.

#### 4. Conclusion

We thus evaluated and made choices among various techniques spanning all steps required to study differential gene expression in banana fruit. Our results highlight two good, reliable and practical storage methods that preserve RNA *in situ* before extraction without requiring ultra-low temperature. We also identified a RNA extraction method yielding high-quality RNA suit-

able for gene expression profiling. To illustrate this, we used the extracted RNA successfully to generate cDNA-AFLP differential expression profiles. Thus, it is possible to study gene expression in any banana fruit tissue, whatever the maturity stage, by applying all the procedures selected in our work.

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**La combinación de un método original que permita mantener la expresión del ARN *in situ* junto con un método eficaz para extraer el ARN permite estudiar la expresión de genes en cualquier tejido de banano.**

**Resumen — Introducción.** La extracción del ARN es un paso previo para estudiar la expresión de los genes en la banana y para comprender los cambios que intervienen en respuesta al entorno. Los métodos estándar de extracción del ARN no son eficaces con plantas como el banano, que presentan elevados niveles de compuestos fenólicos, de hidratos de carbono u otros compuestos ligados con y/o coprecipitan el ARN. **Material y métodos.** Se compararon entre cinco y siete métodos de extracción del ARN. Asimismo se compararon otros cuatro métodos de almacenamiento de tejidos de corona. Se empleó la técnica de cDNA-AFLP para asegurarse de que el ARN extraído poseía la calidad suficiente para aplicaciones moleculares y de que la expresión del ARN no sufría alteraciones tras almacenamiento *in situ*. **Resultados y discusión.** El método del borato caliente modificado resultó ser el mejor método de extracción del ARN mostrando unos elevados rendimientos de gran calidad; y, ofreciendo a la vez ARN no degradado a partir de los tejidos de la corona, la cáscara de los frutos así como de la pulpa en todas las etapas de maduración. El ARN obtenido mediante este método presentó suficiente calidad para las aplicaciones moleculares tales como el cDNA-AFLP que ofrece resultados altamente reproducibles. La liofilización de los tejidos frescos así como la conservación de tejido en la solución tampón de borato caliente, dos originales métodos de almacenamiento, parecen ser apropiados para la conservación del ARN *in situ* en ausencia de temperatura ultra baja. El ARN obtenido fue de calidad, no degradado, y utilizable para todas las aplicaciones. El perfil de expresión del genoma obtenido por la técnica de cDNA-AFLP permaneció intacto por estos métodos de almacenamiento de los tejidos colectados. **Conclusión.** Mediante la aplicación de todos los procedimientos sugeridos en este estudio es posible almacenar y estudiar la expresión de genes en cualquier tejido de banano, independientemente de su estado de madurez, sin afectar el nivel de expresión del ARN.

**Bélgica / *Musa sp.* / banano / liofilización / ARN / almacenamiento / extracción**