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A SIMPLE AND EFFICIENT METHOD FOR DNA EXTRACTION FROM RABI SORGHUM [*Sorghum bicolor* (L.) MOENCH]"

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ABSTRACT
Sorghum [*Sorghum bicolor* (L.) Moench] is an important source for bio-energy and having significant contributions in agriculture. Accordingly, plant breeders are working for different strategies for genetic improvement *i.e.* breeding for higher yield, improved grain quality, and biotic and abiotic stress tolerance in sorghum. For genetic improvement four major biotechnological tools are now emerging e.g. molecular markers, gene identification and cloning, genetic engineering and gene transfer technology to integrate desirable traits into the sorghum genome, and genomics and germplasm databases. Genomic DNA extraction is the basic prerequisite for all of these tools. We made several modifications to the available CTAB method to isolate genomic DNA from sweet sorghum leaf tissues. Higher concentration of NaCl (5 M) in CTAB extraction buffer improved the DNA yield and quality by preventing the sample from becoming viscous during the sample grinding. The yield of DNA ranged from 432-569 ng from 200 mg of leaf tissue. Proteinase k and RNase A treatment properly removes the protein and RNA from DNA. An absorbance value of 1.8 at A260/A280 indicates that the DNA is free from RNA and protein contamination. Three times chloroform:isoamyl alcohol (24:1 v/v) wash resulted in a good quality of DNA. Pre-chilled ethanol being useful for DNA precipitation due to its volatile nature and ideal for small and medium scale DNA extraction. PCR analysis using SSR primers shows a consistent and reliable amplification

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is an important source for bioenergy production. Sorghum breeding programs that focus on developing advance breeding lines that can withstand and adapt to water stress and various biotic factors is more necessary than earlier. In recent days a major focus of breeder is on genome analysis of sorghum for their improvement and it requires a large quantity and pure DNA (Gilchrist *et al*., 2006; Tan and Yiap, 2009). DNA extraction from any plant tissue comprises of three major steps *i.e*. lysis of tissue, separation of DNA from other cellular components, and isolation of pure DNA. Extraction of large quantity and quality DNA from a tissue is a challenging task in genome analysis. Because of the fact that, the method of DNA extraction varies from crop to crop, a slight optimization is required in the protocol of DNA extraction. Moreover, the stage and type of tissue greatly affect the quality and quantity of DNA. Fresh, young and tender tissues are preferred as good source for DNA isolation than mature one. The polysaccharides and many types of secondary metabolites present in tissue affects the DNA purification (Ali *et al*., 2019; Kale *et al*., 2020). The presence of polyphenols, a powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most genome analysis (Mace *et al*., 2003).

A variety of DNA extraction methods and kits are available, earlier Sathelly *et al.* (2014) have reported a DNA extraction protocol from sweet sorghum. However, we got better results using our modified protocol as compared to that of Sathelly *et al*. (2014) the problem associated with the available commercial kits are their high cost and low yields of DNA (Amani *et al*., 2011; Abdellaoui *et al*., 2011; Xin and Chen, 2012). DNA isolation from plant cells surrounded by rigid polysaccharide cell walls involves complicated steps and requires benchtop laboratory equipment (Paul *et al*., 2020). Hence, present research work was carried out with an approach to design a simple, economical and efficient protocol for DNA extraction from rabi sorghum.

MATERIALS AND METHODS

Plant material

Present investigation was carried out at State Level Biotechnology Centre, Mahatma Phule Krushi vidyapeeth, Rahuri in the academic year 2020-2021. Seven rabi sorghum genotypes *i.e.* M 35-1, Phule Anuradha, RSLG-2422, RSV 1876, RSV 1910, RSV 2371 and RSV 1988 used for the study. The genotypes were obtained from Sorghum Improvement Project, Mahatma Phule Agricultural University, Rahuri.

Reagents

-1 M Tris-HCl (pH 8.0)

-5 M Sodium Chloride (NaCl)

-0.5 M Ethylenediaminetetraacetic acid (EDTA) (pH 8.0)

-Isopropyl alcohol (isoprpopanol)

-70% ethanol

-Chloroform-isoamyl alcohol (24:1 v/v)

-Ethidium bromide (EtBr) (10 mg/ml)

(Table 1)

Buffers

-3% (w/v) Cetyl trimethylammonium bromide (CTAB)

-TE buffer (pH 8): 1 M Tris-HCl, 0.5 M EDTA

-1x TBE buffer: 0.9 M Tris, 0.9 M Boric acid, 25 mM

EDTA

(Table 2)

DNA Isolation

i.Preheat (65ºC) the extraction buffer comprises of 1 M Tris-HCl (pH 8), 5 M EDTA (pH 8), 3% (w/v) CTAB, 10 μl proteinase K (20 mg/ml), and 1% (v/v) β-mercaptoethanol (added freshly) kept in water bath at 65ºC for about 15 min. Add preheated extraction buffer to each microcentrifuge tube (2 ml).

ii.Fresh leaf samples (200 mg) were collected from three weeks old germinated seedlings from each line and grinded to make fine powder in liquid nitrogen with the help of pre- chilled mortar and pestle.

iii.This powder was immediately transferred to centrifuge tubes (2 ml) containing pre-warmed extraction buffer and was mixed by inversion.

iv.The homogenate was incubated at 65ºC for about 60 min with frequently swirling.

v.Centrifuge the tubes at 10,000 rpm for 10 min.

vi.Transfer the supernatant into another autoclaved centrifuge tube (2 ml).

vii.To the supernatant equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed gently by inversion for 15 min.

viii.Centrifuge the tubes at 10,000 rpm for 10 min at 4ºC and collect the supernatant in centrifuge tube (1.5 ml) and using wide bored tip, carefully transfer the upper aqueous phase (supernatant), which contains DNA to a new centrifuge tube (1.5 ml).

ix.Repeat the extraction steps (vii and viii).

x.The supernatant then treated with RNase A, mix twice for 30 sec, and incubated at 37ºC for about 1 hour to remove any RNA present in the sample.

xi.The equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube, mix gently by inversion, and incubate at room temperature for about 15 min.

xii.Centrifuge the tubes at 12,000 rpm for 10 min and transfer the supernatant into newly labelled autoclaved centrifuge tube (1.5 ml).

xiii.To the supernatant double volume of chilled absolute alcohol was added and mixed gently by inversion.

xiv.Tubes were incubated at 4ºC for overnight to precipitate the DNA.

xv.The precipitated DNA was collected after centrifugation at 10,000 rpm for 10 min.

xvi.DNA pellet was washed 2-3 times with 70% ethanol, air dried and dissolved in 100 μl of TE (1 M Tris-HCl, 0.5 M EDTA, pH 8) buffer.

xvii.The samples were stored at -20ºC.

Note: The first centrifugation steps during extraction were carried out at RT to avoid precipitation of DNA with CTAB.

Quantitative and Qualitative analysis

Quantification

The concentration of DNA obtained was quantified using Nanodrop spectrophotometer (ND-1000). The purity of DNA from protein and polysaccharide contamination was assessed by estimating the absorbance ratio at A260/A280 .

Quality check

The quality of DNA was determined through agarose gel electrophoresis using 0.8% agarose gel in 1 X TBE buffer for 30 minutes at 80 V. The agarose gel stained with ethidium bromide (EtBr) to check DNA quality. The gel were photographed under gel documentation system (Alpha Innotech Corporation, USA)

SSR marker and PCR

The genomic DNA extracted by present method was used as template for SSR (Simple Sequence Repeat) marker analysis. PCR amplification were performed following PCR reaction: 25 μl contained 2 μl (50 ng/$\grave{i}$ l) of template DNA, 2.5 μl of 10X PCR buffer, 3.3 μl of 15 mM MgCl2, 1 μl of 10 mM dNTPs, 1 μl (10 picomol) of each primer, 0.5μl Taq DNA polymerase (1 U/μl), and 13.7μl of sterile water.

DNA amplification

DNA amplification was performed in a thermal cycler (Eppendorf Mastercycler, Eppendorf, USA) by using the following thermal cycler condition: initial denaturation step at 94ºC for 5 min (1 cycle); followed by 35 cycles each at 94ºC for 1 min (denaturation), 61ºC for 1 min (annealing), 72ºC for 1 min (extension), followed by one final extension at 72ºC for 10 min. DNA was amplified with SSR primer X txp 278 containing forward (5' GGGTTTCAACTC TAGCC TACCG AAC TTCCT3')and reverse (5'ATGCCTCATCATCATGGTTCG TTTTGCTT 3') primer, respectively.

RESULTS AND DISCUSSION

In this study the critical modifications are made in the basic CTAB (Doyle and Doyle, 1987) DNA extraction protocol. Firstly we added a higher concentration of NaCl (5 M) and 1% PVP in CTAB extraction buffer which helped to improve the DNA yield and quality by preventing the sample from being viscous in nature during sample grinding. The same findings were reported by Sathelly *et al*. (2014). Ali *et al*. (2019) used the high concentration of phenol binding reagent (PVP) and NaCl to remove polyphenols and polysaccharides, respectively. Second optimization was made to increase the amount of EDTA (0.5 M) that efficiently neutralizes the divalent cations required for DNase activity and thus protects the DNA from degradation. Similar outcome was reported by Sathelly *et al*. (2014). Thirdly, addition of a higher concentration of Tris-HCl (1 M) facilitates constant pH during extraction with CTAB buffer. Furthermore, the chloroform extraction step was performed before precipitating the DNA, which improves the DNA quality and accordingly the yield by efficient removal of

Table 1: Preparation of stock solutions for DNA extraction

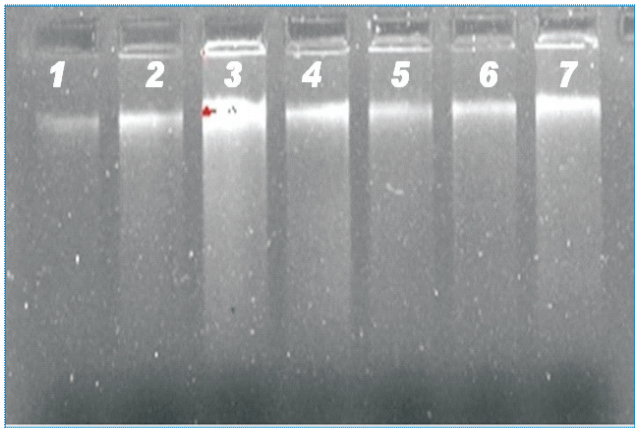
Sl. No.	Solution	Method of Preparation
1	1M Tris HCl (pH 8.0)	Dissolved 12.11g Tris base (Merck) in 80 ml distilled water. Adjusted pH to 8.0 by adding concentrated HCl and NaOH pallet. Adjust volume to 100 ml. Dispensed to reagent bottle and sterilized by autoclaving.
2	0.5M EDTA (pH 8.0)	Dissolved 7.31 g EDTA di Sodium salt (SRL) 80 ml distilled water. Adjusted pH to 8.0 by adding NaOH pellets and Conc. HCl. Adjusted volume to 100 ml. Dispensed to reagent bottle and sterilized by autoclaving.
3	5M NaCl	Weighed 29.92 g NaCl (SRL) added 50 ml of distilled water and mixed well. When the salts get completely dissolved, adjusted the final volume to 100 ml. Dispensed in to reagent bottle and autoclaved.
4	70% Ethanol	Taken 70 ml of ethanol; mixed with 30 ml of distilled water. Dispensed to reagent bottle and stored at room temperature.
5	Chloroform: Isoamyl alcohol (24:1 v/v)	Measured 48 ml of chloroform and 2 ml of isoamyl alcohol. Mixed well and stored in reagent bottle in room temperature. (Use freshly prepared)
6	Ethidium Bromide (10 mg/ml)	Ready to use from AMERESCO

Table 2: Preparation of buffers for DNA extraction

Sl. No.	Buffer	Method of Preparation
1	CTAB Extraction buffer (3%)	Measured 1 ml of 1M Tris HCl (pH 8.0), 2.8 ml of 5M NaCl, and 1 ml of 0.5 M EDTA (pH 8.0). Mixed with about 4 ml of hot distilled water, added 0.3 g (W/V) CTAB (AMRESCO) and 0.1 g (W/V) PVP (HI-MEDIA) to this. Dispensed to reagent bottle. Just before use, added 100 μl (1%) ^{2}- mercaptoethanol.
2	TE buffer (0.1mM), 100 ml	Taken 1 ml of Tris HCl (1M), 200 μl of EDTA (0.5M). Mixed with 99 ml of sterile distilled water taken in a reagent bottle, mixed thoroughly, autoclaved and stored at room temperature.

Table 3: Quantity check of isolated genomic DNA by using Nanodrop

Sl. No.	Name of genotype	Stock concentration of DNA (ng)	OD A260/ A280
1	M 35-1	569.2	1.87
2	Phule Anuradha	455.1	1.81
3	RSLG-2422	432.2	1.85
4	RSV 1876	472.4	1.86
5	RSV 1910	526.9	1.87
6	RSV 2371	461.6	1.83
7	RSV 1988	518.4	1.88

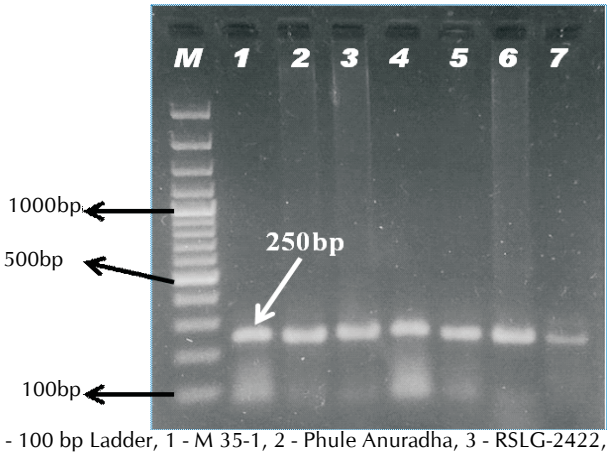


(1 - M 35-1, 2 - Phule Anuradha, 3 - RSLG-2422, 4 - RSV 1876, 5 - RSV 1910,6 - RSV 2371 and 7 - RSV 1988)

Figure 1: Electrophoretic pattren of DNA samples showing sharp, distinct and clear bands of seven *rabi* Sorghum genotypes

polysaccharides, lipids and other non-polar substances. The fifth modification was precipitation of DNA with ethanol instead of cold isopropanol. As, ethanol is considered as volatile in nature and because of this the ethanol was used in DNA precipitation and ideal for small and medium scale DNA extraction. Sathelly *et al*. (2014) observed that DNA precipitation with is opropanol leaves a high salt concentration and was very difficult to remove. Enhancing the time-period of RNase A treatment for DNA from 30 to 60 min facilitates proper removal of RNA from the sample. Similar observation was made by Rasu *et al*., 2013. Finally, addition of chloroform and iso-amyl alcohol (24:1) after RNase A treatment improves DNA quality by efficiently removing proteins from the DNA sample. Incorporating the said modifications led to the extraction of high quality genomic DNA without contaminants *i.e*., RNA and protein from rabi sorghum young and old leaf tissues.

This method was modified from basic CTAB DNA extraction protocol with some critical modifications yielded high quality of DNA from both young and old rabi sorghum leaf tissues. The total DNA yielded was 569.2 ng (M 35^{-1}) and 432.2 ng (Phule Anuradha) from 200 mg of rabi sorghum leaf tissues. Present findings are similar to that of Grewal *et al*. (2013). The absorbance of isolated DNA was 1.8. at A260/A280 (Table 3). This result of absorbance from isolated DNA at A260/A280 indicated that there is not much more RNA and protein contamination in the extracted DNA. Ali *et al*. (2019) observed that the ratio of UV absorption at A260/A280 was 1.8 for a pure DNA, any increase in it indicates RNA contamination and conversely, the presence of protein (largely) decreases the value. Similar findings were reported by Varma *et al*. (2007) and Kale *et al*. (2020). The quantity of DNA was determined on the absorbance at A260/A280 ratio, and quality based upon the banding pattern observed on 0.8% agarose (Figure 1). The results obtained from agarose gel electrophoresis revealed that the improved DNA extraction method produces high quality genomic DNA without shearing and RNA contamination (Figure 1). Similar results were reported by Gadakh *et al*. (2017) in Sugarcane. The DNA isolated can directly be used without any purification for further PCR reaction. The suitability of DNA isolated as a template in PCR



(M - 100 bp Ladder, 1 - M 35-1, 2 - Phule Anuradha, 3 - RSLG-2422, 4 - RSV 1876, 5 - RSV 1910, 6 - RSV 2371 and 7 - RSV 1988)

Figure 2 : SSR amplification pattern obtained with sorghum cultivars

amplification reaction was analyzed by using SSR primers and results showed that consistent and reliable amplification product at 250 bp (Figure 2).

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product at 250 bp. This protocol of DNA extraction is highly suitable for extracting high quality genomic DNA from plants with high levels of polysaccharides and polyphenolics without blending commercial kits.

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