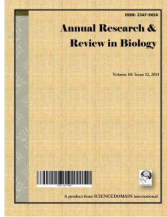

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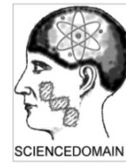

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Evaluation of *Passiflora edulis* Leaf Sample Storage Methods on RNA Quality and Suitability for Use in RT-PCR Assays

F. M. Munguti[1,2*], D. C. Kilalo[3], M. W. Macharia[4], E. N. Magiri[2], J. K. Kinyua[2] and T. A. Holton[4]

[1]Kenya Plant Health Inspectorate Service, P.O.Box 49421-00100, Nairobi, Kenya.
[2]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, P.O.Box 62000-00200, Nairobi, Kenya.
[3]Department of Plant Science and Crop Protection, University Nairobi, P.O.Box 29053-00625, Nairobi, Kenya.
[4]Biosciences, East and Central Africa-International Livestock Research Institute, Hub, P.O.Box 30709-00100, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all the authors. Author DCK designed the study. Author FMM carried out the laboratory experiment and wrote the first draft. Authors MWM and TAH supervised the laboratory experiments and offered technical advice. Authors ENM and JKK reviewed the experiment design and made a follow-up on the experiment. All the authors read, reviewed and approved the final manuscript.

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ABSTRACT

It is prerequisite to extract Ribonucleic Acid (RNA) with high quality and integrity in order to carry out molecular biology studies in any plant species. Samples collected from remote fields require preservation before being processed for RNA extraction and downstream process like RT-PCR,

**Corresponding author: E-mail: munguti.florence@gmail.com;*

real time PCR and genome-wide expression studies. It is therefore important to identify efficient and reliable sample storage methods that stabilize RNA, protecting it from the activities of RNAse in intact samples before analysis. This study was designed to evaluate the effect of different storage conditions for passion fruit leaves on RNA quality and suitability for RT-PCR for two time-points; one week and two weeks post-harvest. Passion fruit leaf samples with suspected viral symptoms were collected from the field and stored using FTA® cards, RNAlater solution, cold ice followed by transfer to -80°C freezer, drying on silica gel and drying in between the sheets of newsprints (as herbarium). The samples were kept for 1 and 2 weeks before RNA extraction and subsequent semi-quantitative RT-PCR to amplify the housekeeping genes AtropaNad and *Cowpea Aphid Borne Mosaic Virus* (CABMV); one of the major viruses causing passion fruit woodiness disease in Kenya. Good RNA yield and quality were obtained from samples stored in silica gel for 1 and 2 weeks after collection similar to -80°C frozen samples a choice preservation method by many laboratories all over the world. Further results confirmed that RNA extracted from samples stored in silica gel was fit for RT-PCR amplification. This study shows that RNA of good yield and quality that is useful for downstream applications can be obtained from passion fruit leaf samples stored in silica gel.

Keywords: Preservation methods; RNA quality; RNA integrity; passion fruit leaves.

1. INTRODUCTION

RNA quality has been considered one of the most critical components for the overall success of molecular biological applications like RT-PCR [1-4]. The ability to retain RNA integrity within sampled plant tissues is dependent on the sample preservation method as well as RNA recovery protocol. This is challenging especially for samples collected in remote areas [5]. Ribonucleic acid (RNA) can be degraded by the presence of cellular and environmental endogenous ribonucleases during transit if the storage conditions are not ideal [6]. Therefore, identification of a suitable technique for collection of samples in the field and subsequent preservation in the laboratory is crucial.

Different plant sample storage methods have been described for preservation of materials both pre- and post-processing to maintain the integrity of RNA in tissues and cells from their origin to the place of analysis. FTA® card is a chemically treated filter paper designed for the collection and room temperature storage of biological samples for subsequent nucleic acid analysis. It is impregnated with denaturing agents that act to lyze cellular material, and to fix and preserve the nucleic acids within the matrix [7]. FTA® cards can be used for any sample: blood, bacteria, viruses, plant, materials, microorganisms [8]. [5] reported the effective use of FTA®cards for sampling and retrieval of DNA and RNA viruses from plant tissues and their subsequent molecular analysis. Herbarium is another storage method that involves placing the samples in between sheets of newsprint and drying the material.

Maintaining samples in cold storage like -20°C, -80°C or snap freezing in liquid nitrogen is commonly used in many equipped laboratories. Freezing prevents the activity of nucleases that degrade RNA. The method can be quite successful when the plants are nearby and in easily accessible localities. [9] reported the method as convenient to process samples at a later date. However, the integrity of RNA is compromised using this method particularly when collecting samples from locations far away from the laboratories due to possibilities of shipment delays and suboptimal packaging. Additionally, frozen samples need to be homogenized quickly to avoid rapid RNA degradation during thawing of the previously frozen samples.

Preservation of plant tissues by silica gel desiccation has been reported to be effective for many species with rapid rates of desiccation following the use of proper leaf material to silica gel ratio [10,11]. Samples can be stored in silica gel at room temperature with occasional changing of the silica gel for proper drying of the materials. Although some species do not respond well to this type of sample preservation, the technique is generally simple and efficient since at dehydrated state, RNA is less susceptible to chemical or enzymatic degradation.

There are new technologies that help preserve RNA at room temperature, such as RNAlater®

(Ambion, Carlsbad, CA, USA) and RNA stable® (Biometrica, San Diego, CA,USA), which keeps isolated RNA in anhydrobiosis at room temperature for weeks [9]. However, these methods are costly and need to be available in the laboratory at the moment of use.

RNA is an extraordinarily fragile molecule that is rapidly degraded in samples unless they are preserved by appropriate treatment post-harvest. RNA quality may also be impaired in samples stored for a long time or under sub-optimal conditions [12]. It is therefore expected that collection and storage conditions will influence the survival of the RNA from RNAses. In the present study, we evaluated the effects of different sample preservation methods on the quality of the RNA obtained from virus infected passion fruit leaf samples for further cDNA synthesis and RT-PCR amplification. The methods included using FTA® cards, RNAlater solution, cold ice followed by transfer to -80°C freezer, drying on silica gel and drying the leave samples in between the sheets of newsprints (as herbarium). RNA from freshly obtained samples was used as a control. The study was aimed at determining the most suitable method that would be convenient and reliable for handling of passion fruit samples collected from farmers fields to the laboratory while aiming to achieve successful diagnostic applications.

2. MATERIALS AND METHODS

Passionfruit leaf samples with typical virus-like symptoms showing different levels of wrinkling, mosaic, chlorosis, vein clearing and ring spots were collected from the field. Sampling was done uniformly by collecting three young and topmost leaflets and stored using five different methods. The different preservation methods tested include herbarium, -80°C freezer, RNAlater, FTA cards and use of silica gel. For herbarium, leaf samples were arranged on a piece of plain newsprint and covered with two extra sheets of newsprint. The sheets containing the samples were placed on herbarium press. The samples were stored at room temperature in the laboratory. Samples stored at -80°C freezer were collected in Zip Lock sealable polythene bags (size 12 x 8 cm), put in a cool box with ice packs before snap freezing in a freezer. For silica gel, leaf samples were harvested into Zip Lock bags and silica gel (Sigma S-5631) was added in the ratio of one gram of leaf material to approximately 10 grams of silica. The Zip Lock bags were shaken to distribute the silica gel between the layers of leaves for sufficient drying. The samples were stored at room temperature with regular checking for dryness after every 12 hours. After the samples were sufficiently dried, they were removed from the silica gel and stored at room temperature in the laboratory.

Sampling onto FTA cards was done by gently pressing the leaf material on FTA® cards and allowing them to air-dry for one hour. The FTA cards were transferred to the laboratory and kept at room temperature until used for RNA extraction. For RNAlater, sampled leaflets were sliced with a sterile blade before immersing in RNAlater solution. Approximately one gram of leaf material was used per 5 ml of the RNAlater solution (Ambion, Inc. Austin, TX). The materials in RNAlater were transferred to the laboratory and kept at 4°C awaiting RNA extraction. Fresh samples were collected for immediate homogenization as a control.

2.1 Total RNA Extraction

All samples were subjected to total RNA extractions following the Trizol method [13]. For samples stored in -80°C, silica gel and in herbarium, approximately 100 mg of leaf sample was ground to a fine powder in liquid nitrogen using mortar and pestle. Samples in FTA®cards were processed by making a 3 mm diameter punch from the chlorophyll-stained regions and placed in a sterile microtube for subsequent RNA extraction. Samples stored in RNAlater were processed by first removing the leaves with a sterile forceps into a clean microtube, centrifuging at 12,000 xg for 1 minute to remove the excess RNAlater solution and ground in liquid nitrogen into a fine powder. Eight hundred microlitres of TRIzol-reagent (Sigma, USA) was added into each of the sample in a sterile microtube with shaking moderately for 30 seconds. The homogenate was incubated at room temperature for 5 minutes and centrifuged at 12,000 xg for 10 minutes at 4°C. The supernatant was transferred to a fresh microtube and 200 µl chloroform added to remove the proteins, lipids and DNA. The upper aqueous layer containing RNA was transferred to a fresh microtube and RNA precipitated using 250 µl of isopropanol followed by 250 µl of 1.2 M NaCl/0.8 M sodium citrate and centrifuged at 12,000 xg for 10 minutes. The precipitated RNA pellet was washed with 1 ml of 75% ethanol and the RNA was briefly air-dried. RNA pellet was dissolved in 30 µl of nuclease-free water and kept at -80°C freezer till use.

2.2 Estimation of RNA Quantity and Quality

The quantity and purity of RNA extracted from the samples was estimated using Nanodrop ND-2000C spectrophotometer (Thermo scientific, Wilmington, DE, USA). The nanodrop measurements were based on the optical density (OD) readings at 260 and 280 nm. To evaluate the quality/integrity of RNA, denaturing (formaldehyde) gel electrophoresis was carried out. Samples were prepared for loading by mixing 4 µl of 200 ng RNA per sample with 6µl deionized formamide, 1 µl of 10x loading dye and 1 µl gel red (1:100) (Biotium, Hayward, CA, USA) as described by [14]. The same was done for the RNA marker. The samples were denatured by heating for 5 mins at 65°C on a heater block and immediately chilled on ice for 5 minutes before loading onto the already cooled 1.2% gel. The gel was run at 100 volts for 40 mins and visualized under UV transilluminator.

2.3 Semi-quantitative RT-PCR

First strand complimentary deoxyribonucleic acid (cDNA) was synthesized with 1 µg of total RNA using Maxima First Stand cDNA synthesis kit for RT-PCR (Thermo Scientific) according to the manufacturer's instructions. The cDNA was stored at -20°C until use.

Semi-quantitative polymerase chain reaction (PCR) was conducted with Accupower PCR premix (Bioneer Corporation, Daejeon, Korea) using internal control AtropaNad2- gene -specific primers. The primer sequences were: forward 5'-GGACTCCTGACGTATACGAAGGATC-3' and reverse 5'-AGCAATGAGATTCCCAATATCAT-3', amplifying a 188 bp product. Polymerase Chain Reaction amplification was done using Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Cycling conditions consisted of initial denaturation of 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, annealing at 55°C for 40 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes. The PCR products were separated by gel electrophoresis in a 2% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) and visualized under UV transilluminator.

To evaluate the effect of the different methods on virus detection, semi-quantitative polymerase chain reaction (PCR) was conducted with specific primers for *Cowpea Aphid Borne Mosaic Virus* (CABMV); one of the causal viruses of passion fruit woodiness disease in Kenya [15]. The primer sequences were: forward 5' CACCAGAGCATCAAAGACACAGCTCA3' and reverse 5' CAGCTCAGTAAATGGTTTGAGGCCA3' amplifying 500 bp product. Accupower PCR premix (Bioneer Corporation, Daejeon, Korea) was used to perform the PCR in a 20 µl reaction. 2 µg of cDNA in a 20 µl reaction was used with a final concentration of 1X buffer, 1.5 nM Mgcl2, 250 µM dNTPs, 1 µl of 5 µM each forward and reverse primer for (CABMV) and I U Taq polymerase. Amplification was carried out in an Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following profile; Initial denaturation of 94°C for 5 minutes and 35 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 40 seconds and final extension of 72°C for 7 minutes. The PCR products were visualized by 2% agarose gel stained with GelRed (Biotium, Hayward, CA, USA). 1 kb plus DNA ladder (Thermoscientific) was also loaded to the wells.

2.4 Data Analysis

Data analysis was performed using SAS (Statistical Analysis systems) version 9.3. Two-way ANOVA was carried out and the Fisher's Least Significant Difference (LSD). Comparison of Means Test was used to test for any significant differences (at P = .05) in the ratio of $A_{260/280}$ and RNA quantity among the different storage methods for week 1 and 2.

3. RESULTS AND DISCUSSION

3.1 Measuring RNA Quality and Purity

To assess the presence and purity of extracted RNA, the ratio of absorbance at 260 and 280 nm is usually used. A $_{260/280}$ ratio greater than 1.8 is usually considered acceptable indicator of good quality RNA [1], although this ratio does not guarantee pure RNA. If the ratio is appreciably lower, it is an indication of the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. Ratios lower than expected could indicate that additional cleaning is necessary [4]. From our RNA extraction results as shown in the Table 1, all the extracted RNA gave $A_{260/280}$ nm absorbance ratio of between 1.7±0.23 and 2±0.06. The mean $A_{260/280}$ values for samples stored in freezer and

in silica gel for 1 and 2 weeks were not significantly different (*P*=.05) and were within the acceptable range of 1.8-2.0. The mean absorbance ratio for samples stored in RNAlater, FTA card and Herbarium were significantly lower (*P*=.05) than the expected $A_{260/280}$ ratio of 2, indicating presence of contaminants. RNA quality based on A $_{260/280}$ was maintained in samples stored in silica gel and Freezer across the two weeks hence silica gel provides an alternative for conventional cold storage which is usually costly and extremely time sensitive.

3.2 Assessment of RNA Integrity by Electrophoresis

Use of intact RNA sample is key for successful applications of molecular biology techniques including diagnostics. The integrity of 28S and 18S ribosomal RNA is used as the measure of RNA integrity and the lack shows a smear in the agarose gel, indicating that the total RNA is degraded. Visual inspection of the gel images was used to confirm the presence of bright, clear bands corresponding to the 28S and 18S ribosomal subunit, providing qualitative assessment of the rRNA integrity. Intact RNA was obtained in samples stored using silica gel, – 80°C freezer and RNAlater as evident by the two sharp bands corresponding to 18S and 28S rRNA on denaturing 1.2% agarose gel electrophoresis (Fig. 1). These clear distinct bands were comparable to the ones obtained from RNA from freshly homogenized sample (control) as seen in Fig. 1C. This was found to be related to the $A_{260/280}$ ratio values of RNA obtained for samples preserved in silica gel and -80°C freezer (Table 1), as there was no significant difference (*P*=.05) among these values (Table 1). Additionally, this was an indication that the protein, polyphenol and polysaccharide contamination was low in the RNA obtained from leaf samples preserved in silica gel and -80°C freezer, with $A_{260/280}$ ratio values that was at 2.0, [16]. According to the $A_{260/280}$ ratio values RNA obtained from the leaf samples preserved in silica gel and -80°C freezer, had good quality and integrity. In fact, the bands obtained in silica gel were stronger than the bands on -80°C freezer and comparable to the bands on freshly extracted RNA (Fig. 1C). These results also demonstrated that RNA isolated from samples stored in the herbarium and FTA cards was not of good integrity as evident by the smearing and absence of distinct bands (Fig. 1).

3.3 RNA Suitability for RT-PCR

The quality of RNA is very important for RT-PCR. According to [17], RNA contaminants can affect the PCR reaction by inhibiting the action of polymerase. It is therefore necessary to perform PCR using gene specific primers to confirm possible genomic DNA contamination before diagnostic application. RT-PCR for housekeeping gene; AtropaNad2.1a/2b specific primers targeting ndhB plant internal gene was carried out. Amplification targeting NADH dehydrogenase region at 188 bp (Fig. 2) was evident from all the samples stored in silica gel, followed by samples stored in -80°C freezer, and then RNALater. This was indicated by the observation of clear band signals. Faint signals of amplification products were also visible in the samples stored in FTA cards and herbarium which had earlier indicated a moderately to complete RNA degradation in the denaturing gel electrophoresis (Fig. 1). This was an indication that even samples with moderate RNA degradation could still lead to some significant PCR amplification (Fig. 2).

Table 1. Concentration and quality of RNA isolated from five different storage methods of leaf samples for 1 and 2 weeks post-harvest

Storage method	RNA concentration (ng/µl)		RNA quality (Absorbance values at $A_{260/A280}$)	
	Week 1	Week 2	Week 1	Week 2
FTA cards	830.3±166.02	954.5±441.46	1.8±0.12	1.7±0.14
Herbarium	1156.9±156.99	434.6±423.39	2±0.03	1.7±0.14
RNA later	271±164.99	927.5±341.19	1.7±0.23	1.9±0.05
-80°C freezer	1161.9±145.42	4171.8±365.24	2±0.06	2±0.02
Silica gel	1447.6±236.44	2336.1±100.00	2±0.01	2±0.03

Each data is the average of three independent extractions of each sample and the ± SD

RT-PCR was carried out for amplification of *Cowpea Aphid Borne Mosaic Virus* (CABMV) coat protein gene using specific primers. Amplification was evident for samples stored in silica gel giving specific product at expected band size of 500 bp (Fig. 3). The rest of the methods gave amplification in either one or two samples which could be related to the low quality of the RNA template or possible inhibition of the RT-PCR by RNA contaminants obtained during RNA extraction.

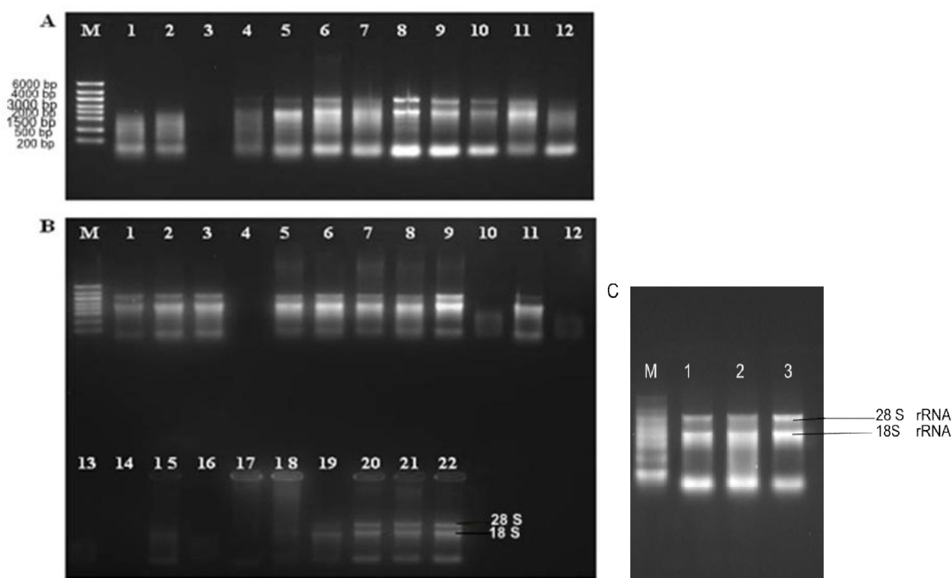

Fig. 1. Total RNA isolated from passion fruit leaf samples from five different preservation methods after one (A) and two (B) weeks of storage and C (Fresh samples prior to storage) as assessed by denaturing agarose gel electrophoresis. 200 ng RNA per sample was used per lane. For 1 week of storage (A), lanes 1 and 2 (FTA cards), lanes 3 and 4 (herbarium), lanes 5, 6 and 7 (-80°C freezer), lanes 8, 9 and 10 (silica gel) and lanes 11 and 12 (RNAlater). For 2 weeks storage (B) lanes 1-3 (-80°C freezer), lane 4 (blank), lanes 5-9 (RNAlater), lanes 10-13 (FTA cards), lanes 14-18 (herbarium) and lanes 19-22 (silica gel). Lane M represents RiboRuler RNA ladder (Thermoscientific) for both A, B and C

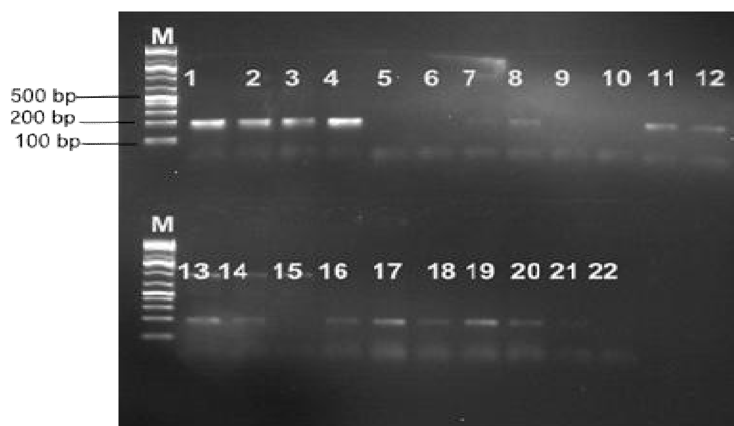

Fig. 2. PCR products for plant internal control gene using AtropaNad2.1a/2b primers. Lanes 1-4 (silica gel), lanes 5-8 (Herbarium), lanes 9-12 (FTA cards), Lanes 13-16 (RNALater), Lanes 18-21 (-80°C freezer), lane 22 (blank) and lane M represents 1 kb plus DNA ladder (Thermoscientific)

Fig. 3. PCR amplifications products using virus specific primers for CABMV detection. Lanes 1-3 (silica gel), 4-6 (herbarium), 7-9 (FTA cards), 10-12 (RNAlater), 13-15 (-80°C freezer), 16 (positive control) and 17 (negative control). Lane M represents 1 kb plus DNA ladder (Thermoscientific)

In this study, the PCR analysis demonstrated that the RNA obtained from leaf samples stored in silica gel and -80°C freezer were well suited for downstream applications, such as gene amplification, which can be further used in studies involving analysis of gene expression. However, silica gel would be preferably a method of choice for samples destined for virus detection, thus providing alternative to cold chain logistics especially where samples need to be collected and transported from remote areas.

4. CONCLUSION

Our study demonstrated that silica gel can be used for collecting and preserving passion fruit leaf samples from fields far from the processing laboratories for RNA extraction.RNA quality and integrity was maintained for 2 weeks of storage at room temperature and still remained competent for subsequent molecular applications like cDNA synthesis and RT-PCR. The information obtained from this study is relevant to other researchers involved in collection of samples from remote locations to the laboratory facilities. The ability to collect and preserve samples in a simple and convenient manner allows analysis of samples that would otherwise prove difficult. Such methods are invaluable for disease diagnostics and epidemiological studies, particularly in developing countries where it is difficult to provide a cold chain from sample collection to processing.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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