Biotechnology Journal International

20(2): 1-7, 2017; Article no.BJI.33145
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Comparative Analysis of DNA Extraction Methods in Two Popular Varieties of Finger Millet (*Eleusine coracana*) from Ethiopia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ABL and STH carried out all experimental work, data acquisition and analysis, literature search. Author STH was responsible for study concept, designing and coordinating the research and supervising the work. Author STH contributed to writing and manuscript preparation. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2017/33145
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history/22300

Original Research Article

Received 31st March 2017
Accepted 2nd June 2017
Published 15th December 2017

ABSTRACT

Aims: The aim of this investigation was to standardize the genomic DNA isolation protocol to obtain high quality and quantity DNA for genomic analysis. To accomplish this task, dry leaves of finger millet which are rich in polysaccharides and polyphenols and seeds were utilized as study group.

Methodology: Two popular finger millet varieties of Ethiopia (Tadesse and Degu) were selected and obtained from Hawassa Agricultural Research Institute, Hawassa, Ethiopia. DNA isolation was carried out by two most popular and reliable methods i.e. Cetyl Trimethyl Ammonium Bromide (CTAB) and Dodecyl Trimethyl Ammonium Bromide (DTAB) method with little modifications. The quality of DNA obtained through the two methods was comparatively evaluated.

Results: The CTAB method proved its superiority over DTAB. The purity of extracted DNA was

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excellent as evident by A_{260}/A_{280} ratio ranging from 1.76 to 1.78, which also suggested that the preparations were sufficiently free of proteins and polyphenolics/polysaccharide compounds. However, the DTAB method failed to extract sufficient quantity and quality of genomic DNA for further genomic analysis.
Conclusions: Based on our study, our protocol can be useful for other difficult cereal crops in the future.

Keywords: CTAB; DTAB; Eluesine coracana; genomic DNA.

1. INTRODUCTION

Finger millet (*Eleusine coracana* (L) Gaertn) is an allotetraploid crop with a basic chromosome number of nine and genome composition AABB (2n = 4 x = 36) [1]. It is an important food crop cultivated widely in arid and semi-arid regions of the world due to its resistance to pests and diseases, especially in East Africa, India and in other Asian countries including Sri Lanka and China [2]. The African native variety, finger millet, likely originated from the highlands of Ethiopia and Uganda. Mainly subsistence farmers grow the crop, which serves as a food security crop because of high nutritional value and excellent storage qualities and its importance as a low input crop [3]. Ethiopia is one of the major producers of finger millet in addition to Uganda, India, Nepal and China [4].

The problem of cereal species in genomic DNA isolation is the presence of high phenolics, polysaccharides and other secondary metabolites content that contaminates DNA and interferes with subsequent analysis [5]. The most preferable and frequently used method to extract DNA from polysaccharide and polyphenol-rich leaves is the CTAB (Cetyl Trimethyl Ammonium Bromide) method developed by Doyle and Doyle [6] and the most cost effective method is the Dodecyl Trimethyl Ammonium Bromide (DTAB) by Mannen et al. [7]. However, the later method cannot be used to extract DNA from dried and mature leaf of finger millet which is rich in polysaccharide and polyphenol-rich leave [8]. Therefore, standerdization of CTAB and DTAB method is needed to obtain pure DNA from dried and mature leaf of finger millet for further molecular analysis.

An array of DNA isolation protocols have been optimized and used in various combinations to isolate quality DNA from plants for analyses [9]. Isolation of plant genomic DNA is a fundamental requirement for most genomic characterization and mapping procedures involving the use of genetic markers, and for the identification and isolation of plant genes for further molecular study. The degree of purity and quality of the DNA isolated varies from application to application. For example, high molecular weight and pure DNA is a prime prerequisite for the production of genomic DNA libraries, which is screened for plant gene sequences and for the determination of genetic markers such as RFLPs, RAPD [10].

Genetic exploration of finger millet is important to support its breeding program. One of the significant steps in any molecular biology activities is DNA isolation to produce high quality DNA for further analysis. Leaves of finger millet, as other cereal crops, contain high concentration of polysaccharides and polyphenols. These compounds can interfere with enzyme activities in subsequent molecular analysis. The aim of this study was to perform comparative analysis of DNA extraction method to obtain high-quality genomic DNA by CTAB and DTAB methods from mature leaf and seed of finger millet. Established CTAB and DTAB extraction buffers with varying concentrations of cetyl trimethylammonium bromide, polyvinyl pyrrolidone, Tris-HCl, $NaCl_2$ and EDTA, were tested in this study were tested in this study [11].

2. MATERIALS AND METHODS

2.1 Plant Materials

Freshly harvested, mature and healthy leaves of two popular varieties of finger millet (*E. coracana*) i.e. Tadesse and Degu were collected from the experimental field of Hawassa Agricultural Research Institute, Hawassa, Ethiopia, both varieties are popular and known to be cultivated by 70% of Ethiopian farmers [11].

2.2 Reagents and Chemicals

2.2.1 CTAB extraction buffer consisted of 1-3% CTAB, 5 M NaCl, 50 mM EDTA, and 100 mM Tris HCl

DTAB extraction buffer contained 5-10 volume of 8% DTAB. Stock solutions of the different CTAB

and DTAB extraction buffer components were prepared by standard procedures. Other reagents and materials used were 1-3% β-mercaptoethanol, chloroform, 100% isopropanol at 4°C, 70% and 100% absolute ethanol in sterile ddH_2O, 3 M NaCl, 7.5 M NH_4Cl, 10 mg/mL RNase and Tris-EDTA (T10E1) buffer were also utilized for DNA extraction. All the chemical and reagents used were of molecular biology grade and procured from Hi-Media Ltd., Mumbai, India.

2.3 Genomic DNA Isolation

2.3.1 Optimized CTAB based DNA isolation protocol

Doyle and Doyle [6] and Clarke [12] method was adopted for optimization of DNA isolation protocol by varying the concentration of CTAB extraction buffer containing Tris-HCl, EDTA, NaCl, PVP and β-mercapthoethanol. CTAB solution (700 µl for each samples) was poured in a 15 ml tube and 0.2 volume of 3% β-Mercapto-ethanol was added. β -Mercapto-ethanol was stored at 4°C. Aliquot of CTAB in 1.5 ml Eppendorf tube was heated in water bath at 65°C for 90 minutes. 50 mg matured, dried leaves materials per sample were weighed and pulverised thoroughly using a clean mortar and pestle. The powder was transferred into an Eppendorf tube containing warm CTAB (700 µl) solution immediately. The powder was allowed to dissolve and the sample was incubated at 65°C for 90 minutes followed by centrifugation at 5000 rpm for 5 minutes. The supernatant was transfer to fresh Eppendorf tube. Another aliquot of CTAB solution (700 µl) was added to the tissue pellet and stirred slightly with 1000 µl pipette tip; incubated for 90 min at 65°C and centrifuged at 5000 rpm for 5 minutes and supernatant (only clear liquid) was transferred in a new eppendorf tube. The same procedure is repeated for a third extraction and each fraction proceeds with the next step and was treated separately. To each tube, 600 µl Chloroform was added and shaken carefully for two minutes upside down. This chloroform step was carried out immediately and the samples were shaken thoroughly by holding the top of Eppendorf tubes for approximately 5 minutes and incubated at 65°C for 90 minutes. Then the tubes were centrifuged at 5000 rpm for 5 minutes and transferred the supernatant in another Eppendorf tube. The chloroform extraction step was repeated to make sure that all impurities were removed before proceeding to the next step. Cooled Isopropanol (4°C), approximately 2/3rd of the solution volume was added and shaken carefully by inversing the Eppendorf cap. In most cases, DNA became visible as white threads. The tubes were frozen at -20°C for 2 hours and centrifuged at 5000 rpm for 10 min followed by aspirating liquid using 200 µl yellow tips (without touching pellet). 200 µl of 70% ethanol was added to the pellet and washed the inner of the tube surface by turning the cap followed by centrifugation at 5000 rpm for 5 minutes. Ethanol was aspirated using 200 µl yellow tips and the DNA-pellet was air dried at room temperature for 15 minutes. The pellet was dissolved in 100 µl TE and stored at 4°C. Heat treated 10 µg/ml RNase A was added to a final concentration of the solution pellet followed by mixing, and incubating at 37°C for 2 hours. Then after; 200 µl cooled 7.5 M NH4Cl solution (stored at 4°C) was added and mixed carefully followed by adding cooled 200 µl of 100% ethanol and stored at -20°C for 2 hours followed by centrifugation at 5000 rpm for 30 minutes. The inner cap surface was rinsed with 200 µl of 70% ethanol followed by centrifugation at 5000 rpm for 10 minutes and dissolved aspirated liquid and dried pellet in 100 µl TE at room temperature. Repeated the same steps with adding cooled 200 µl of 3M NaCl solution (4°C) by mixing with the addition of 100% ethanol and followed by 200 µl of 70% ethanol, centrifugation and aspiration. The pellet was air dried at room temperature and resuspended in 100 µl TE buffer. All the centrifugation steps were carried out at room temperature to avoid precipitation with CTAB, DNA degradation to yield maximum quantity and quality of DNA.

2.4 DTAB DNA Isolation Protocol

The DTAB DNA extraction method of Gustincich et al. [13] as described by Manen et al. [7] was adopted. The clean, mature leaves and seeds were weighed. To keep liquid volumes within the capacity of one microfuge tube, not more than 50 mg dry weight at environmental moisture was used per extraction. Too much material relative to the extraction buffer may produce poor results. While vortexing, shaking, or inverting the samples in microfuge tubes throughout the procedure, pressure was kept on the lids to prevent accidental opening, as sand, heat, chloroform and detergents tend to weaken the tubes' seal. Same procedures and solutions were used as with CTAB method. DTAB method was used to standardize the DNA isolation protocol by varying the concentration of DTAB extraction buffer containing Tris-HCl, EDTA, NaCl, PVP, β-mercapthoethanol with varying time of

incubation. The differences between CTAB and DTAB protocol is the surfactant concentration, CTAB has a hydrocarbon (-CH2-) chain length of sixteen (as its name suggests) whereas, DTAB has a hydrocarbon (-CH2-) chain length of twelve.

2.5 Determination of the Yields: Genomic DNA Quantity and Purity

DNA yield and purity was determined by spectrophotometric analysis by checking the optical density (OD) in a UV spectrophotometer (Shimadzu, Japan) at 260 and 280 nm. DNA purity was determined by calculating the absorbance ratio at $A_{260/280}$.

2.5.1 Statistical analysis for spectrophotometric measurement

The present investigation was carried out with four replications with two treatments. DNA Yield (µg) = DNA Concentration × Total Sample Volume (ml) [14]. The amount of DNA was quantified by using the following formula:

$$\text{DNA concentration (µg/µl)} = \frac{\text{OD260 x 50 (dilution factor) x 50 µg/ml}}{1000}$$

The final results were calculated by using mean of total replicates.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 DNA extraction by CTAB

DNA isolation is a primary and critical step for molecular analysis of any plant species. Among all the tested protocols, CTAB method yielded convincing results. The incubation time duration is determined by the choice of tissue as generally leaf cells are easier to crack than seed cells, and it is easier for DNA to release from leaf cells than from seeds. Whereas from the leaf samples, the purity ratio of DNA obtained was 1.78 (Table 1). In another variety Degu, the ratio of purity of DNA recorded almost close i.e. 1.77 and 1.76 for leaf and seed, respectively (Table 1). The CTAB method proved its superiority over DTAB for DNA isolation, especially for Degu compared with Tadesse. The purity ratio was recorded almost touching to standard value of 1.8.

3.1.2 DNA extraction by DTAB method

Fresh and young leaf are the first choice to obtain good-quality DNA. However, mature leaves contain higher quantities of polyphenols and polysaccharides, which make it very difficult to isolate DNA of good quality. However, availability of young leaves for the molecular studies is quite challenging for some species due to early DNAse activity. Keeping in view, the present investigation was designed to optimize the protocol to obtain better quality DNA even from dried and mature leaf samples. No DNA fragmentation due to shearing of DNA during extraction procedure was seen in any of the samples and results were reproducible. DTAB method of DNA isolation did not yield purified DNA from both the varieties (Table 2). An effort has also been made with varying concentration of RNase with DTAB. The concentration has been increased by two fold in addition to DTAB (2 µl RNase per 100 µl DTAB, 3 µl RNAase per 100 µl DTAB; results not shown) but it failed to produce genomic DNA with standard purity. The purity ratio of DNA from Tadesse variety recorded 1.59 and 1.55 (Table 2) from leaf as well as seed respectively. Similar results were also recorded with variety, Degue. The DTAB method was not proved to be promising to isolate the genomic DNA. The purity ratio of DNA was measured was 1.57 and 1.54 from leaf and seed respectively (Table 2).

Table 1. UV-Vis Spectrophotometric DNA analysis of two finger millet varieties by using CTAB isolation method

Plant Part	DNA sample (ng/µl)	Tadesse	Degu
		$A_{260/280}$ nm	$A_{260/280}$ nm
Leaf	50	1.78	1.77
Seed	50	1.77	1.76

* Values are expressed as mean

Table 2. UV-Vis Spectrophotometric DNA analysis of two finger millet varieties by using DTAB isolation method

Plant Part	DNA sample (ng/µl)	Tadesse	Degu
		$A_{260/280}$ nm	$A_{260/280}$ nm
Leaf	50	1.59	1.57
Seed	50	1.55	1.54

** Values are expressed as mean*

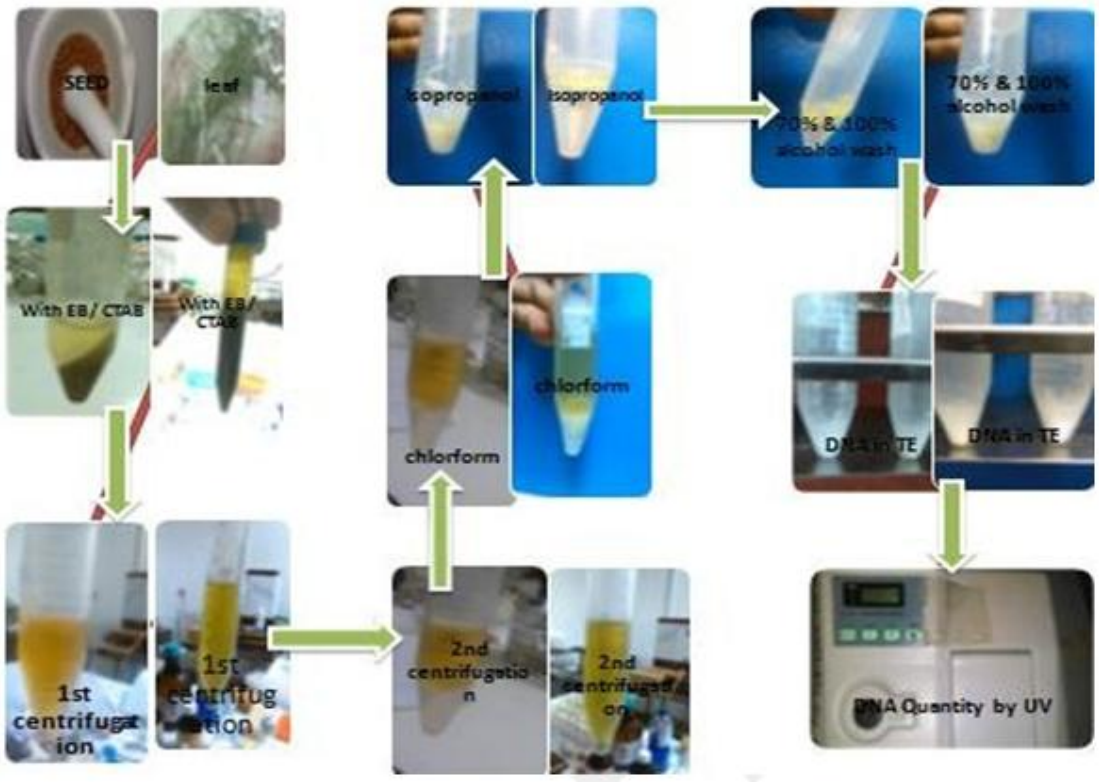

Fig. 1. Steps of genomic DNA isolation method from seeds and dried mature leaves of *Eleusine coracana*

3.2 Discussion

DTAB method of DNA isolation did not show promising results for both the varieties i.e. Tadesse and Degu as evident by the presence of ratio of purity of DNA (Table 2). We encountered many difficulties from the very first step of cell lysis to DNA separation in the supernatant and subsequent reactions when the DTAB DNA extraction method Gustincich et al. [13] as described by Manen et al. [7] was followed. The amount of DNA obtained with these protocols was very low, and the quality was poor for most of the samples. A_{260}/A_{280} ratio was less than 1.6, that is, below the optimal limit of 1.8 [6] making the DNA no amenable for further studies, the probable reason may be the type(s) of nucleic acid present in it. The RNase concentration of 10 ug/ml was adequate to remove RNA polysaccharides, polyphenols (anti-oxidants) and secondary metabolites (rich in iron and fiber with high calcium contents). Polyphenols and polysaccharides bind to nucleic acids during DNA isolation and interfere with subsequent reactions [15,16]. The most preferable and frequently used method to extract DNA from polysaccharide and polyphenol-rich leaves is DTAB method [7]. However, it cannot be used to extract DNA from mature leaves which are rich in polysaccharide and polyphenol [8]. Therefore, some modification in DTAB method is recommended.

The CTAB method described by Doyle and Doyle [6] gave better quality DNA yield from the studied plants. Hence, this method was considered for the purpose of standardization at varying concentration of Tris-HCl, β-mercaptoethanol, NaCl, and PVP. Purity of extracted DNA was excellent as evident by A_{260}/A_{280} ratio ranging from 1.76 to 1.78 (Table 1), suggesting that the preparations were sufficiently free of proteins and polyphenolics/polysaccharide compounds. These results are at par with the findings of Syamkumar et al. [17].

CTAB is generally used as detergent to separate out polysaccharides. Similarly, NaCl concentration greater than 1.5 M removes the polysaccharides [17]. The finding of Pirttila et al., [16] also support the results of present investigation showed that as increasing in the

concentration upto 3% of PVP, β-mercaptoethanol proved best to increase the yield and purity of DNA but similar protocol using DTAB method failed to yield the DNA. Use of potassium acetate removes most of secondary metabolites and polysaccharides from the DNA resulting in better yield of high-molecular weight DNA [18]. Several workers like Couch and Fritz [19], Chaudhry et al. [20] have recommended the use of PVP with molecular weight of 10,000 at 2% (w/v) to address the problem of phenolics. The PVP with low molecular weight has less tendency of precipitating with the nucleic acids as compared to PVP with high molecular weight thus yielding sufficient amount of polyphenol-free DNA. The spectrophotometric measurements at A_{260} and A_{280} nm of the DNA obtained from the modified protocol gave an absorbance ratio (A_{260}/A_{280}) of 1.76-1.78 (Table 1) indicating pure DNA. Clarke [12] reported absorbance ratio 1.75-1.8 of the genomic DNA isolated from cereals and woody plants by using CTAB method.

It is important to note that the A_{260}/A_{280} ratio is an indicator of purity [21] rather than a precise answer for DNA extraction protocol. Pure DNA and RNA preparations are expected to have A_{260}/A_{280} value of >1.8 and >2.0 and are subjected to the extinction coefficients of nucleic acids at absorbance of 260 nm and 280 nm [22]. Even though the A_{260}/A_{280} ratio is relatively insensitive to change, it is useless when DNA/protein mixtures are tested experimentally. Thus, probable use of this protocol becomes apparent when nucleic acids are purified from plant tissue. Tissue samples and to lesser extent whole cells has high protein content than that of nucleic acid on a weight basis and purification of samples at A_{260}/A_{280} ratio represents an enrichment of nucleic acid that could be as much as one million fold rather than that of proteins.

Several factors influence A_{260}/A_{280} ratios. For example, measurement of wavelength at 260 nm is very near to the peak of absorbance spectrum for nucleic acids, on the other hand absorbance measurement at 280 nm is located in a portion of the spectrum that possesses a very steep slope. As a result, very small differences in the wavelength in and around 280 nm will greatly affect in the A_{260}/A_{280} ratio in comparison to small absorbance differences at 260 nm. Consequently, different instruments results slightly different way on the same solution due to the variability of wavelength accuracy between instruments. Individual instruments, however, must produce consistent results. Concentration of samples can also affect the results, as dilute samples will have very little difference between the absorbance at 260 nm than that of 280 nm. The type(s) of protein present in a mixture of DNA and protein can also alter the A_{260}/A_{280} ratio. Thus, with very small differences, the detection limit as well as resolution of the instrument measurements begins to become much more significant.

4. CONCLUSION

A comparison of two most commonly used DNA extraction methods in cereal was conducted with slight modification to obtain high quantity and quality of DNA in two varieties of finger millet. In present study, the purity ratio of DNA by CTAB method was found to be 1.76 to 1.78 that is highly applicable as a pure from both the varieties. Thus, the modified CTAB method produced best results for DNA extraction from finger millet. DTAB method failed to yield purified DNA as it showed purity ratio of 1.5-1.6. The present protocol avoids the use of expensive liquid nitrogen and environmentally hazardous phenolic compounds, thus found to be safe and eco-friendly for DNA extraction in finger millet.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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