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An Optimised Cetyltrimethylammonium Bromide (CTAB)-Based Protocol for Extracting RNA from Young and Old Cassava Leaves

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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Study Protocol

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ABSTRACT

Ribonucleic acid (RNA) integrity, quality and quantity are critical in most plant molecular studies. Extracting high quality RNA from cassava leaves and other recalcitrant plant tissues are difficult due to the presence of polysaccharides, polyphenols and other secondary metabolites that often co-precipitate with the final RNA extract. This is an optimised a CTAB-based method that suitably extracts RNA from the polysaccharide-rich cassava leaves. The modifications were introduced into a version of the CTAB protocol as described by Gasic and colleagues [1]. The changes included an increased rate or use of Extraction Buffer (EB) for every gram ground leaf tissue (20 ml EB per 1 gram tissue), incubation of the Tissue-EB and Chloroform: Isoamyl alcohol (24:1) mixture at a lower water-bath temperature of 50°C and all centrifugation steps carried out at 4°C. In addition, the EB contained a higher concentration of soluble polyvinylpyrrolidone (PVP-K-30). The pH of sodium acetate was lowered to 5.2 and a final two-step high molarity (10M) Lithium Chloride (LiCl) precipitation was applied. Ethyl alcohol concentration was raised to 100%. The modified CTAB method produced RNA of high concentration (>1.0 µg), high A260:A280 and A260:A230 ratios

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(> 2.0) and high integrity (distinct and visible 28S rRNA and 18S rRNA bands) from young and old cassava leaves, compared to RNA (from the same leaf tissues) generated by several other published methods or commercial kits. The protocol is efficient, simple, and reproducible and is therefore recommended for RNA extraction from metabolite-rich cassava leaves or plants with similar tissues.

Keywords: CTAB; polysaccharides; polyphenols; RNA extraction protocol; cassava.

1. INTRODUCTION

Purification of Ribonucleic acid (RNA) of high quality and quantity is a pre-requisite and an essential step for many molecular techniques [1,2,3,4]. However, isolating suitable RNA remains problematic especially from recalcitrant plant species or tissues with high levels of phenolic compounds and/or polysaccharides [5]. The extracted RNA from these plant species are often of poor quality and too low for further downstream application [5,6]. Cellular components that inhibit high quality and quantity RNA isolation include endogenous RNases, polysaccharides, polyphenols, proteins, lipids and other secondary metabolites [7,6,4]. Phenolic compounds readily oxidise to form covalently linked quinones and easily bind proteins and nucleic acids resulting in high molecular weight complexes [7,8]. Polysaccharides tend to co-purify and co-precipitate with the RNA in the presence of alcohols or low ionic strength buffers [2,5,9]. Polysaccharide contamination hinders re-suspension of the precipitated RNA, interferes with absorbance-based RNA quantification, and may inhibit enzymatic manipulations, poly $(A)^+$-RNA isolation as well as electrophoretic migration [10]. Endogenous ribonucleases reduce the integrity of the RNA, particularly when the amount increases, such as during senescence, wounding, or pathogen attack [11,12,13]. Homogenisation triggers inevitably the mixture of RNA and endogenous RNases [10].

The above-mentioned contaminants can occur at various concentrations depending on the plant species and organs that are considered for nucleic acid extraction. A number of CTAB-based methods have been developed for RNA extraction from tissues containing high levels of polysaccharides and phenols [14,15,16]. The successes of yielding suitable RNA from cassava leaves using these methods have proved unreliable or have not been reported. The readily available commercial kits such as RNeasy plant kit (Qiagen), TRIzol Reagent (Sigma) and Concert Plant RNA Reagent (Invitrogen) have been successfully applied to extract RNA from cassava, but can be a costly option especially when a large number of RNA extractions are needed. The difficulty of obtaining RNA of high quality and quantity from cassava can be attributed to the high concentration of polysaccharides, phenolic compounds, proteins, and other secondary metabolites in the leaves. This paper describes an optimised protocol that provides the high quantity and quality RNA from cassava leaves. This procedure is modified from a version of the CTAB-based method as described by Gasic and colleagues [1].

2. MATERIALS AND METHODS

2.1 Cassava Leaves

Leaves of the cassava model genotype TMS 60444 were used in the extraction of RNA using a modified CTAB protocol as well as four other methods, obtained from literature (Table 1) for comparison purposes. Genotype TMS 60444 was first established and grown under greenhouse conditions located in Lindau-Eschikon, Zürich, Switzerland (47°26'N, 8°40'E, 540m asl) [17]. The plants were grown for four months before utilisation of its leaves for RNA extraction. Young leaf tissues constituted the three top most fully expanded leaves, while three leaves at the mid-stem were considered old leaf tissues.

2.2 Extraction Buffer (EB)

The EB was modified to include CTAB (2%), PVP K-30 (2%), Tris-HCl (100 mM; pH 8.0), EDTA (25 mM), NaCl (2 M), Spermidine (0.5g/l; free acid-HRS), 2% β-mercaptoethanol (added just before use), and Sterile RNase-free H_2O. Other reagents included Chloroform: Isoamyl-alcohol (24:1), Lithium Chloride (LiCl; 10 M), Sodium acetate (3 M; pH 5.2), and Ethyl alcohol (100%). The modified EB was then used to isolate RNA which was then contrasted with RNA extracted using protocols or commercial kits sourced from literature (Table 1).

Table 1. Main component of extraction buffers of the other four protocols (from literature review) that were also used to extract RNA and their final RNA extract compared with those of obtained from the modified CTAB-based method

#	Protocol / Kit	Main component of extraction buffer
1	RNeasy Plant Minikit Method	Guanidine Isothiocyanate
2	Total Nucleic Acid + DNase Treatment	Proteinase-K & RNase-free DNase 1 (Promega)
3	TRIzol Reagent Method	Phenol and Guanidine isothiocyanate
4	Reilly method [18]	Sodium Lauryl-sarcosine

2.3 RNA Isolation Procedure

(1) Cassava leaves were homogenised in liquid nitrogen with a pestle and mortar to a fine powder. Liquid N_2 was constantly added to the tissue during grinding to prevent thawing.
(2) The frozen powdered tissues were then quickly transferred to a pre-chilled 50 ml falcon tube containing EB at a rate of 20 ml of the extraction solution per 1 g tissue.
(3) The mixture was vortexed briefly and incubated on ice for 5 minutes. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added.
(4) The sample was then heated in a water bath at 50°C for 15 minutes and then centrifuged at 5000 rpm, RT, for 10 minutes.
(5) The resulting supernatant was transferred to fresh a 50 ml falcon tube, where an equal volume of Chloroform: Isoamyl alcohol (24:1) was again added, mixed, vortexed and centrifuged as described above.
(6) The resultant supernatant was transferred to a fresh 50 ml tube, 0.25 volumes LiCl (10 M) were added and the mixture was incubated overnight at 4°C.
(7) The sample was then centrifuged at 5000 rpm, RT, for 20 minutes the resultant supernatant decanted and the precipitated pellet was dried by inverting the tubes for 10 minutes on a kimwipe.
(8) The pellet was then re-suspended in 250 µl sterile RNase-free H_2O and transferred into a 2 ml micro-centrifuge tube where 250 µl LiCl (10 M) was added.
(9) The mixture was flicked to mix and the RNA precipitated by incubating on ice for 2 ½ hours. The sample was then centrifuged at 13 000 rpm, 4°C, for 10 minutes.
(10) The resultant supernatant was decanted; the RNA pellet re-suspended in 250 µl sterile RNase-free H_2O and 25 µl sodium acetate (3 M; pH 5.2) and 1 ml 100% ethyl alcohol were added.
(11) The mixture was then incubated at -20°C for 60 minutes and then centrifuged as described above.
(12) The resultant supernatant was decanted and the RNA pellet vacuum dried in a SpeedVac for 3 minutes. The dried RNA pellet was then re-suspended in 100 µl sterile RNase-free H_2O.

Note: The extraction of RNA using the modified CTAB method (as described in the procedure above) and the other four protocols was replicated five times i.e. on the three young and three old leaves of individual plants of cassava genotype TMS 60444.

2.4 Analysis of RNA

The total quantity and purity of the final RNA extract was determined using a spectrophotometer (NanoDrop®ND-1000, Technologies Inc.). Analysis of Variance (ANOVA) was carried out on the concentration (ng/µl) and the spectrophotometric absorbance ratio (A_{260}:A_{280} and A_{260}:A_{230}) values and the means compared or separated using the Fisher's protected Least significant differences (Lsd) test at 5% probability level. The PROC ANOVA procedure of Genstat Discovery Edition 3 was (Lawes Agricultural Trust Rothamsted Experimental station, UK) was followed.

The spectrophotometric ratios A_{260}:A_{280} and A_{260}:A_{230} are used to indicate protein and polysaccharide (+ polyphenols) contaminations respectively [19]. The integrity of the total RNA was verified by separating the fragments on 2% non-denaturing Agarose gels using electrophoresis [20].

3. RESULTS

Variation in specific leaf tissue A_{260}:A_{280} and A_{260}:A_{230} ratios were observed. For instance, the modified CTAB protocol had consistently more than 2.0 of the A_{260}:A_{280} and A_{260}:A_{230} ratios in

both young and old leaves compared to other methods whose ratios were less than 2.0 and inconsistent between the leaves (Table 2). Specifically, methods that had less than 1.8 of the A_{260}:A_{280} and A_{260}:A_{230} ratios in both leaves included TRI®$_{ZOL}$ Reagent, Reilly method [18] and RNeasy Kit (Table 2). Although the A_{260}:A_{280} ratio of total Nucleic Acid + DNase method was 1.94 and 1.83 in young and old leaves respectively (all above recommended 1.8), the same method exhibited 1.38 and 1.26 of A_{260}:A_{230} ratio in young and old leaves (Table 2).

RNA concentration in ng/µl from the five tested protocols varied significantly between and within leaf tissues (Table 2). For example, the more than 1000 ng/µl synthesised from young and old leaves via modified CTAB method was significantly higher (*P≤0.05*) than amounts of RNA from the other four protocols. In young leaves, RNeasy kit produced mean of 161 ng/µl of RNA, while the remaining three methods (TRI®$_{ZOL}$ Reagent, Total Nucleic Acid + DNase and Reilly method) yielded more than 250 ng/µl (Table 2). Variation in RNA concentration from old leaves was also computed. These included 66, 100, 212 and 248 ng/µl of RNA respectively extracted from RNeasy Kit, Reilly method [18], Total Nucleic Acid + DNase and TRI®$_{ZOL}$ Reagent kit (Table 2). Generally, RNeasy Kit produced the least amount of total RNA and modified CTAB method had the highest RNA concentration from both leaves (Table 2).

RNA integrity was determined through 2% Agarose gel electrophoresis (Fig. 1). Bands corresponding to 28S rRNA and 18S rRNA were more distinctly visible in RNA isolated using the modified CTAB method compared to the remaining four protocols (TRI®$_{ZOL}$ Reagent, RNeasy Kit, Total Nucleic Acid + DNase & Reilly method) that exhibited none of these bands (Fig. 1).

4. DISCUSSION

The effectiveness of the modified CTAB-based method and four other protocols (from literature) in purifying RNA of high quality, quantity and integrity from polysaccharide rich cassava leaves was tested. The success of an RNA isolation procedure is judged by the quantity, quality and integrity of the isolated RNA [21]. In this experiment, all the tested protocols permitted the extraction of RNA from both young and old leaf tissues. The RNA quality was measured by means of spectrophotometric ratios that relate differences in absorption spectra maxima of pure RNA, A_{max} = 260 nm, proteins, A_{max} = 280 nm, and polysaccharides, A_{max} = 230 nm [19]. Pure RNA should have an A_{260}:A_{280} ratio between 1.9–2.1 and an A_{260}:A_{230} ratio of 1.8–2.3. These ratios varied in the five protocols that were tested therefore indicating differences in RNA purity levels from both young and old cassava leaves.

The above results indicated that, with the exception of the modified CTAB method, RNA samples from the other four protocols were significantly contaminated with polysaccharides, phenolic compounds and proteins as shown by their low A_{260}:A_{280} and A_{260}:A_{230} ratios i.e. all less

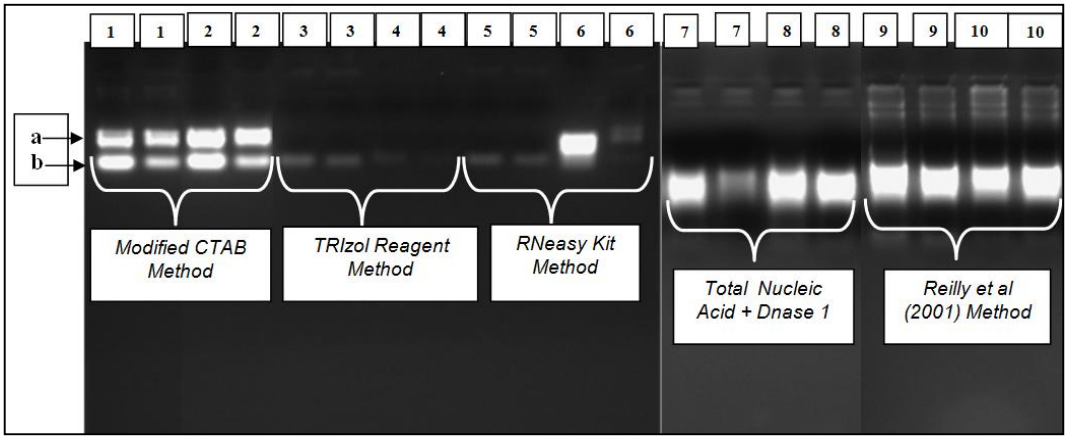

Fig. 1. Agarose gel electrophoresis (2%) showing integrity of RNA extracts red using five protocols. *Band (a) = 28S rRNA; Band (b) = 18S rRNA;* Young leaves = samples 2, 4, 6, 8 & 10; Old leaves = samples 1, 3, 5, 7 & 9

Table 2. Purity and concentration of total RNA extracted from young and old cassava leaf tissues using different protocols and kits

Leaf tissue	Protocols	A_{260}:A_{280}	A_{260}:A_{230}	concentration (ng/µl)
	TRI®$_{ZOL}$ Reagent	1.52^{s}	1.23^{k}	420^{c}
Young leaves	Total Nucleic Acid + DNase	1.94^{r}	1.38^{j}	617^{b}
	Reilly method [18]	1.18^{t}	0.68^{m}	284^{ce}
	RNeasy Kit	1.56^{s}	0.93^{l}	161^{de}
	Modified CTAB	2.19^{q}	2.21^{i}	1793^{a}
	TRI®$_{ZOL}$ Reagent	1.42^{w}	1.24^{o}	248^{g}
Old leaves	Total Nucleic Acid + DNase	1.83^{v}	1.26^{o}	212^{gh}
	Reilly method [18]	1.18^{x}	0.40^{p}	100^{gh}
	RNeasy Kit	1.41^{w}	0.44^{p}	66^{hh}
	Modified CTAB	2.16^{u}	2.20^{n}	1078^{f}
	Lsd at P≤0.05 for leaf tissues	***0.11***	***0.14***	***160.2***

Means followed by the same letter in the A260:A280; A260:A230 and yield columns are not significantly varied (P>0.05). For each tissue, five samples were extracted and measured during three independent experiments

than 1.8. The A_{260}:A_{280} and A_{260}:A_{230} ratios from modified CTAB method were more than 2.0. Although RNA produced by the Total nucleic acid + DNase method from young leaf tissues was protein-free (A_{260}:A_{280} > 1.8), the sample was still significantly contaminated with phenolic compounds (A_{260}:A_{230} < 1.8). Additionally, the modified CTAB method produced RNA of high concentration from both young and old cassava leaf tissues compared to the other four protocols that generated RNA of low concentration. These results showed that the RNA from the modified CTAB method was not only sufficient in concentration, but was also free from contamination by polysaccharides, phenolic compounds or proteins. Using a closely related (but not a replica) CTAB based method, Gasic et al. [1] obtained similarly high quantity and quality RNA from various apple tissues rich in polyphenols and polysaccharides. The successful extraction of RNA from cassava leaves using the modified CTAB-based method can be attributed to modifications introduced on the original procedure.

The changes included using 20 ml EB (instead of 10 ml) for every 1 gram ground tissue. This not only improved the 'capture' of the RNA from the starting material, but also the efficiency of separating organic and aqueous phase after centrifugation process. The tissue-EB and chlorophyll: Isoamyl alcohol mixture was incubated in a water-bath pre-warmed at 50°C instead of 60°C. The lowered incubation temperature reduced the chances of RNA degradation, as shown by the distinctly visible 28S rRNA and 18S rRNA bands (Fig. 1). Similar findings have been reported by Alemzadeh et al. [6] who observed that a lower temperature during RNA extraction was effective in isolating high-quality non-degraded RNA from phenolic-rich tissues of eelgrass.

Finally, a two-step (overnight and $2^{1}/_{2}$ hrs) precipitation (instead of single) with LiCl (2.5 M) was introduced into the protocol. This further eliminated polysaccharides and thus improved and increased the purity and yield of final RNA [2,19]. In addition, the reagents used to constitute the EB of the modified CTAB method also contributed to the extraction of a high quantity and quality of RNA from both cassava leaf tissues. Landolino et al. [19] also reported that an improved EB, precipitation procedure and a final clean-up step differentially remove contaminating metabolites.

CTAB is a detergent that preserves the integrity of nuclear and organelle membranes yielding total RNA with lower concentrations of un-spliced heteronuclear transcripts, as well as an increased RNA-to-DNA ratio [22,23]. The (PVP) K-30 (soluble) in the EB improved sequestration and elimination of phenolic compounds [4,24] and inclusion of low spermidine concentration deterred co-isolation of the RNA with polysaccharides and phenolics [14]. The high molarity of NaCl (5 M) and the strong reductant β-ME in the EB increased the solubility of polysaccharides, reducing their co-precipitation with RNA in later steps of the protocol and denatured ribonucleases and other contaminating proteins that are released during tissue disruption and homogenisation [19,25,26]. The high molarity LiCl not only differentially precipitated RNA from admixture with DNA, but it also increased RNA yield and favored

precipitation of larger transcripts over smaller ones [2].

The centrifugation steps were carried out at 4°C because the low temperature reduced RNA degradation and decreased the rate of chemical reactions between nucleic acid and phenolic compounds hence improving elimination of phenolic compounds [4]. RNA purification protocols with CTAB in the EB have been adapted to extract RNA from other polysaccharides and polyphenol-rich plant tissues such as *Arabidopsis siliques*, sweet potatoes, grape berries and other woody plants [4,2,15]. In two studies, isolation and characterisation of cassava catalase expressed during post-harvest physiological deterioration and towards identifying the full set of genes expressed during cassava post-harvest physiological deterioration [27,28], sodium lauryl-sarcosine in the EB was used to extract total RNA from storage roots of cassava cultivar CM2177-2 or leaves for northern hybridisations. However, when the EB containing Lauryl-sarcosine was tested and used to extract RNA from young and old leaf tissues of cassava genotype TMS 60444, the final RNA extract was of low quantity and purity.

5. CONCLUSION

The success of most molecular techniques depends on RNA of high quality, quantity, and integrity. RNA of high A_{260}:A_{280} and A_{260}:A_{230} ratios (more than 1.8), high concentration (more than 1.0 µg) and integrity (distinct and visible 28S and 18S rRNA bands) are preferred. Based on these requirements, we recommend the adoption and employment of the optimised CTAB-based method to extract RNA of sufficient quantity and quality from metabolite-rich cassava leaves. The protocol is efficient, simple, and reproducible. The protocol is efficient, simple, and reproducible and can be used to extract RNA from other plants with similar metabolite-rich tissues.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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