Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI - 10.26479/2016.0203.09**

COMPARATIVE ASSESSMENT OF DIFFERENT PROTOCOLS FOR ISOLATION OF TOTAL RNA FROM VARIOUS ORGANS OF THE TEA PLANT (*CAMELLIA SINENSIS)*

Afruza Zaman[*1, 2], Madhurjya Gogoi[1, 3], Sangeeta Borchetia[1], Mohan Chandra Kalita[2], R N S Yadav[4] and Tanoy Bandyopadhyay[1]

[1] Department of Biotechnology, Tocklai Tea Research Institute, Tea Research Association, Jorhat - 785008

[2] Department of Biotechnology, Gauhati University, Guwahati - 781014

[3] Centre for Biotechnology and Bioinformatics, Dibrugarh University, Dibrugarh – 786004

[4] Department of Life Sciences, Dibrugarh University, Dibrugarh - 786004

ABSTRACT: Quality RNA isolation in good quantity from plants, such as tea plant, rich in polyphenols, polysaccharides and other interfering compounds, is difficult. The purity and quality of the extracted RNA and the time required for isolation are very important in molecular biological works. In this study, four methods namely, modified Lithium Chloride – Sodium Dodecyl Sulphate (LiCl- SDS) method, Guanidine Hydrochloric Acid (Guanidine - HCl) method and the two commercial kits**:** TRI reagent (Sigma-Aldrich) and HiPer™ Plant RNA kit (Himedia Laboratories) were used in isolating RNA from different organs (leaf, root, flower and seed) of the tea plant (*Camellia sinensis*). Results showed that the quality and quantity of the RNA isolated from different organs of the tea plant using modified LiCl- SDS method was superior to the other methods. The time required for isolation was also very short (~1.5 h). The extracted RNA was found to be suitable for downstream molecular biological works such as reverse transcription, PCR amplification and quantitative real time PCR analysis.

KEYWORDS: Polyphenols, RNA isolation, Roots, Seeds, Tea

***Corresponding Author: Dr. Afruza Zaman** Ph. D.

Department of Biotechnology, Tocklai Tea Research Institute, Tea Research Association, Jorhat - 785008 Department of Biotechnology, Gauhati University, Guwahati – 781014.

*Email Address: zamanafruza@gmail.com

© 2016 Life Science Informatics Publication All rights reserved

1.INTRODUCTION

Isolation of high quality ribonucleic acid (RNA) from plant tissues is one of the pre-requisites for many downstream molecular biological processes like cDNA library construction, real-time PCR analysis, northern blotting, microarray analysis, etc. However, extraction of high quality intact RNA is one of the major hindrances for functional genomics analysis, particularly in case of tissues which are rich in polyphenols, polysaccharides, lipids etc. [1-4]. It was seen that the RNA yield in those tissues is either low or very poor in quality as the interfering compounds mentioned above binds with the nucleic acids and thereby hamper the specific and adequate precipitation of RNA [5-7]. Moreover, it is very difficult to get the desired amount of RNA concentration where the initial amount of tissue or availability of plant materials are very less like callus and plantlets maintained in *in-vitro* cultures [8].Tea plant [*Camellia sinensis* (L.) O. Kuntze] belonging to the family *Theaceae* is very rich in polyphenols and other secondary metabolites. The extraction of RNA from tea is not as easy as in other plants because of presence of high concentration of polyphenols [6, 9]. The most studied organs of the tea plant are the bud with first and second leaves. A number of protocols for extracting quality RNA especially from leaf of *Camellia sinensis* have been described in earlier works [9, 10]. Compared to leaf, the other organs such as roots and seeds are generally less studied at molecular level. Although certain protocols exist for isolating RNA specifically from roots and seeds [6, 11-13] but are not so effective particularly in case of tea plant. The use of the commercially available TRIZOL reagent was found to be effective in isolating good amount of RNA from young seedlings as well as dry seeds of *Arabidopsis* [14]. However, Bilgin *et al*. [15] had reported the presence of organic contaminants in the RNA extracted using TRIZOL reagent. Conventional methods like use of guanidine-HCl, CTAB and sarcosyl [5, 10, 11, and 16] are used on a regular basis in many research studies but they are not effective in isolating high quality RNA from recalcitrant tissues. In the present work, a convenient and time-efficient protocol was developed to get high amount of good quality RNA from different organs of tea plant, particularly the seeds, which are rich in lipid, polysaccharide and polyphenol content.

2. MATERIAL AND METHODS

Plant material:

Dry seeds, roots, flowers and leaves were collected from the TV1 clone of the tea plant maintained in the germplasm collection centre of Tocklai Tea Research Institute, Assam (Figure 1).The collected samples were immediately immersed in liquid nitrogen and kept in -80^{0}C refrigerator to prevent any kind of enzymatic activity. The seed kernels were first removed to expose the cotyledons. Roots were cleaned with alcohol to remove dirt or soil which may interfere in the whole process.

Reagents and plasticwares:

Modified LiCl–SDS method - RNA extraction buffer [(100 mM Tris-hydrochloride (pH 8.0), 10 mM Ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 200 mM LiCl, 1% SDS and 200 mM β-

© 2016 Life Science Informatics Publication All rights reserved

mercaptoethanol (added just before use)] and resuspension buffer [2 M LiCl, 3 M sodium acetate (pH 5.2)] were prepared in 0.1% (v/v) Diethylpyrocarbonate (DEPC) treated water. In addition, polyvinylpolypyrrolidone (PVPP 1%), phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0), isopropanol and 75% ethanol were also used during the extraction procedure.

Guanidine- HCl method - Reagents used for the guanidine–HCl method were prepared as described by Bandyopadhyay *et al*. [10].

Tri reagent (Sigma-Aldrich) and HiPer^{TM} Plant RNA isolation kit (Himedia Laboratories) were used as per manufacturer's instructions.

All plasticwares and mortar- pestles were incubated overnight with 0.1% DEPC treated water and then autoclaved before use.

RNA isolation procedure:

RNA was isolated using guanidine- HCl method, TRI reagent (Sigma-Aldrich) and HiPer^{TM} Plant RNA isolation kit (Himedia Laboratories). RNA was also extracted according to the LiCl – SDS method of Das *et al.* [6] with certain modifications. This method was optimized for extracting higher amount of RNA particularly from seeds and roots. The detailed procedure of the modified LiCl-SDS method is described below:

(All centrifugations were performed at 14000 rpm at 4^0C)

1. 1 ml of extraction buffer was taken in a 2 ml sterile microcentrifuge tube to which 10 µl β-mercaptoethanol and 10 mg of PVPP were added and the solution was vortexed for homogenous mixing. 100 mg of tissue (each of seed, root, flower and leaf) was weighed and ground to a fine powder in liquid nitrogen using mortar and pestle. The powder transferred to the tube containing the homogenized extraction buffer was mixed together by inverting the tube 8-10 times. The mixture was then incubated at room temperature for 5-10 min.
2. 500 µl of cold PCI was then added to the solution containing the tissue. The tube was again inverted several times for thorough mixing and then centrifuged for 15 min.
3. The mixture after centrifugation separated into 3 phases: an upper aqueous phase, an interface and a lower organic phase. The upper phase containing RNA was transferred to a new 1.5 ml tube to which equal volume of PCI was added for further decontaminating the RNA. The tube was inverted 5-6 times and centrifuged for 10 min.
4. The upper aqueous phase was transferred to a new tube. 0.1 volume of 3M sodium acetate and 1 volume of isopropanol were added and the tube was incubated at -20^0C for 15 min.
5. The tube was again centrifuged for 10 min, the supernatant was discarded and the pellet obtained was washed with 250 µl of 75% ethanol.
6. After discarding the supernatant the pellet was dissolved in 200 µl of resuspension buffer and followed with centrifugation for 10 min.

© 2016 Life Science Informatics Publication All rights reserved

7. The pellet obtained from the above step was again washed with 250 µl of 75% ethanol.
8. The RNA pellet finally obtained was partially air dried and finally dissolved in 50 µl of Nuclease- free water

The modifications done to the method of Das *et al* (2012) is tabulated below:

Original method of Das *et al*	**Modified LiCl-SDS method**
Extraction buffer contains 100mM LiCl	Extraction buffer conatins 200mM LiCl
800 µl extraction buffer used per 100 mg tissue	1ml extraction buffer used per 100mg tissue
No PVPP added	10 mg PVPP added per 1 ml extraction buffer
400 µl PCI added	500 µl PCI added
After addition of isopropanol and sodium acetate, tube incubated for 10 min at room temperature	After addition of isopropanol and sodium acetate, tube was incubated at -20^{0}C for 15 min
No resuspension buffer added	200 µl of resuspension buffer added followed by a washing step with 75% ethanol
30 µl nuclease free water added to the RNA pellet	50 µl nuclease free water added to the RNA pellet

RNA analysis and estimation:

The RNA extracted from all the tissues separately by employing the above mentioned four methods, were electrophoresed in a 1% agarose gel stained with ethidium bromide to check the integrity of the isolated RNA. Quantity and purity of the RNA was estimated in a Biophotometer (Eppendorf, Hamburg, Germany). Purity was measured in terms of protein and carbohydrate contamination by taking absorbance ratios A260/280 and A260/230 respectively.

cDNA synthesis and PCR amplification:

The extracted RNA was first treated with DNaseI enzyme to remove any contaminating DNA which might interfere in downstream applications. The DNA free RNA was then reverse transcribed using the SMARTScribe reverse transcriptase (Clontech) to generate single stranded cDNA. This cDNA was further used for full- length amplification of Dihydroflavonol 4-reductase (DFR) gene using gene-

specific primers (Forward primer - 5'ATACATATGAGGAGGTACAGCTATGAAAGACTCTGTT 3' and Reverse primer – 5' GTCGTTTTACAGGATCCTTAAACCTTGTTGCCATT 3') to determine the quality of the cDNA synthesized from the RNA.

Quantitative Real – Time PCR (qRT-PCR) analysis:

The cDNA, reverse - transcribed from the RNA obtained by the modified LiCl – SDS method, was further used for qRT – PCR analysis to determine the level and quality of amplification. A fragment

© 2016 Life Science Informatics Publication All rights reserved

of DFR gene was amplified using gene-specific primers (Forward primer – 5' TGCAGAGAGAAGGGTTTGCT 3' and Reverse primer – 5' AAGGCAAGGCACCAATACAC 3').

3. RESULTS AND DISCUSSION

Prior to using the present modified protocol, several protocols like the guanidine- HCl method [10] together with commercially available HiPer™ Plant RNA isolation kit (Himedia Laboratories) and TRI Reagent (Sigma- Aldrich) were tested for comparing their efficiency in isolating RNA from various organs of the tea plant. In case of the guanidine – HCl method, 16.5 µg and 18.48 µg of RNA was isolated from 100 mg of leaves and flowers respectively. The RNA isolated using TRI reagent showed a yield of 35 µg and 30.3 µg and the HiPer™ Plant RNA isolation kit yielded 20.9 µg and 7.18 µg of RNA from leaves and flowers respectively (Table 1).

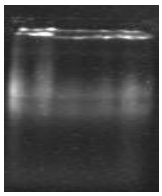
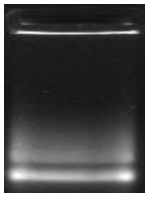
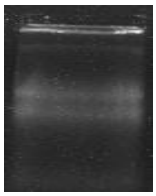
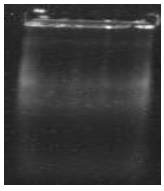
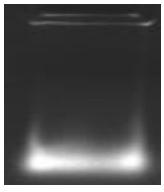
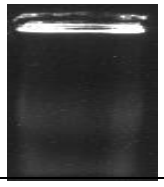
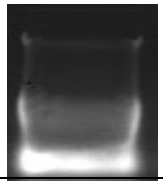
RNA extraction procedure	Samples	RNA yield (µg/ 100mg tissue)	A 260/ 280	A 260/ 230
Guanidine - HCl method	Leaves	16.5	1.63	1.44
	Flowers	18.48	1.75	1.85
	Seeds	Nil	-	-
	Roots	Nil	-	-
HiPer™ Plant RNA isolation kit (Himedia)	Leaves	20.9	1.8	1.78
	Flowers	7.18	1.9	1.92
	Seeds	Nil	-	-
	Roots	Nil	-	-
TRI reagent ® RNA isolation reagent (Sigma)	Leaves	35	1.77	1.5
	Flowers	30.3	1.45	1.43
	Seeds	Nil	-	-
	Roots	Nil	-	-
	Leaves	60.6	1.75	2.03

© 2016 Life Science Informatics Publication All rights reserved

Modified LiCl – SDS method	Flowers	41	1.69	1.57
	Seeds	42.46	1.74	2.38
	Roots	25	1.64	1.85

Table 1 RNA yield and corresponding purity measured by the A 260/ 280 and A 260/ 230 ratios in various organs of tea using different protocols.

The RNA obtained using the above mentioned protocols showed a level of purity ranging from 1.4-1.9 (A260/280 and A260/230). The integrity and quality of the RNA were checked on a 1% agarose gel run at 60 Volts. Visualization of the gel under UV transilluminator showed intact 28S and 18S RNA fragments for leaves and flowers. However, these bands were not visible in case of seeds and roots (Figure 1).

Different parts of the TV1 plant	**Total RNA**		
	Guanidine - HCl method (A)	**HiPer Plant RNA isolation kit (B)**	**TRI reagent (C)**
			
			
			
			

© 2016 Life Science Informatics Publication All rights reserved

Figure 1: RNA isolation from leaves, flowers, seeds and roots of the TV1 clone of the tea plant using (A) Guanidine- HCl method (B) HiPer Plant RNA isolation kit and (C) TRI reagent

The yield of RNA isolated from seed and root samples using the modified LiCl – SDS method was found to be 42.46 µg and 25 µg respectively which was not possible with the other tested methods. RNA yield from leaves and flowers were also found to be comparatively higher (60.6 µg and 41 µg respectively) than the yield from other protocols (Table 1). The quality of RNA checked on a 1% agarose gel showed that the RNA was intact and without any smear (Figure 2). DNase I treatment effectively removed contaminating genomic DNA present in the RNA sample as evident from the 1% agarose gel (Figure 2). The absorbance ratios of A260/ 280 and A260/ 230 were in the range of 1.4-1.9 and 1.4-2.3 respectively indicating good quality and pure RNA. The whole process was completed within a short duration of 1.5 h.

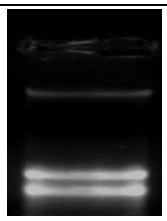
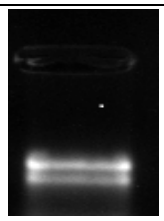
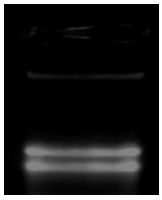
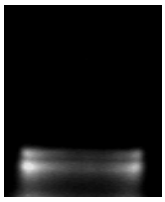
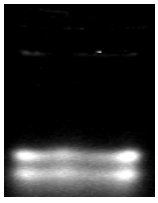
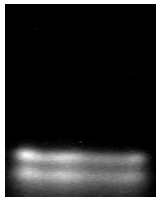
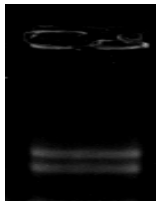
Different parts of the TV1 clone of the teaplant (A)	Total RNA	
	Before DNaseI treatment (B)	After DNaseI treatment (C)
Leaves		
Flower		
Seed		
Root		

Figure 2: RNA isolated from different organs of the TV1 clone of the tea plant using the modified LiCl- SDS method

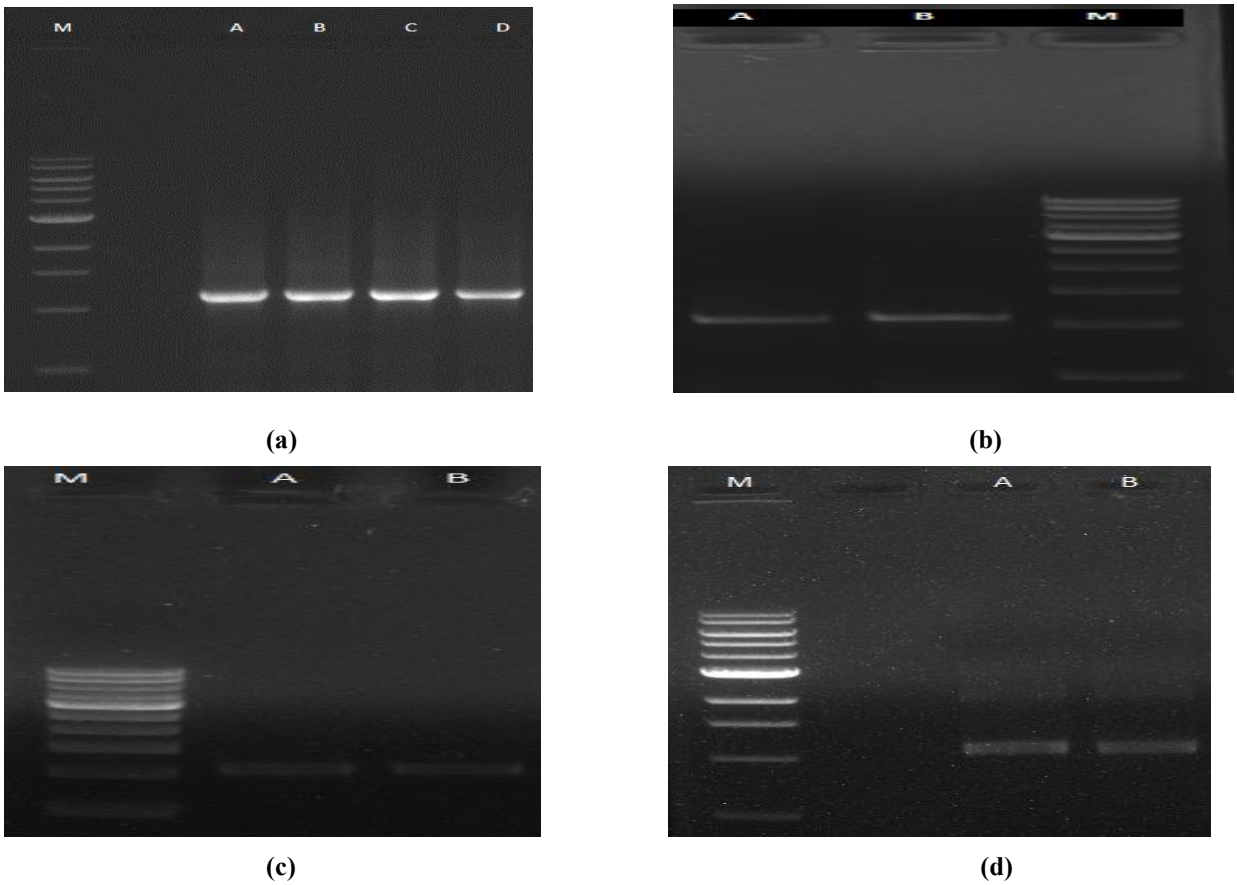
© 2016 Life Science Informatics Publication All rights reserved

(A) Various parts of the TV1 clone used for isolation of RNA (Leaves, flower, seed, root)

(B) Isolation of intact total RNA from the various organs

(C) RNA as appeared after DNase I treatment

(D) cDNA was prepared from the RNA, separately isolated using all the four methods and used as template for amplification of DFR gene fragment which showed desired amplicon band (Figure 3). As RNA isolation was not possible from the seed and root samples using guanidine-HCl method, TRI reagent and HiPer plant RNA isolation kit (Figure 1), so cDNA could not be prepared and PCR was not performed.



(a) **(b)**

(c) **(d)**

Figure 3: PCR Amplification of the DFR gene. **Lane M** represents 500 bp ladder. (a) Amplification of leaf, flower, seed and root respectively (**Lane: A- D**) from the cDNA derived from the modified LiCl – SDS method of RNA isolation. (b), (c), (d) Amplified cDNA of leaf and flower respectively (**Lane: A and B**) derived from the RNA isolated using TRI reagent, Guanidine – HCl and HiPer Plant RNA isolation kit in respective order. The cDNA analysed through qRT-PCR, showed a single melting peak for all the samples signifying single PCR product. Figure 4 shows the melting peak of DFR gene amplicon using gene specific primers.

© 2016 Life Science Informatics Publication All rights reserved

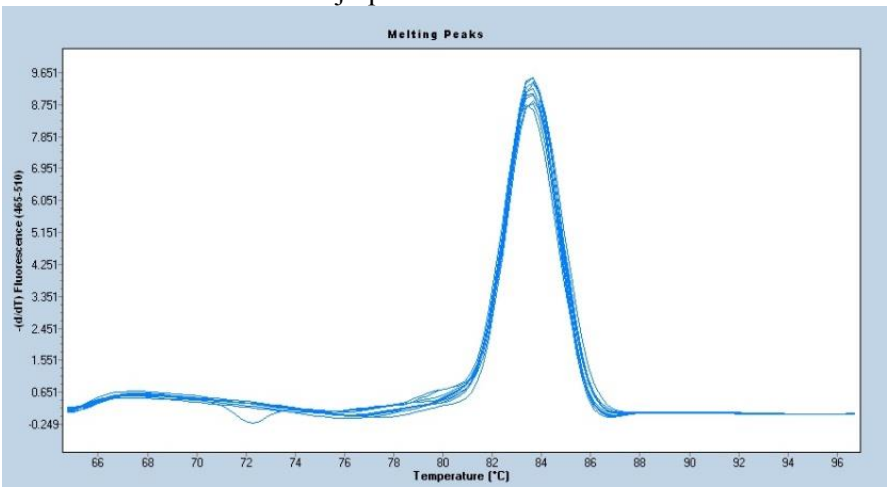


Figure 4: Melting peak of DFR gene amplicon obtained in qRT – PCR using gene specific primers. The template cDNA was derived from the RNA synthesized using the modified LiCl- SDS method.

In this study, the LiCl – SDS method of RNA isolation [6] was further standardized to extract a higher amount of pure RNA from the tea plant irrespective of plant parts used. Tea seeds, in particular, are a repertoire of storage compounds like polysaccharides, lipids and proteins which serve as a major hindrance in quality RNA isolation. Although RNA isolation from seeds of other plants has been reported [2, 11, 12], tea seeds have not been much subjected to RNA isolation studies. Tea plant, as a whole, contains an exceptionally high amount of polyphenols which get readily oxidized to quinones during the extraction process. The quinones covalently bind to nucleic acids hampering the adequate isolation of RNA [17]. Addition of polyvinylpyrrolidone (PVP) results in the formation of a complex with the polyphenols which gets separated from the nucleic acids. But PVP, in the presence of phenol, can irreversibly bind with the poly- A tail of mRNA and cause precipitation losses [5]. On the contrary, use of PVPP as a substitute for PVP in RNA isolation was reported in certain plants to arrest the oxidation of released polyphenols into quinones [17, 18]. In this study, use of insoluble PVPP in the extraction buffer resulted in more efficient removal of polyphenols in the initial centrifugation step as compared to soluble PVP [5, 19]. Inclusion of a two- step LiCl treatment of different concentrations in this method resulted in selective precipitation of total RNA. Earlier reports also showed that LiCl can specifically precipitate RNA from DNA, proteins, carbohydrates or other contaminants [20]. Increase in concentration of LiCl from 100 mM [6] to 200 mM in the extraction buffer [9] together with the incorporation of a resuspension step consisting of 2 M LiCl and 3 M sodium acetate [10] resulted in effective precipitation of total RNA from the contaminating polysaccharides and proteins. In addition, the time and temperature of incubation were optimized to get the desired yield of total RNA as evident from values in Table 1. Real- time PCR experiments require precision at every step where the cDNA, used as a template, needs to be synthesized from high quality mRNA. However, cDNA is normally prepared from total RNA where the abundance of mRNA is quite low and there may not be any templates for reverse transcription upon purification of mRNA. Thus, the protocol for isolating

© 2016 Life Science Informatics Publication All rights reserved

total RNA should be effective to get high quality and high quantity of mRNA. The integrity of the total RNA isolated in this study was checked by the presence of intact 28S and 18S bands of ribosomal RNA. The single stranded cDNA synthesized from the RNA in all the tissues were successfully amplified using the DFR gene which resulted in desired bands. Generation of good amplification curves and single melting peak in Real- Time PCR analysis using this cDNA as a template was a validation of the quality of the cDNA as well as the RNA from which it was prepared.

4.CONCLUSION

The present study attempted to isolate high amount of pure RNA from various organs of the tea plant particularly from the recalcitrant tissues such as seeds and roots and the modified LiCl – SDS method was found to be successful as compared to the other methods used in this study. Extraction of RNA from tea seeds, which was not reported earlier, was also conveniently carried out using this modified method. The short time involved in the extraction process together with the use of easily available chemicals makes this method suitable for routine RNA isolation which can be used for many downstream molecular works such as Real Time PCR analysis, cDNA library construction, etc.

CONFLICT OF INTEREST

The authors declare that no competing financial interests exist.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Tocklai Tea Research Institute for his kind support and encouragement. The authors are thankful to Mr. R. K. Bordoloi, Plant Physiology and Breeding Department, TTRI for his help in collection of plant samples. The authors acknowledge the financial support of Department of Biotechnology (DBT), Govt. of India through their project “Establishment of Institutional Biotech Hubs by DBT under special programme for the North Eastern States of India”.

REFERENCES

1. Kansal R, Kuhar K, Verma I, Gupta RN, Gupta VK, Koundal KR. Improved and convenient method of RNA isolation from polyphenols and polysaccharide rich plant tissues. Indian J Exp Biol. 2008; 46(12): 842- 845.
2. Suzuki Y, Kawazu T, Koyama H. RNA isolation from siliques, dry seeds and other tissues of *Arabidopsis thaliana*. Biotechniques. 2004; 37(4): 542- 544.
3. Prasad P, Kumar J. An RNA isolation protocol for recovery of high quality functional RNA from fungi and plants. Curr Sci. 2012; 102(9): 1257- 1260.
4. Sharma AD, Gill PK, Singh P. RNA isolation from plant tissues rich in polysaccharides. Anal Biochem. 2003; 314(2): 319- 321.

© 2016 Life Science Informatics Publication All rights reserved
Peer review under responsibility of Life Science Informatics Publications

5. Morante- Carriel J, Selles- Marchart S, Martinez- Marquez A, Martinez- Esteso MJ, Luque I, Bru- Martinez R. RNA isolation from loquat and other recalcitrant woody plants with high quality and yield. Anal Biochem. 2014; 452: 46- 53.
6. Das A, Saha D, Mondal TK. An optimized method for extraction of RNA from tea roots for functional genomics analysis. Indian J Biotechnol. 2013; 12(1): 129- 132.
7. Ghawana S, Paul A, Kumar H, Kumar A, Singh H, Bhardwaj PK, Rani A, Singh RS, Raizada J, Singh K, Kumar S. An RNA isolation system for plant tissues rich in secondary metabolites. BMC Res Notes. 2011; 4: 85.
8. Wanqian L, Bochu W, Chuanren D, Biao L. A method for isolating functional RNA from callus of *Dendrobium candidum* contented rich polysaccharides. Colloids Surf B Biointerfaces. 2005; 42(3-4): 259- 262.
9. Lal L, Sahoo R, Gupta RK, Sharma P, Kumar S. RNA isolation from high- phenolic tea leaves and apical buds. Plant Mol Biol Report. 2001; 19(2): 181.
10. Bandyopadhyay T, Bharalee R, Gohain B, Gupta S, Agarwala N, Singh HR, Chakrabarty S, Bhorali P, Kalita MC, Das S. Isolation of functional RNA from heavily infested, wilted and necrotic leaf tissues of tea with high polyphenol content. J Agric Sci Technol. 2012; B2: 121-127.
11. Singh G, Kumar S, Singh P. A quick method to isolate RNA from wheat and other carbohydrate rich seeds. Plant Mol Biol Report. 2003; 21(1): 93a- 93f.
12. Mortaji Z, Afshari RT, Alizadeh H, Samadi BY. RNA isolation and expression from different dormant and after- ripened wheat seed tissues rich in polysaccharides and proteins. Asian J Plant Sci. 2008; 7(2): 201- 206.
13. Muoki RC, Paul A, Kumari A, Singh K, Kumar S. An improved protocol for the isolation of RNA from roots of tea. Mol Biotechnol. 2012; 52(1): 82- 88.
14. Meng L, Feldman L. A rapid TRIzol- based two- step method for DNA- free RNA extraction from *Arabidopsis* siliques and dry seeds. Biotechnol J. 2010; 5(2): 183- 186.
15. Bilgin DD, DeLucia EH, Clough SJ. A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real- time PCR. Nat Protoc. 2009; 4(3): 333-340.
16. Jamalnasir H, Wagiran A, Shaharuddin A, Samad AA. Isolation of high quality RNA from plants rich in flavonoids, *Melastoma decmfidum* Roxb ex. Jack. Aust J Crop Sci. 2013; 7(7): 911- 916.

© 2016 Life Science Informatics Publication All rights reserved

17. Jaiprakash MR, Pillai B, Venkatesh P, Subramanian N, Sinkar VP, Sadhale PP. RNA isolation from high- phenolic, freeze- dried tea (*Camellia sinensis*) leaves. Plant Mol Bio Report. 2003; 21(4): 465-466.
18. Wang X, Xiao H, Chen G, Zhao X. Isolation of high- quality RNA from *Reaumuria soongorica*, a desert plant rich in secondary metabolites. Mol Biotechnol. 2011; 48(2): 165-172.
19. Dash PK. High quality RNA isolation from polyphenol-polysaccharide- and protein- rich tissues of lentil (*Lens culinaris*). 3 Biotech. 2013; 3(2): 109- 114.
20. Barlow JJ, Mathias AP, Williamson R, Gammack DB. A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. Biochem Biophys Res Commun. 1963; 13(1): 61- 66.

© 2016 Life Science Informatics Publication All rights reserved