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AN EFFICIENT REPRODUCIBLE METHOD FOR EXTRACTION OF HIGH QUALITY RNA FROM KODO MILLET (*PASPALUM SCROBICULATUM* L.) LEAVES

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ABSTRACT

Kodomillet (*Paspalum scrobiculatum* L.) is a rich source of novel genes for tolerance to different abiotic stresses as salinity, drought and high temperature. The molecular techniques demands RNA of high quality to isolate stress responsive genes. The leaves of Kodomillet contains high amounts of secondary metabolites along with polysaccharides, these organic components frequently interfere with RNA extraction and purification which in-turn results in poor RNA both qualitatively and quantitatively. Such RNA preparations hinder the downstream molecular biology studies such as cDNA library construction, qRT-PCR and Northern blotting. Since, there are no earlier reports on the isolation of RNA from kodomillet, here we report a reproducible method with slight modifications in the standard sucrose- sodium chloride extraction buffer and lithium chloride ($LiCl_2$) was used for selectively precipitation of RNA in the final step. The isolated RNA proved amenable to carry out the further downstream molecular studies.

KEY WORDS: **Kodomillet, RNA isolation, Lithium chloride and Salinity stress.**

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INTRODUCTION

Crop production and subsequent attainment of maximum yields are highly influenced by environmental factors in addition to management practices. Formers used to do regular agricultural practices (Mechanical irrigation, chemical fertilizers etc.) to overcome the abiotic stresses in agriculture plants and are not successful hundred percent moreover they are not farmer and eco-friendly. Earlier the regular breeding practices found to be more significant and proved to be successful in order to develop abiotic stress tolerant cultivars. But in recent times, the availability of germplasm is becoming a limiting factor for breeding programs. Therefore, it is an immediate and urgent need to search for new genetic sources of environmental stress tolerance. Kodo millet *(Paspalum scrobiculatum* L.*),* is cultivated as an annual crop. It has been cultivated for 3000 years in India, where it is considered as a minor cereal crop except in the Deccan where it is a cereal of utmost importance. The seeds are used as human food grains are ground into meal and used for puddings (Quattrocchi, 2006). In Africa, it is harvested as a wild cereal (FAO/ICRISAT, 1996) and is mainly considered as a famine food (NRC, 1996). Kodo millet is well suited to dry conditions (Galinato et al., 1999) and found to be potentially rich source for the discovery of novel genes which directly involved in abiotic stress tolerance which can be utilized further in developing of transgenic crops. The Plants of Kodo millet release high levels of polyphenols, polysaccharides and RNase in times of stress that make RNA extraction often difficult (Kansal *et al.,* 2008). In a recent study, over 50 phenolic compounds has been identified from kodo, finger, foxtail, proso, little and pearl millets using HPLC and ESI-MSn (Chandrasekara and Shahidi, 2011). Polyphenols are known to be readily oxidized during nucleic acids extraction to form covalently linked quinones that can irreversibly bind to proteins and nucleic acids to form high molecular weight complexes (Loomis,W. 1974). The polysaccharides tend to co-precipitate with the nucleic acids in low ionic strength buffers (Salzman et al., 1999; Reid et al., 2006; Japelaghi et al., 2011). Total RNA isolation in plant species is often complicated by the presence of recalcitrant tissue and organic compounds (Geuna *et al.,* 1998; Salzman *et al.,* 1999; Tai *et al.,* 2004; Yeh *et al.,* 1991). In physiological and biochemical studies of plants, often it becomes important to examine changes in levels of total RNA (or) mRNA of a particular gene. The prerequisite for gene expression studies is the isolation of good quality RNA. The isolation of high-quality RNA depends greatly on the treatment and handling of the tissue prior to RNA extraction. Even though, many methods exist to extract high-quality RNA from plant tissues by using Guanidinium salts or Trizol or CTAB, not all are preferred for mRNA analysis. Surfactant agents (CTAB and SDS) are widely used in RNA isolation to inhibit RNase activity. However, the efficiency of such methods varies depending on the composition of the treated tissues (Chan et al. 2004; Rodrigues et al. 2007). Here Lithium Chloride ($LiCl_2$) protocol is used for RNA extraction of kodo millet tissues. The proposed protocol overcomes the problems associated with polyphenol, polysaccharide and metabolite contamination. This is the first report on extraction efficiency of RNA from kodo millet leaves under abiotic stress condition i.e, salinity. Isolated RNA can be successfully used in downstream processes such as synthesis of cDNA, reverse transcriptase-polymerase chain reaction (RT-PCR) and the construction of suppression subtractive hybridization (SSH) library.

MATERIALS AND METHODS

Seeds of three kodo millet accessions (IC 426676, IPS 351 and IPS 145) were procured from ICRISAT and NBPGR, Hyderabad and sown in pots, filled with red soil and vermi compost in a ratio of 4:1 and were grown under optimal growth conditions. After germination three seedlings per pot were maintained and the plants were irrigated regularly, few plants in

another pot were subjected to salinity (NaCl) stress treatments. The untreated plants were maintained as controls. After completion of saline treatment, the leaves were collected from control and treated plants and frozen immediately in liquid nitrogen then stored at -80°C until further use. Precautions were taken to prepare RNase free materials as per Sambrook *et al*., (1989). Solutions were prepared with 0.1% Diethylpyrocarbonate (DEPC). Tris solution was prepared from sterile RNAse free water and autoclaved before use. All glassware baked at 140°C overnight. Plastic ware certified as RNase and DNase free was used without any additional handling. Oakridge tubes were pretreated with 3% hydrogen peroxide (H_2O_2) for overnight and autoclaved before use.

Isolation and Extraction of RNA

Fresh leaf sample (1.5 g) was taken into a mortar and ground fine powder in liquid nitrogen. 10 ml of extraction buffer was added to each sample (0.1M Tris HCl pH 9.0, 0.25M Sucrose, 0.2 M NaCl and 10 mM $MgCl_2$). 10 ml of phenol (water saturated): chloroform mixture (1:1) v/v, 1 ml of 0.5 M potassium EDTA and 1ml of 20% SDS were added sequentially. The grinded solution was transferred to oakridge tubes 144µl β- mercaptoethanol was added and thoroughly shaken at 4°C for 20 min and centrifuged at 16,000 rpm for 30min.The supernatant was transferred to another oakridge tube. Equal volumes of chloroform and isoamyl alcohol (49:1) was added to the aqueous phase, vortexes and spun at 15,000 RPM for 15 min at 4°C. The aqueous phase was transferred to another oakridge tube and 8M $LiCl_2$ was added to make a final concentration of 3M. It was kept at 4°C for 20 hours and centrifuged at 16,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the precipitate was washed with 5 ml of 2M LiCl then 5ml of 75% ethanol was added to the precipitate and centrifuged at 15,000 rpm for 20 minutes at 4°C .The pellet was air dried and dissolved in 150 µl of DEPC water.

Analysis of RNA

Purity and concentration of RNA was assessed by determining the absorbance of the sample at 260 and 280 nm using a spectrophotometer. Integrity of RNA was evaluated on a 1.5% denaturing formaldehyde-agarose gel electrophoresis stained with ethidium bromide (Sambrook et al. 1989). The bands were visualised and photographed using Gel documentation unit.

First strand cDNA synthesis and RT-PCR

All RNA samples were further treated with RNase-free DNase (Fermentas), according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcribing 2 µg of total RNA with RevertAid First Strand cDNA Synthesis Ki (Fermentas) in a 20 µl reaction using oligo$(dT)_{18}$ primers and RevertAid™ M-MuLV Reverse Transcriptase according to manufacturer's instructions. Reverse transcription was performed at 42°C for 1 hour, followed by 70°C for 5 min. cDNA was diluted 8 times for the use of real-time qRT-PCR reaction. All cDNA were stored at -20°C until further use.

RESULTS AND DISCUSSION

Kodomillet is containing rich protein source when compared to other minor millets. It also contains albumin and globulin. All these components may interrupt the RNA extraction processes and results in poor yield of RNA with degradation. RNA is degraded rapidly by ribonucleases (RNases) and hence, it must be extracted quickly and efficiently (Sambrook *et al.,* 1989). Cellular ribonucleases act quickly and efficiently to degrade RNA upon cell lysis. Therefore, the collected tissue is frozen in liquid nitrogen and stored at -70°C so as to provide the best possible material for miRNA analysis. Moreover, the β mercaptoethanol along with phenol/chloroform might inactivate RNAase by dissociating the disulfide linkages (Venkatachalam *et al*., 1999, Dawson *et al*., 1986, Wang *et al*., 2005). Higher centrifugation speed (g) was maintained for proper

segregation of nucleic acids from proteins and polysaccharides (Salzman *et al*., 1999). Using of 2M lithium chloride solution efficaciously precipitated only the RNA thereby easing the elimination of left over amounts of contaminating proteins and polysaccharides (Barlow *et al*., 1963). The RNA pellet was washed with 75% ethanol twice to eliminate any salt residues present in the sample (Hidayah Jamalnasir, 2013**)**. The precipitated RNA was free from DNA and therefore eliminates the further use of deoxyribonulceases (DNase). For isolating nucleic acids from plants, the general approach was to remove macromolecular contaminants as quickly as possible. The reason to keep the temperature low during RNA extraction is to stop the phenolic compounds not to react with nucleic acids and allowing them to precipitate with other debris after the first centrifugation and keep the supernatant clear. The low temperature not only increased the quantity of RNA but also the quality of RNA. When RNA was isolated by the current method, 360µg of RNA was obtained from 1 g of leaf tissue from different accessions of kodo millet. Salinity stressed leaf samples yielded 280µg of total RNA. The A260/A280 ratio of the RNA extracts indicates the presence of very low amounts of contaminating proteins, polysaccharides and polyphenol compounds (Table 1). Purity of RNA samples (A260/A280 ratio) isolated from the leaves was 1.77, while in case of salinity stressed leaves was 1.7. The observations revealed that the RNA produced from leaf tissues was good and significant, with less or no impurities. The bands corresponding to 28S and 18S rRNA was significantly prominent indicating the suitability of leaf tissues for the isolation of RNA. The main bands were looking bright and the brightness of the 28S was higher than that of 18S band, indicating that the extracted RNA was of high purity (Fig 1), with clear distinction and poor degradation. Further RNA isolated from all the accessions was reverse transcription. Further, this single stand cDNA was used as template using the gene specific primers of actin gene an expected amplicon of 350 bp was successfully amplified from all RNA preparations indicating the high quality of RNA and agreeable for downstream applications such as RT-PCR, qRT-PCR and cDNA library construction (Fig 2). Several methods have been developed for the extraction of high-quality RNA from different plant species and tissues. In Kodo millet, $LiCl_2$ protocol was successful for the isolation of RNA from leaves. All the reports observed were found to be first of its kind in kodo millet plants. All accessions have produced the mandatory amounts of RNA, signifying that it can be further constructive in RT–PCR studies.

Table 1
Total RNA yield isolated from kodomillet

Sr. No	Variety	RNA yield (µg/g tissue)	RNA purity A260/A280
1	IC 426676	320±11	1.77±0.013
2	IPS 351	360±25	1.8±0.02
3	IPS 145	340±10	1.73±0.01
4	Salinity Stress Leaf	280±20	1.7±0.02

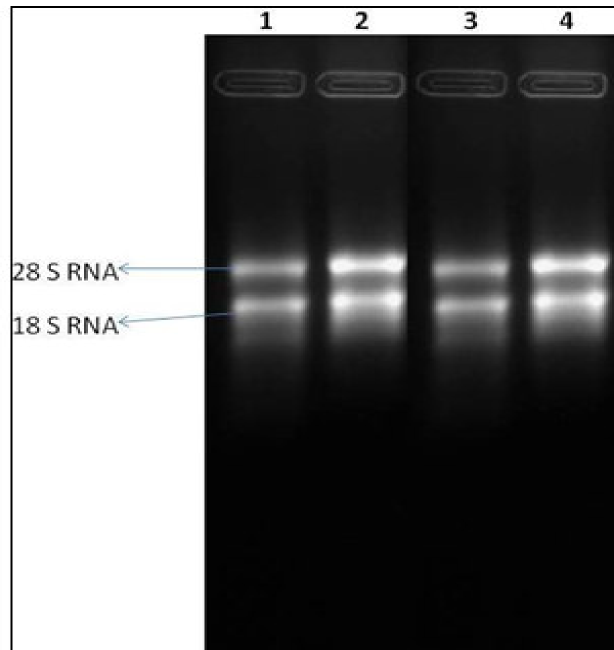

Figure 1
The intact mRNA in kodomillet leaf samples

Figure 2
Actin amplification products of cDNA

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