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Simple and Efficient Protocol for RNA and DNA extraction from Rice *(Oryza sativa L.)* for Downstream Applications

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Abstract

Plant DNA and RNA extraction is difficult due to the presence of metabolites that interfere with isolation procedures and downstream applications. In the present study DNA and RNA extraction protocol standardized from rice (Oryza sativa L.). Isolation of DNA using Triton-X-100 based extraction method with PVPP treatment efficiently removes metabolites and yield with high quality DNA from rice coleoptiles.RNA isolation *methods based on TRIzol was suitable for rice leaf whereas in seed, Tris-HCl (pH 9.0) with β-mercaptoethanol protocol gave best results. The isolated DNA and RNA proved to be suitable for PCR and RT-PCR amplification, respectively. The technique is reproducible and can be applied for PCR based markers identification and gene expression studies.*

Keywords: *Oryza sativa L, DNA and RNA,* Triton-X-100 and TRIzol.

Introduction

In last few years, plant molecular biology has expanded enormously. Molecular markers are now widely used to track loci and genome regions in several crop-breeding programmes. PCR-based markers analysis and gene expression studies are increasing exponentially. In these studies high-quality and intact DNA and RNA is the keystone for performing PCR and RT-PCR based research. However, individual species and organs and/or tissues of plants can behave differently during extraction of DNA and RNA due to difference in metabolic activities.

Rice *(Oryza sativa L.)* is a rich source of polysaccharides. These polysaccharides co-precipitate during RNA and DNA extraction and also known to inhibit polymerase activity[1]. A good extraction protocol should be simple and high yielding. Many protocols have been tried for DNA isolation in rice material like, the CTAB (Cetyl Trimethyl Ammonium Bromide) based method, urea-phenol method, SDS-Potassium acetate method, SDS mini-prep method and commercial kits based protocols, modified CTAB method[1-4]. Similarly, for RNA isolation like CTAB method, SDS (Sodium dodecyl sulphate) and GT (Guanadine thiocyanite) methods were used by the researchers to isolate total RNA free from polysaccharide and polyphenolics rich plants like rice, mango, banana, maize, pecan, pear leaves, grapes and potato tubers[5-8] .

The prime objective of this study was to isolate genomic DNA from rice leaves with good quality and to set an isolation method suitable for pure DNA which can be stored for a long period and lasts for several PCR reactions for downstream applications. DNA isolation was performed using protocol established in our lab with some modifications. This protocol is giving best results in many plants like, cotton, barley, brinjal, tomato, rose, banana, and a range of medicinal plant species, with minor changes.

DNA isolation protocol followed by disruption of the cell membrane to release the DNA in the extraction buffer by using detergents such as SDS with Triton-X-100[9] have proven better in many plant systems. This method combines enrichment and maintenance in an osmotically supplemented buffer with PVPP to complex polyphenolic compounds followed by selective precipitation of nucleic acids. The broken cell was treated with EDTA as it has affinity towards divalent ions like Ca_2^+, Mn_2^+, and $Mg2^+$. EDTA is mostly used in extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases. Plant crude extracts contains high amount of RNA, protein, pigments and polysaccharides which is main crucial component to separate DNA[10]. RNase is used for removing RNA and most of the proteins are removed in phenol and chloroform treatment. Polysaccharide-like contaminants are, however, more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes[11]. NaCl at concentrations of more than 0.5 M, together with detergent is known to remove polysaccharides[12,13.] Polymerase chain reaction has found wide applications in genomic studies. For reproducible PCR results, conversely, the quantity and quality of DNA play an important role.

The reliable quality of RNA molecule is a basic requirement for subsequent gene expression experiments. TRI Reagent (Trizol T924) is a ready-to-use reagent for the isolation of total RNA from cells and tissues of plants. Trizol is a reagent, made up of mono-phasic solution of phenol and guanidine isothiocyanate developed by Chomczynski and Sacchi in 1987. The efficacy of

the methods depends on types of the chemical properties of the material used. Here, two RNA extraction protocols were used: (i) based on Trizol reagent to isolate RNA from leaf and (ii) protocol according to Guifeng Wang (2011) with some changes was used for efficient extraction procedure to isolate RNA.

The yield and quality of the obtained DNA as well as RNA obtained were consistently good, as confirmed by spectrophotometric analysis and separation on agarose gel. Both, DNA and RNA extraction protocols were found to be reliable for downstream applications.

Material and Methods

DNA isolation: Plant Materials: Total 19 varieties of Rice (*Oryza sativa)* were collected from local framers of Vapi (20.3667° N, 72.9000° E) and Valsad (20.63°N 72.93°E) regions or commercially available (table-1) were used for DNA isolation in this experiment. Rice seeds were surface sterilized with 0.1% $HgCl_2$ and rinsed with distilled water. Sterilized seeds were soaked in water in a Petri-dish lined with 5 layers of filter papers and incubated at 27°C for 8 to 10 days. After 15days, the coleoptiles and leaves were crushed in liquid nitrogen and stored at -20°C, till used.

Table-1
Selected varieties for DNA isolation and their purity and concentration

NO	Variety	Conc. ug DNA/gm	260/280
1	Desi-7	10.61	1.75
2	Local	17.73	1.84
3	ARIZE-Tej	14.30	1.77
4	Suruchi MRP 5629	15.31	1.77
5	Arize -6201	4.68	1.63
6	801H	70.51	1.6
7	807H	26.35	1.5
8	835H	20.12	1.4
9	837H	54.21	1.4
10	BIHARI	49.37	1.4
11	GR-3	4.47	1.2
12	GR-4	28.65	1.3
13	GR-11	11.72	1.1
14	KHADSHI	18.39	1.4
15	JAYA	121.27	1.5
16	PANKAJ	21.65	1.4
17	TAYCHHUN	68.12	1.4
18	8744H	43.72	1.4
19	US10	11.56	1.66

DNA isolation protocol: Approximately, 800 mg of developing rice coleoptiles and leaves were grinded in liquid nitrogen along with 400 mg PVPP, by adding 8 ml of freshly prepared and pre-heated Triton-X-100 extraction buffer (0.03 M Tris-Base (pH 9.1); 0.2 M NaCl.; 2 % (w/v) SDS; 0.5 % (w/v); Triton-X-100; 0.02 M DTT; 0.1 M EDTA/NaOH (pH 9.0); ddH_2O to final volume 100 mL. DTT was added freshly in extraction buffer before DNA isolation) and further incubated at 65 °C in water bath for 30 min. Then, 2.68 ml of the 5 M KAc (Potassium acetate pH 6.5 with glacial acetic acid) were added to each tube and incubated on ice for 30 min and centrifuged at 18500g at 4°C for 20min. To the supernatant, 0.5 volumes of IPA were added to precipitate DNA by mixing gently. It was kept at 37°C for one hour and at -20°C for 10min followed by centrifugation at 18500g at 4°C for 15min. DNA pellet was air dried and suspended in 1 ml of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). Protease treatment (1.0 µg/µL stock) for 30min at 37C was given and then centrifuged at 15000g at 4°C for 10min. RNA contamination was removed by 8 µL RNase treatment (2.0 µg/µL stock) for 2hr at 37°C followed by 30min at -20°C and centrifuged at 1500g at 4°C for 15min. Further, DNA purification was done using once P:C (Tris saturated Phenol (pH >7.8): Chloroform, 50:50, v/v), twice C:I (Chloroform: Isoamyl Alcohol, 24:1, v/v) treatment. To the extract 0.1 volumes of 3 M NaAc (Sodium acetate pH 5.2 with acetic acid glacial) and 2 volume of absolute alcohol was added to the supernatant to precipitate the DNA and incubated at -20°C for 30 min then centrifuged at 18500g at 4°C for 15min. The DNA pellet was washed with 1 ml of 70 % chilled ethanol, air dried and re-suspended in 200 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0).

RNA isolation: Plant Materials: For isolation of RNA, rice seedlings grown in lab and field were used as described below.

Lab experiment: Three varieties (Tej, local and Arize -6201) were used. The rice seeds were surface sterilized with 0.1% $HgCl_2$ and rinsed with distilled water. Sterilized seeds were soaked in water and grown aseptically in half strength MS media, 10-12 days old coleoptiles were used for RNA isolation.

Field experiment: Three varieties (PAC801, PAC807 and PAC835) were grown and maintained at the Vikarm farm, Vapi, Gujarat (20.3667° N, 72.9000° E). After self-pollination, immature seeds and their flag leaves were collected during seed development stage at regular intervals.

Protocol: RNA isolation protocol: The samples and labware were washed with 0.1% DEPC water. Approximately, 200mg sample was crushed into fine powder in liquid nitrogen with mortar and pestle, 1 ml Trizol (Sigma) in 2 ml eppendorf was taken and incubated for 10 min at 37^0C. The mixture was centrifuged at 12000g for 10 min at 4^oC. 200 µL chloroform was added in the supernatant and incubated it for 3-5 min at 37 ^{o}C. The sample was again centrifuged at 15000g for 10 min at 4^oC. Equal volume of P: C: I was added to the supernatant and mixed in MIXMATE (Eppendorf) for 2 min, followed by centrifugation at 10000g for 5min at 4^oC. The RNA was precipitated from the aqueous phase with equal amount of isopropyl alcohol. The samples were first incubated at 37^oC, later at -20°C for 10 minutes and centrifuged at 15000g for 10min at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The pellets were air dried for 10-15min and dissolved in 30 µL Ribo reserve solution (Amresco^{TM}) (RNA storage solution). Obtained RNA samples were stored at -20°C.

Protocol: II: RNA isolation protocol: The collected spikes and their flag leaf of each of the three varieties were subjected for RNA isolation at regular time intervals. The seeds and flag leaves were crushed into fine powder in liquid nitrogen. To 200mg sample, 0.5ml RNA extraction buffer (100mM Tris-HCl (pH 9.0), mixed with 1% β-mercaptoethanol (v/v) just before use and stored at room temperature) was added. The sample was properly mixed and incubated for 10 min at 37°C. Freshly prepared 20% SDS (sodium dodecyl sulphate) (w/v) was added to 20 µL suspension, mixed and incubated for 10 min at 37°C. Mixture was then centrifuged at 12000g for 15 min at 4°C. Resulted supernatant was carefully transferred in a new RNase free 2 mL tube. Another tube containing approx 0.4mL mixture was added with double the volume of TRIzol. It was mixed in MIX MATE for 30sec at 1000g and incubated for 10min. 240 µL chloroform was added for and mix for 1min at 1000g. It was centrifuged at 15000g for 10min at 4°C. The aqueous phase was transferred to a fresh tube and equal volume of chilled isopropanol was added, mixed and precipitated at -20°C for 30min. This was centrifuged at 12000 g for 10 min at 4°C. The pellet was gently re-suspended in 200µL DEPC-treated H_2O and equal volume of phenol: chloroform (1: 1) (phenol is saturated with 0.1 M citrate buffer, pH 4.3 ± 0.2) was added, mixed thoroughly and centrifuged at 12000 g for 10 min at 4°C. The aqueous phase was carefully transferred to a fresh tube. An equal volume of chloroform was added and mixed thoroughly with a vortex then centrifuged at 15000g for 10 min at 4°C.The aqueous phase was carefully transferred to a fresh tube, 1/10 volume of 3 M sodium acetate (pH 4.8, 40µL) and two volumes of ice-cooled ethanol (0.8mL) was added and mixed by inversion several times, and precipitated at −20°C for 45 min to 60 min. The sample was centrifuged at 13000 g for 20 min at 4°C. Supernatant was removed and 700µL of pre-cooled 70% ethanol was added and centrifuged at 15000g for 10 min at 4°C. The supernatant was discarded and the pellet was allowed to dry at room temperature for 15-20 min.30µL of Ribo reserve solution (Amresco™) was added and stored in -20°C.

cDNA synthesis: cDNA synthesis was done using Applied Bio-systems® High capacity cDNA transcription kit according to manufacturer's manual and product stored at -20 °C .

DNA and RNA quantification: The quality and quantity of the DNA/RNA extracts were assessed using the µQuant microplate reader, Bio-Tek instruments incorporation, USA. The concentration was determined at A260 nm Contamination due to phenol/carbohydrates and proteins was determined by ratios A260/A230 and A260/A280, respectively[16].

Downstream application: The quality of the extracted DNA and RNA was evaluated using two procedures: gel electrophoresis and PCR amplification. RNA samples were first denatured with formamide and mixed with gel loading dye at 65°C for 5 min and chilled for 10min in -20°C. The samples were loaded on a 1.2% agarose gel, stained with ethidium bromide and visualized with UV light. Consecutively to check the efficiency and reliability of the method, extracted DNA was amplified with (ISSR-3) UBC814, (CT)8A, 48 Ta (°C) and RNA converted to cDNA and amplified with ADP-*glucose pyrophosphorylase small sub unit* (*OsAGPS1*) F: GTGCCACTTAAAGGCACCATT R: CCCACATTTCAGACACGGTTT , 52.9 Ta (°C). PCR reaction mixture consists of total 12 µL and carried out in 3 stages with primer annealing at T_a. Electrophoresis of samples was carried out on agarose gel.

Results and Discussion

DNA isolation methodology: The DNA was isolated from 19 varieties. Isolation method has given comparative good result for long term storage of DNA. The result showed that the DNA isolated by this method gave clear and sharp bands on 1.2% agarose gel (figure-1). DNA purity A260/280 ratio and concentration is given in (table-1). The average DNA concentration was 32.2 µg/gm and purity range between1.4 to 1.84. While some varieties GR-3, GR-4 and GR-11 purity range between 1.1-1.3, which was comparatively low among all varieties but the average concentration was 14.94 µg/gm of GR-4 variety, which was higher among other three varieties. Few varieties like Local, Khadshi, PAC 835H, Pankaj, PAC 807H, GR4, PAC 8744H, Bihari, PAC 837H, Taychhun, PAC 801H and Jaya gave average 45 µg/gm of DNA concentrations. 800mg of rice tissue was required for this method. The quantity and the quality of the DNA extracted by this method were high enough to perform more than 150 PCR reactions. Thus, this protocol gave suitable result for downstream application and bulk of PCR reactions.

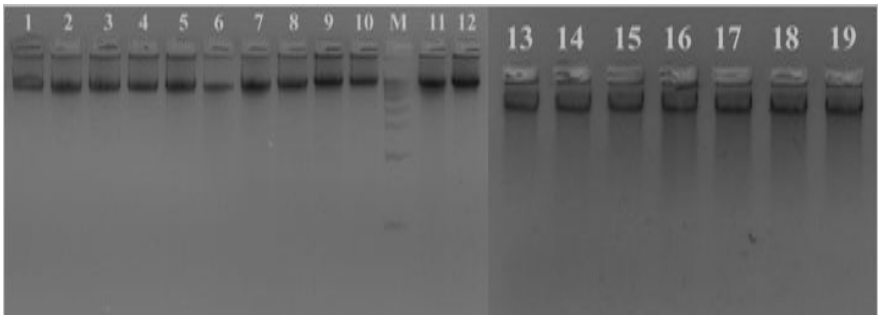

Figure-1
1.2% agarose gel electrophoresis of Genomic DNA isolated from 19 selected rice Varieties

The three main contaminants associated with plant DNA that can cause considerable difficulties while conducting PCR experiments: polyphenolic compounds, polysaccharides and RNA. Inclusion of sodium chloride (NaCl) with the lysis buffer has been used for removing polysaccharides[1]. In addition to cell wall material and sugar or starch content, plant cells contain an enormous (species specific) array of polysaccharides. Removal of these has led to many laboratories developing species specific protocols with some modification according to the requirements. Triton-X-100 and SDS lyses were utilized in this method which led proteins and polysaccharides to get trapped in large complexes coated with Sodium dodecyl sulfate. Addition of KAc participates in precipitation of these complexes by replacing sodium ions with potassium ions [10, 17]. PVPP was used to purge polyphenols and it promoted precipitation of the phenolic compounds[18] .The protocol proposed here first precipitates the DNA using isopropanol. RNase treatment is the generally accepted method to remove RNA; however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification [19]. The phenol/chloroform extractions were needed to remove major protein, phenolic, and cell debris contaminates[12]. Thus, residual proteins and other cellular contaminants were removed or separated from the DNA using phenol/chloroform. It was noticed that some contaminants that inhibit PCR could not be removed with phenol/ chloroform extraction 20 . Described protocol for DNA isolation method was found suitable for isolation of reasonably pure DNA in sufficient amount from rice leaves that can be stored for a longer duration and lasting for several PCR reactions.

However, the bottleneck still exists due to the efficiency and the stability to PCR amplification, here ISSR-3 primer was checked on rice varieties (figure-3.1). The DNA extracts were maintained for more than two years without deleterious effect at -20°C.The efficiency and reliability of the present procedure may be suitable for various plants. This DNA isolation method is efficient for different plant species, including wheat, cotton, barley, brinjal, tomato, rose, banana, and a range of medicinal plant species, in our laboratory.

RNA isolation methodology: RNA isolation from plant tissues, however, may be quite challenging. Some plant tissues, such as fruits, storage tubers, and seeds, contain a large amount of storage proteins, polysaccharides, and polyphenolics or other secondary metabolites that would co-precipitate with the RNA and constitute the major obstacle of RNA isolation (Ling-Wen Ding, 2008). The methods based on TRIzol and/or guanidinium isothiocyanate have been widely used for RNA extraction. These methods mostly used in various systems such as plants, animals and bacteria. However, cereal endosperm contains very high levels of starch, which causes the solidification of samples in such RNA extraction buffers. Some plant tissues with a high level of starch can also hinder the resuspension of precipitated RNA and contaminate the RNA due to co-precipitation, affecting its storage, quality and quantity of isolates.

In the present study, two methods were subjected for RNA isolation; the one followed more for various systems in laboratories was Trizol reagent based method and in other was extraction buffer was used with Trizol reagent based protocol. Trizol method gave good separate bands on 1.2% gel electrophoresis of coleoptiles sample (figure-2), but this protocol failed while isolating RNA from developing seeds at 14 DAF (Day after Flowering). The main problem of the TRI reagent method was that it made sticky and viscous extraction buffer which was not separated in to two different layers on applying high centrifugation for long period of time. The TRI reagent showed the solidification of crushed plant material at first attempted to isolate RNA from developing rice seeds. The solidification of seed samples in the TRI reagent buffer was a serious problem affecting the final quality of the extracted RNA. This type of solidification problem also reported by Guifeng Wand, *et al* (2011), higher-strength SDS (20%) with lysis buffer and β-mercaptoethanol resolved this problem.

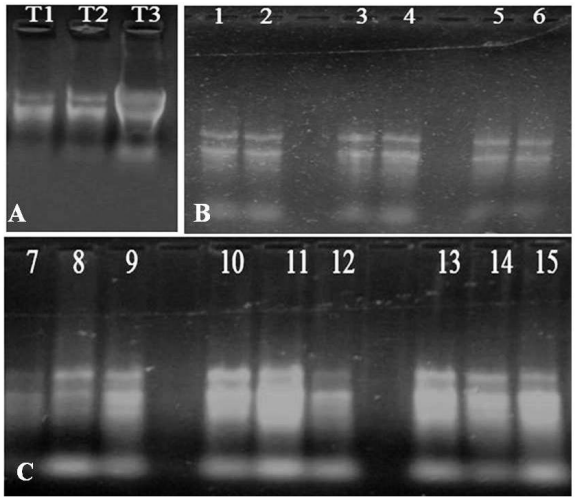

Figure-2

Total RNA from rice (A) RNA extracted with Trizol method from coleoptiles, (B) mature flag leaf of PAC-801(lane:1 at 1DAF and lane:2 at 5DAF), PAC-807(lane:3 at 1DAF and lane:4 at 5DAF) and PAC-835(lane:5 at 1DAF and lane:6 at 5DAF) and (C) total RNA from seed using Tris-HCl trizol protocol PAC-801(lane:7 at 1DAF, lane:8 at 5DAF and lane:9 at 9DAF), PAC-807(lane:10 at 1DAF, lane:11 at 5DAF and lane:12 at 9DAF) and PAC-835(lane:13 at 1DAF, lane:14 at 5DAF and lane:15 at 9DAF),on 1.2% agarose gel electrophoresis

In this protocol seed homogenate was suspended in an alkaline Tris buffer (pH 9.0) with a presence of β-mercaptoethanol prevented RNA degradation, probably by inhibiting the endogenous ribonuclease [21]. The precipitation period with equal volume of isopropanol in aqueous phase at -20 °C was increased from 20min to 30-35min. Phenol **:** Chloroform treatment to remove impurities was performed twice which resulted in intact RNA. The incubation period for next precipitation was increased from 2h to 6-8h at -20 °C that gave appropriate RNA

precipitates. Dissolving RNA pellet in 30 µL Ribo-reserved solutions served to store RNA for long time at least 6 months. The extraction of total RNA gel was shown in Figure: 2, with 1.2% agarose gel electrophoresis with formamide gave impacted denatured RNA. Average purity was more than 1.7 for all three varieties but few stages of DAF gave low purity (table-2).

Table-2
RNA isolation using Tris-HCL Trizol protocol , PAC-801, PAC-807 and PAC-835 varieties seed RNA Purity and Yield

Varieties	Stage	DAF	Purity260/280 ratio	RNA ug/gm
	1S	**1**	1.74	155.41
	2 S	**5**	1.84	278.01
	3 S	**9**	1.88	818.73
	4 S	**12**	1.37	22.13
PAC801	5 S	**16**	1.7	439.3
	6 S	**19**	1.76	131.05
	7 S	**23**	1.75	135.37
	8 S	**26**	1.53	47.17
	9 S	**30**	1.61	71.12
	1S	**1**	1.49	29.60
	2 S	**5**	1.24	8.05
	3 S	**9**	1.86	609.60
	4 S	**12**	1.67	309.17
PAC807	5 S	**16**	1.61	506.45
	6 S	**19**	1.69	654.37
	7 S	**23**	1.61	522.28
	8 S	**25**	1.69	500.07
	9S	**29**	1.92	398.98
	1S	**1**	1.29	9.39
	2 S	**5**	1.1	4.29
	3 S	**9**	1.54	33.39
	4 S	**12**	1.8	152.26
PAC835	5 S	**16**	1.75	228.99
	6 S	**19**	1.31	177.76
	7 S	**23**	1.82	223.1
	8 S	**26**	1.74	182.71
	9 S	**29**	1.77	145.31

Modified TRI reagent RNA extraction protocol was found to be efficient and effective for rice seeds containing high levels of starch. The method presented here is effective in isolation of RNA from seed and leaf tissues. Moreover, this method reduces sticky gel like formation. Commonly used reagents like alkaline Tris buffer, β-mercaptoethanol and high concentration of SDS, enhanced the separation and prevented clumping of seeds during extraction. Phenol (pH 4.3 ± 0.2) saturated with citrate buffer gave good quality of RNA.

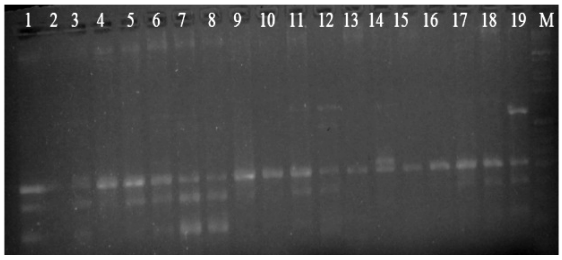

Figure-3.1
ISSR amplification profile for primer 3 on 2% gel electrophoresis of rice. Numbers represent the varieties code and M represent high range ruler)

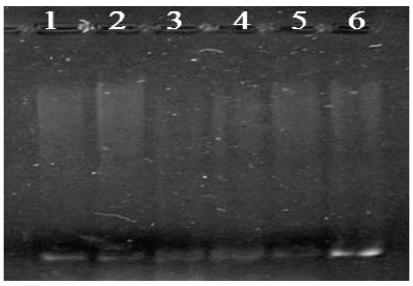

Figure-3.2
PCR amplification of ADP-*glucose pyrophosphorylase small sub unit* on 3% gel electrophoresis of synthesized cDNA, (Lane: 1 and 2 PAC801 (2S, 3S), Lane: 3 and 4 PAC835 (2S, 3S) and Lane: 5 and 6 PAC807 (2S, 3S))

Conclusion

High content of polysaccharides must be removed in DNA and RNA isolation of rice tissues. In DNA isolation Triton X-100 buffer gave good quality and purity of DNA with sharp band pattern on agarose gel electrophoresis. Isolated DNA can be stored for long time at -20^{o}C. 1 µL DNA of 50mg/ µL had been used for PCR amplification.

In DNA and RNA protocol precipitation steps were given twice for utmost pure isolation. RNA pellet was dissolved in Ribo resolved and stored in -20 ^{o}C. RNA converted in to cDNA and further used for (1 µL of RNA means 40 mg/ µL) stock PCR and RT-PCR based analysis. RNA method is suitable for varied rice materials like leaf, seed and may probably for other starchy plants.

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