



## An optimized method for the isolation of high-quality functional RNA from polyphenol and polysaccharide-rich *in vitro* cultured cells of tea

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### Abstract

The tea plant is rich in polyphenols which attribute to its quality and flavor. However, it also hinders the isolation of high-quality RNA from tea samples. Furthermore, tea's callus and cells in suspension cultures are rich in polysaccharides and polyphenols, which act as a bottleneck during functional RNA isolation, hampering its quality and yield immensely. In this study, a lithium chloride-based isolation procedure teamed with phenol-based extraction of RNA was optimized to overcome these hurdles. The method yielded high RNA (175.8-260.8 µg/g FW) with  $A_{260/280}$  ranging from 1.82-2.08 and  $A_{260/230}$  ranging from 1.83-2.02, indicating that the isolated RNA was free of protein, polyphenol, and polysaccharide contaminants. The isolated RNA was also found to be suitable for downstream processing such as reverse transcription, PCR and qRT-PCR analysis. Therefore, this method can be used for the isolation of high-quality RNA from *in vitro* cultured cells of tea with high polyphenol and polysaccharide content, and also open avenues for future research in molecular biology related to callus and suspension cultures of tea.

**Keywords:** tea, *Camellia sinensis*, *in vitro* culture, RNA isolation, callus, polysaccharide-rich

### Introduction

Tea is the second most consumed beverage in the world. Its popularity is based on its astringent flavor and aroma. This property is rendered to tea by the high polyphenol content that the plant possesses. The two main varieties of tea plants are the China-type (*Camellia sinensis*) which have smaller leaves and are found in central China and Japan, and the Assam-type (*Camellia assamica*), which have broad leaves and grow mainly in northeast India and Szechuan and Yunnan provinces in China [1]. Tea, being rich in polyphenols, has been a problematic sample as far as isolation of high-quality RNA is concerned [2, 3]. Although, various methods for the isolation of good quality RNA from tea leaves and other plant parts have been developed eventually [4-6]. However, *in vitro* cultured cells of tea rich in polysaccharides and polyphenols hinder the appropriate isolation of RNA, resulting in poor quality and low yield. Additionally, polyphenols and polysaccharides cause the degradation of RNA [7]. Various methodologies [3, 6, 8-10], including commercial kits and Trizol (Sigma, USA), have been attempted to isolate RNA from cultured tea cells. However, functional RNA isolation from *in vitro* cultured tea cells using the above techniques failed to yield high-quality RNA in adequate amounts. Hence, the present study aimed to develop a reliable and convenient method for isolating high-quality total RNA from polyphenol and polysaccharide-rich *in vitro* cultures of tea with high yield.

### Materials and Methods

#### Reagents and Chemicals

Murashige and Skoog (MS) medium, 6-benzylamino purine (BAP), agar,  $\beta$ -mercaptoethanol, polyvinylpyrrolidone

(PVPP), carbendazim, pectinase, sodium acetate, potassium acetate, isopropyl alcohol, ethylenediaminetetraacetic acid (EDTA), and Tris HCl were purchased from Himedia (Mumbai, India) and 2,4-dichlorophenoxyacetic acid (2,4-D), lithium chloride, sodium dodecyl sulfate (SDS) and ethanol were purchased from Sigma (MO, USA). Rio's and Synergy system's (Millipore, MA, USA) purified water was used to prepare growth hormone solutions and the growth media.

#### Plant Material

The Assam-type cultivars S.3A/1 and T.3E/3 and the China-type cultivars HV39 and CPI were selected for the study. We collected leaves and flowers of the selected cultivars from the experimental plot of Tocklai Tea Research Institute, Tea Research Association (TRA), Jorhat, Assam, India. The samples were put into ice immediately after plucking to reduce polyphenol oxidase activity.

#### Surface Sterilization

The collected samples were washed repeatedly with few drops of a mild detergent such as Tween 20. The leaves and flowers were then treated with 0.3% carbendazim, followed by treatment with 0.1% mercuric chloride ( $HgCl_2$ ) for 10 minutes under a sterile environment. Finally, thorough rinsing of the samples with sterile water was carried out before inoculation.

#### Callogenesis and Suspension Culture Development

Leaf and flower samples, after sterilization, were cut into smaller pieces and inoculated into the growth medium containing modified Murashige and Skoog (MS) medium

[11] with BAP and 2, 4-D (0.5-2 mg/L). The sample plates were kept in the dark for callus initiation. After 17-22 days, callogenesis occurred; the time required differing with cultivar and sample. The samples were sub-cultured every three weeks into fresh medium for sustainable growth and maintaining the calli's friability.

For suspension culture development, 1 g of cells from each sample was treated with 0.5% pectinase for 12 hours for digesting the middle lamella of cells in clusters. The cells were then rinsed with sterile water, transferred to 100 mL modified MS broth containing 1 mg/L 2, 4-D and BAP each, and kept under orbital shaking at 120 rpm.

### Total RNA Isolation

Cells weighing 100 mg were crushed using liquid nitrogen with the help of mortar and pestle. Twenty milligrams (20 mg) of activated PVPP were added to each sample during crushing to inhibit polyphenol oxidase activity. Samples were then transferred to sterile microcentrifuge tubes containing 1 mL extraction buffer (0.005M EDTA and 0.2M Tris-HCl at pH 8, 0.09M LiCl, and 1% SDS) and 10  $\mu$ L  $\beta$ -mercaptoethanol and vortexed briefly. An equal volume of PCI (phenol: chloroform: isoamyl alcohol, 25:24:1 v/v) was added to each tube, followed by thorough mixing by repeatedly inverting the tubes. After an incubation of 10 minutes at room temperature, the tubes were centrifuged at 10000 rpm for 20 minutes at 4°C. This led to the separation of the contents in each tube into three different phases – the upper aqueous phase (consisting of the nucleic acids), the interphase (consisting of the debris), and the lower organic phase (consisting of proteins). The upper aqueous phase was transferred to a fresh microcentrifuge tube, and 0.1 volumes 3M sodium acetate and commensurate chilled isopropyl alcohol were added to each tube incubated at -20°C for 45 minutes. After incubation, the tubes were centrifuged at 14000 rpm for 20 minutes at 4°C. The supernatant from each tube was decanted, and 0.5 mL nuclease-free water was added to each tube to dissolve the pellet, followed by centrifugation at 10000 rpm for 10 minutes at 4°C. The supernatant from each tube was transferred to a fresh microcentrifuge tube and then an equal volume of 8M LiCl was added for selective RNA precipitation. After an incubation period of an hour at -20°C, the tubes were centrifuged at 14000 rpm for 20 minutes at 4°C.

Further discarded supernatant from each tube and 100  $\mu$ L 3M potassium acetate and 250  $\mu$ L chilled ethanol were added to each tube and incubated at -20°C for 45 minutes. After incubation, the tubes were centrifuged at 14000 rpm for 20 minutes at 4°C. The RNA obtained as the pellet was washed with 3M LiCl and freshly prepared 80% ethanol. After quick drying, the pellets were dissolved in 20  $\mu$ L nuclease-free water.

### RNA Quantification

RNA isolated from the different samples was assessed for quality through electrophoresis in a 1.2% denaturing agarose gel stained with ethidium bromide at 80V for 15-20 minutes. Quantification and purity check of the isolated RNA was done using a Biophotometer (Eppendorf, Germany). Absorbance ratios  $A_{260/280}$  and  $A_{260/230}$  served as measurements of purity in terms of protein and carbohydrate contamination respectively.

### First-Strand cDNA Synthesis and PCR Amplification

Each sample was treated with RNase-free DNaseI enzyme for 15-20 minutes at 37°C to remove any contaminating DNA present that might interfere with downstream processing. The DNA-free RNA was then reverse transcribed using the Verso cDNA synthesis kit (Thermo Fischer Scientific, USA) according to the manufacturer's guidelines to synthesize single-stranded cDNA. cDNA was synthesized using an equal concentration of mRNA from each sample for the reverse transcription process.

After its synthesis, the cDNA samples were tested for their quality by PCR amplification of a segment of the 18S rRNA of tea using specific primers (Forward primer- 5'CGCTGGCACCTTATGAGAAATC3' and Reverse primer- 5'ACCTGGTAAGTTTCCCCGTGTT3').

### qRT-PCR Analysis

The cDNA reverse transcribed from the RNA using the present method was used for qRT-PCR analysis to determine the quality and amplification level. A fragment of the reference eukaryotic Initiation Factor 4 $\alpha$  (eIF-4 $\alpha$ ) gene of *Camellia sinensis* was amplified using gene-specific primers (Forward primer- 5'CTGGTCAAATCGGAAATGGTTATG 3' and Reverse primer- 5'GACCTCCTGTCAATCTTGGTTAG 3').

## Results and Discussion

### Total RNA Yield and Quality

The integrity of the total RNA isolated using the present method was ascertained from the presence of intact 25S and 18S rRNA bands (Figure 1) as visible under a UV transilluminator. The isolated RNA's purity was deciphered from the  $A_{260/280}$  ratio, ranging from 1.82-2.08, indicating little or no contamination with proteins, and  $A_{260/230}$  ratio, which ranged from 1.83-2.02 indicating minimal contamination with polyphenols and/or polysaccharides. Total RNA concentration obtained in flower samples ranged from 175.8-199.7  $\mu$ g/g FW, while that in leaves ranged from 182.2-260.8  $\mu$ g /g FW. This is comparable to data reported in other plants using various methods [12-14].

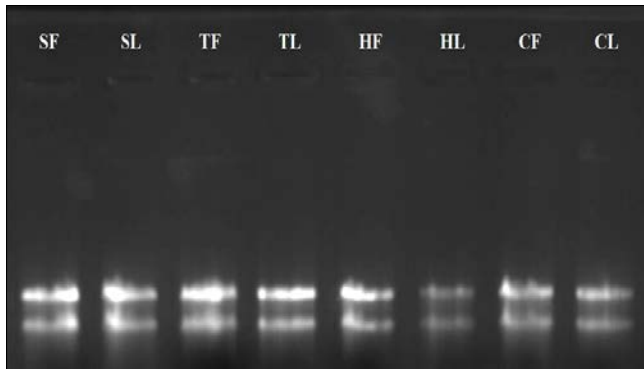
**Table 1:** Biophotometer readings for evaluation of RNA quality and quantity

Sample	Cultured cells from	Total RNA ( $\mu$ g/g FW)	Absorbance ratios	
			$A_{260/280}$	$A_{260/230}$
S.3A/1	Flowers	194.5	2.01 $\pm$ 0.08	1.85 $\pm$ 0.02
	Leaves	260.8	1.93 $\pm$ 0.02	1.99 $\pm$ 0.03
T.3E/3	Flowers	179.4	1.88 $\pm$ 0.04	1.83 $\pm$ 0.01
	Leaves	182.2	1.98 $\pm$ 0.07	1.86 $\pm$ 0.04
HV39	Flowers	175.8	2.02 $\pm$ 0.04	1.84 $\pm$ 0.04
	Leaves	237.7	1.82 $\pm$ 0.02	1.93 $\pm$ 0.05
CP1	Flowers	199.7	2.08 $\pm$ 0.02	2.02 $\pm$ 0.06
	Leaves	214.0	1.99 $\pm$ 0.04	1.87 $\pm$ 0.08

\* Data is expressed as the mean of two samples  $\pm$  standard deviation.

The tea plant is rich in polyphenols (30-35%) [15, 16], which generally hampers RNA yield and quality during its isolation. Nonetheless, in time, various protocols have been developed which, to some extent, have been successful in overcoming this impediment during the extraction of good quality RNA from tea plant parts [2-4, 17]. However, these new methods have also not yielded good quality and quantity of RNA from *in vitro* cultured tissues of tea such as callus and cells from suspension cultures. Moreover, the

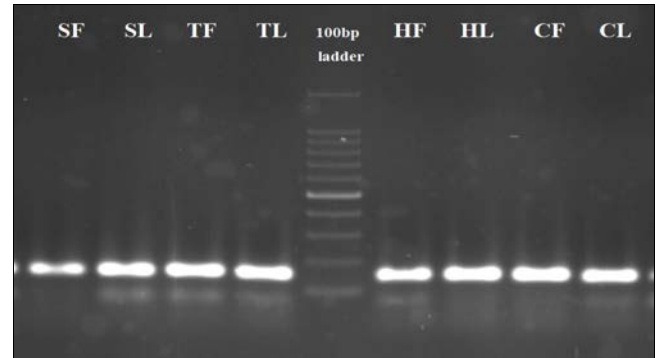
standard TRI reagent (Sigma-Aldrich) and HiPer™ Plant RNA isolation kit (Himedia, India) were found to be ineffective in isolating good quality RNA. This was due to the high concentration of polysaccharides in the cultured cells, in addition to high polyphenols, which posed a fresh challenge. The low yield and quality of RNA from cultured cells in tea was primarily due to complex formation between polysaccharides and polyphenols with the RNA, hampering its isolation. This method tried to overcome this bottleneck by applying phenol extraction coupled with potassium acetate, followed by lithium chloride, which precipitates RNA explicitly.



**Fig 1:** Ethidium bromide-stained 1.2% agarose gel showing distinct 25S and 18S rRNA bands from *in vitro* cultured cells of tea. Lanes 1-8 denote total RNA isolated from cultivars S.3A/1, T.3E/E, HV39, and CP1 flowers and leaves

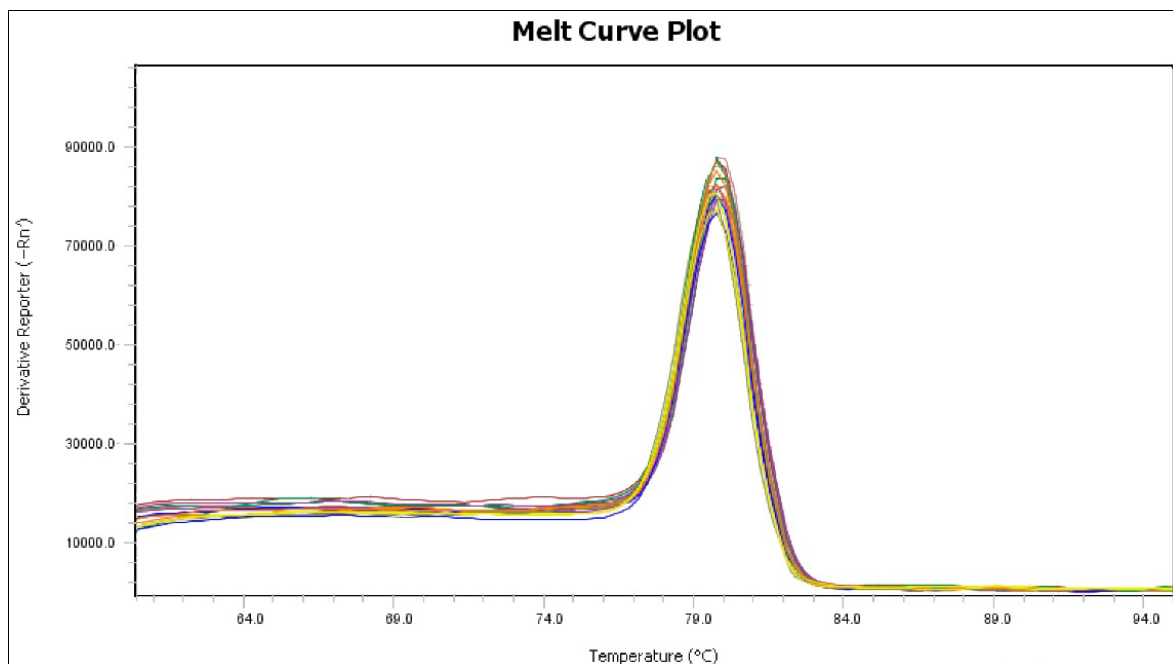
### cDNA Synthesis and Quality Assurance

First-strand cDNA synthesis using the Verso cDNA synthesis kit (Thermo Fischer Scientific, USA) was confirmed by the amplification of 18S rRNA fragment with a single sharp band of the expected size 145bp (Figure 2).



**Fig 2:** A single sharp band formed by the amplification of a segment of 18S rRNA gene (145 bp) using the isolated RNA.

The expression of the reference gene eIF-4 $\alpha$  using all the cDNA samples through qRT-PCR was carried out in triplicates. Melt curve analysis revealed a single melting peak signifying a specific amplified product (Figure 3). The quality of isolated RNA, and hence the cDNA, is crucial for the reproducibility of qRT-PCR results [18]. Specific and reproducible results in the present qRT-PCR assay indicate the high quality of RNA isolated using the present method free from polyphenol and polysaccharide contamination.



**Fig 3:** Melting peak obtained by qRT-PCR of the reference gene eIF-4 $\alpha$  using gene-specific primers.

### Conclusion

The present study aimed to optimize a method for the isolation of total RNA from *in vitro* cultured cells of tea. This method is a reliable and high-throughput method for the isolation of high-quality total RNA from tea callus and cells in suspension, which are rich in polysaccharides in addition to their high polyphenol content. This rapid and straightforward method resulted in high yield and quality total RNA, which was suitable for downstream processes such as qRT-PCR analysis.

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### Conflicts of Interest

The authors declare that there is no conflict of interest.

\*This work is a part of the Ph.D. dissertation of the first author.

## References

- Mendilcioglu K. Tea growth techniques. Ege University Agricultural Faculty, 2000, 43.
- Lal L, Sahoo R, Gupta RK, Sharma P, Kumar S. RNA isolation from high- phenolic tea leaves and apical buds. *Plant Molecular Biology Reporter*, 2001;19(2):181.
- Das A, Saha D, Mondal TK. An optimized method for extraction of RNA from tea roots for functional genomics analysis. *Indian Journal of Biotechnology*,2013;12(1):129-132.
- Muoki RC, Paul A, Kumari A, Singh K, Kumar S. An improved protocol for the isolation of RNA from roots of tea. *Molecular Biotechnology*,2012;52(1):82-88.
- Deka D, Zaman S, Namdev R, Phukon M, Borthakur A, Kalita R *et al.* Efficient protocol for isolation of high-quality RNA from tea without using liquid nitrogen for molecular analysis. *Indian Journal of Biotechnology*,2016;15:81-84.
- Zaman A, Gogoi M, Borchetia S, Kalita MC, Yadav RNS, Bandyopadhyay T. Comparative assessment of different protocols for isolation of total RNA from various organs of the tea plant (*Camellia sinensis*). *Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences*,2016;2(3):95-107.
- Birtic S, Kranner I. Isolation of high-quality RNA from polyphenol-, polysaccharide- and lipid-rich seeds. *Phytochemical Analysis*, 2006: 17:144-148
- Shirzadegan M, Christie P, Seemann JR. An efficient method for isolation of RNA from tissue cultured plant cells. *Nucleic Acids Research*,1991;19(21):6055.
- Sah SK, Kaur G, Kaur A. Rapid and reliable method of high-quality RNA extraction from diverse plants. *American Journal of Plant Sciences*,2014;5(21):3129-3139.
- Khosravi H, Deljou A, Monfared SR. High quality RNA isolation from leaf, shell, root tissues and callus of Hazelnut (*Corylus avellana* L.). *Journal of Nuts*,2017;8(2):89-95.
- Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*,1962;15:473-497.
- Wanqian L, Bochu W, Chuanren D, Biao L. A method for isolating functional RNA from callus of *Dendrobium candidum* contented rich polysaccharides. *Colloids and Surfaces B: Biointerfaces*,2005;42:259-262.
- Li B, Wang B, Tang K, Liang Y, Wang J, Wei J. A simple and convenient approach for isolating RNA from highly viscous plant tissue rich in polysaccharides. *Colloids and Surfaces B: Biointerfaces*,2006;49:101-105.
- Gupta S, Verma AK, Chand L. Comparative study of different protocols for isolation of RNA from polysaccharides and polyphenolics rich plant tissue. *International Journal of Plant Physiology and Biochemistry*,2011;4(2):23-26.
- Erturk Y, Ercisli S, Sengul M, Eser Z, Haznedar A, Turan M. Seasonal variation of total phenolic, antioxidant activity and minerals in fresh tea shoots (*Camellia sinensis* var. *sinensis*). *Pakistan Journal of Pharmaceutical Sciences*,2010;23(1):69-74.
- Massounga Bora AF, Ma S, Li X, Liu L. Application of microencapsulation for the safe delivery of green tea polyphenols in food systems: Review and recent advances. *Food Research International*,2018;105:241-249.
- Bandyopadhyay T, Bharalee R, Gohain B, Gupta S, Agarwala N, Singh HR *et al.* Isolation of functional RNA from heavily infested, wilted and necrotic leaf tissues of tea with high polyphenol content. *Journal of Agricultural Science and Technology B*,2012;2:121-127.
- Cankar K, Stebih D, Dreo T, Zel J, Gruden K. Critical Points of DNA Quantification by Real-Time PCR- Effects of DNA Extraction Method and Sample Matrix on Quantification of Genetically Modified Organisms. *BMC Biotechnology*,2006;6:37.