IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

COMPARISON OF DNA EXTRACTION METHODS AND PCR AMPLIFICATION OPTIMIZATION FOR THE SCOT MARKER IN FINGER MILLET

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Abstract

The Poaceae family includes finger millet (Eleusine coracana L. Gaertn), which is a staple crop in Africa and India. It can withstand greater stress than other crops and offers nutrients such as calcium, zinc, and protein. Here DNA separation was required for molecular characterisation using Scot (Started Codon Targeted) markers. As a result, the CTAB approach was the most effective for obtaining DNA. So we devised a quick and effective strategy. Due to the crop's presence of polyphenol and PCR inhibitors, an easy CTAB (Cetyl trimethyl ammonium bromide) approach for isolation of gDNA from finger millet was developed based on four modified protocols. The CTAB method's first protocol outperformed a modified CTAB method. a different hand For Scot, we optimised the PCR amplification. According to the band position, the result was monomorphic. Furthermore, we created a protocol for the isolation of gDNA from finger millet among four protocols, which was a convenient and easy approach for extracting DNA from finger millet while reducing the number of processes and chemicals. As an easy and convenient approach for DNA extraction in finger millet, the first protocol outperformed the others. This approach will bring new insight into research in addition to being effective for DNA isolation. We have developed a procedure for extracting DNA from finger millet using the CTAB method, which is a simple and convenient method.

INTRODUCTION

The allotetraploid finger millet (Eleusine coracana L. Gaertn) has a basic chromosome number of 9 and a genomic makeup of AABB (2n = 4x = 36). It is one of the most significant minor crops in the world. In over 25 countries, including Africa and South Asia, finger millet is a staple food grain. The crop is essential in Africa and India's dry, semi-arid, hilly, and tribal regions. The adaptation characteristic in Finger millet has been found to provide it an advantage over other cereal grains in stressful situations. It is well-known for its stress resistance and nutritional value (Parvathi et al., 2019), and each 100 g of finger millet provides 12.6 mg of iron, 410 mg of calcium, and 290 mg of phosphorus (Antony et. al., 2018). Finger millet has a high content of calcium, tryptophan, methionine, fibre, and sulfur-containing amino acids (Hittalmani et al., 2017). When compared to other grain-based diets like rice and sorghum, finger millet protein concentration ranges from 7 to 14 percent, with high methionine and all necessary amino acids (National Research Council, 1996). It is also advised for diabetes people due to its low glycaemic

index and high fibre content (Chandra et. al., 2016; Antony et al., 2018). Major research in plant biotechnology, plant breeding, and genomics has permitted significant improvements in grain crops. More advanced genomics research has contributed to the large-scale identification of plant genes or geneproducts. Understanding osmoregulation and storage proteins, as well as the genetic traits of high nutritional value and drought resistance in finger millets, are all appealing systems for genomiv level research. It can also act as a gene pool for several critical features in biotic and abiotic stress resistance genes. To discover genetic variation and prepare the library, DNA extraction is required. For genomic and molecular research, high-quality DNA is necessary (Tripoidi and Festa, 2021; Chandra et al., 2016).

The presence of compounds that interfere with DNA separation processes and downstream applications such as DNA restriction, amplification, and cloning makes DNA extraction challenging in diverse plants. The rapid advancements in molecular marker technology have opened the door to their usage in high-value crops such as finger millets (Sood et al., 2016). There are no technologies for genomic investigations involving the identification of alleles or genes. Muza et al. (1995) used Southern blot hybridization using gene probes to classify 26 finger millet lines from Africa and India into cytotype groups. Its genomic studies were used in sequence analysis and phylogenetic links between E. coracana subspecies were investigated (Hilu, 1988). The genetic complexity of Gramineae species (Eleusine coracana, Pennisetum) has been studied by a number of researchers.

Leza et al. (2017) and Deshpande et al. (2017) did studies on comparative study of DNA extraction (1980). The CTAB approach was found to be superior than the DTAB method by Leza et al. (2017). The beginning material, simplicity of handling, time and labour required for isolation, ultimate quantity, and quality of genomic DNA are all aspects to consider when choosing a DNA isolation process (Gupta et.al.2020). To isolate genomic DNA from a vast population of plants, a lot of functional genomics research is needed.

The current study describes a modified methodology for extracting and isolating DNA from tissues with high quantities of polysaccharides based on the hexadecyltrimethylammonium bromide (CTAB) approach. This is necessary in order to reduce the amount of chemicals, time, and stages required for high-quality DNA extraction. A procedure for isolating high-quality DNA will have a wide range of applications, including mapping, fingerprinting, and TILLING.

MATERIALS AND METHODS

In the year 2021, young finger millet leaves (Sample numbers 33 and 6) were gathered from cup culture at Banda Agriculture University and Technology, Banda, Uttar Pradesh (India). Mention the chemical source and grade utilised in this investigation. The CTAB approach (Tripoidi and Festa, 2021; Umar et al., 2017; Joshi et al., 2020) was used to extract DNA from finger millet leaves, with some changes to reagents, centrifuge rpm, and times. The CTAB extraction buffer contained 2% CTAB, 5 M NaCl, 5 Mm (millimolar) ETDA, and 100 Mm Tris HCl. 1-3 percent -mercaptoethanol, sodium acetate, 100 percent isopropanol, 70 and 100 percent ethanol, chloroform, and isoamyl alcohol (24:1) were also utilised, as well as TE buffer (Tris EDTA, pH 8.0) and 10% SDS (Sodium dodecyl sulphate).

Modified protocols for genomic DNA isolation

1) In a preheat (650 °C) CTABbuffer, young leaves (200 mg) were crushed with sterile motor postal. 2 ul of 1 percent -mercaptoethanol were added after the leaves were crushed and vortexed for 1-2 seconds. Now it was stored for incubation at 65 oC for 1 hr. An equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm at 25 oC for 10 min. Two distinct phases have been noted. The supernatant was decanted and transferred to a fresh tube with caution. The procedure starts with the addition of chloroform:isoamyl alcohol (24:1) and ends with the decantation of the supernatant. A 600 ul supernatant was utilised for the precipitation, which was made with isopropanol (400 ul) and kept at -200C for 1 hour. The supernatant was centrifuged at

8000 rpm at 40°C for 10 minutes. Nucleic acid was collected twice during the above processes, at the start of washing with 70% ethanol at 5000 rpm for 5 minutes. The pellet was dried and dissolved in 60 ul of 1X TE buffer after harvesting. We moved on to the next steps, which included using a spectrophotometer and gel electrophoresis to quantify the amount of gDNA.

2) 2) We followed the first protocol above the steps indicated in the first protocol. However, after adding chloroform, we changed the following: Isoamyl alcohol (24:1), then centrifuge for 10 minutes at 10,000 rpm. We added 180 ul sodium acetate and 400 ul isopropanol, then held it at -20 oC for 1 hour. It was then centrifuged at 8000 rpm for 10 minutes at 4 0C. Nucleic acid was collected at the start of the washing procedure with 70% ethanol at 5000 rpm for 5 minutes. Finally, similar to the first procedure, we dried the DNA pellet and dissolved it in TE buffer.
3) The third technique followed the same steps as the first, but with a 10% SDS/CTAB buffer mixture. Except for two 70 percent ethanol time steps, we followed the identical methodology as the first. Except for the DNA pellet that sank to the bottom of the tube, all of the wasted liquid was discarded. The pellet was then dried before being dissolved in 60ul of 1X TE buffer.
4) In the fourth procedure, we added 10% SDS to CTAB buffer, followed the identical first protocol as before, but the chloroform: isoamyl alcohol step was changed (C: I). We didn't utilise chloroform; instead, we used isoamyl alcohol (24:1). After incubation, we added isopropanol (400 ul) and then washed with 100 ul of 70% ethanol. As a result, the DNA pellet was dried at room temperature for 2 hours before being dissolved in 1X TE buffer (60 ul).
5) The SCOT primer was optimised for determining isolated DNA by PCR amplification for SCOT marker PCR (CAACAATGGCTACCACCA). NanoDrop was used to determine the DNA concentration (Thermofisher). Using a PTC-100 Thermocycler, all PCR reactions were completed in 96-well plates in under 10 minutes (MJ Research Model PTC100). PCR buffer (Promega), 0.5 U Taq polymerase (Promega), and 0.5 um primer were used in the reaction. Each response has 20 ng of DNA in it (Callord et al., 2009). Thermo Scientific's 100 bp plus ladder was used. A conventional PCR cycle was utilised, which included an initial denaturation phase at 94 0C for 3 minutes, followed by 35 cycles of 94 0C for 1 minute, 500C for 1 minute, 72 0C for 2 minutes, and a final extension step of 72 0C for 1 minute. TAE buffer was used to separate all products on a 1.5 percent agarose gel, and the gel was stained with EtBr.

RESULTS AND DISCUSSIONS

This result was seen in Finger Millet based on four types of modified steps in the CTAB Method procedure, as shown in Figs. (1), (2), (3), and (4). The following were the basic steps: 1) lysis of the cell wall As illustrated in Table 1, the five basic procedures are: 2) DNA separation, 3) DNA precipitation, 4) Purification of Isolated DNA, and 5) DNA dissolving. Edwards et al. (1991) employed CTAB buffer in combination with SDS, both of which are cation surfactants and lysis buffers. The third and first extraction reagents, on the other hand, are C & I. The first precipitation reagent was isopropanol, while the second precipitation reagent was 70% ethanol. Umar et al. (2017) and Joshi et al. (2017) provided the foundation for this experiment (2020). We selected two-samples, such as samples 36 and 6, which are depicted as bands A and B in all of the figures. All of the explanations are provided below.

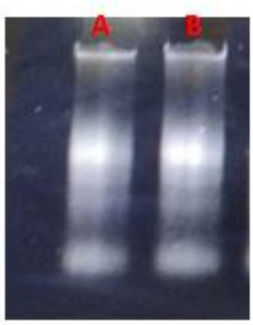

Fig.1. This represented result of protocol. 1

When compared to other bands, Fig. 1 was the first to be determined using gel electrophoresis, and it showed a good and sharp band. We did not utilise RNase in this approach, hence the DNA band below the DNA band showed RNA and protein contamination in the image.

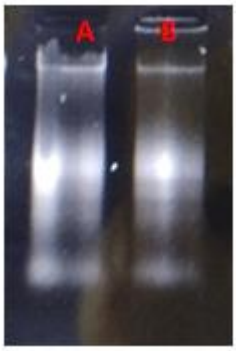

Fig.2. This represented result of proceed protocol .2

Protocol 2's result is given in Fig. 2. We were resolved to use gel electrophoresis to isolate the gDNA band. The band performed better than the others (Figs. 3 and 4), but not as well as Fig. 1.

Fig.3. This represented result of proceed protocol 3

The following protocol was used to represent it as an isolate gDNA in Figure 3: The strength of the gDNA band was exhibited in Figure 3. As a result, we discovered that the separated gDNA bands were A and B, with the B band being superior to the A band. As a result, the Figs. 1 and 3 results were satisfactory.

Fig.4. This represented result of protocol 4.

The outcome of the preceding technique is shown in Figure 4. 4. We used isoamyl alcohol to replace the chloroform (24:1). After incubation with CTAB buffer at 65°C for 1 hour, isopropanol was added directly. The results were depicted as gDNA bands, with the A band being superior to the B band.

Table.1.Comparative study of four Protocols

S. No.	**Steps of Protocol-I**	**Steps of Protocol-II**	**Steps of Protocol-III**	**Steps of Protocol-IV**
1	Taken 200mg young leaves	Taken 200mg young leaves	Taken 200mg young leaves	Taken 200mg young leaves
2	Crushed in 2ml CTAB buffer	Crushed in 2ml CTAB buffer	Crushed in 2ml CTAB buffer and add **10%SDS**.	Crushed in 2ml CTAB buffer
3	Add 2ul of 1% β-Merceptoethanol	Add 2ul of 1% β-Merceptoethanol	Add 2ul of 1% β-Merceptoethanol	Add 2ul of 1% β-Merceptoethanol
4	Vortex for 1-2 sec	Vortex for 1-2 sec	Vortex for 1-2 sec	Vortex for 1-2 sec
5	Keep it for incubation for 1 hour at 65^0C	Keep it for incubation for 1 hour at 65^0C	Keep it for incubation for 1 hour at 65^0C	Keep it for incubation for 1 hour at 65^0C
6	Add equal amount of Chloroform: Isoamylalcohol (24:1)	Add equal amount of Chloroform: Isoamylalcohol (24:1)	Add equal amount of Chloroform: Isoamylalcohol (24:1)	-
7	Centrifuge at 10,000rpm at 25^0C for 10 min	Centrifuge at 10,000rpm at 25^0C for 10 min	Centrifuge at 10,000rpm at 25^0C for 10 min	Centrifuge at 10,000rpm at 25^0C for 10 min
8	Transferred to	Transferred to	Transferred to	Transferred to

	supernatant in new tube	supernatant in new tube	supernatant in new tube	supernatant in new tube
9	Again repeat C:I steps	Again repeat C:I steps	Again repeat C:I steps	-
10	Centrifuge at 10,000rpm at 25^0C	Centrifuge at 10,000rpm at 25^0C	Centrifuge at 10,000rpm at 25^0C	-
11	600ul Supernatant transferred in new tube and add chilled isopropanol (400ul)	600ul Supernatant transferred in new tube and add chilled isopropanol (400ul) and **Sodium acetate (180ul).**	600ul Supernatant transferred in new tube and add chilled isopropanol (400ul)	600ul Supernatant transferred in new tube and add chilled isopropanol (400ul)
12	Kept it for 1hr at -20^0C	Kept it for 1hr at -20^0C	Kept it for 1hr at -20^0C	Kept it for 1hr at -20^0C
13	Proceed for centrifuge at 8000rpm at 4^0C for 10min	Proceed for centrifuge at 8000rpm at 4^0C for 10min	Proceed for centrifuge at 8000rpm at 4^0C for 10min	Proceed for centrifuge at 8000rpm at 4^0C for 10min
14	Discard the supernatant, except pellet	Discard the supernatant, except pellet	Discard the supernatant, except pellet	Discard the supernatant, except pellet
15	Add70% ethanol (100ul)for washing	Add70% ethanol (100ul)for washing	Add70% ethanol (100ul)for washing	Add70% ethanol (100ul)for washing
16	Centrifuge at 5000rpm at 4^0C for 5 min	Centrifuge at 5000rpm at 4^0C for 5 min	Centrifuge at 5000rpm at 4^0C for 5 min	Centrifuge at 5000rpm at 4^0C for 5 min
17	Again repeat 70% ethanol steps and centrifuge at 5000rpm at 4^0C for 5 min	-	-	-
18	Dry pellet	Dry pellet	Dry pellet	Dry pellet
19	Dissolve in 60ul TE buffer	Dissolve in 60ul TE buffer	Dissolve in 60ul TE buffer	Dissolve in 60ul TE buffer

Otherwise, all of the finger millet procedures yielded satisfactory results for gDNA isolation. All of the figures (1, 2, 3, 4) and table.1 were compared (yellow colour indicated modified steps, asterisks indicated eliminated stages). Protocol I produced a fantastic outcome in Fig.1. In comparison to the other bands, I demonstrated good intensity and crispness, as per protocol. As a result, the first protocol was better to all others. Comparative DNA extraction methods were investigated by Scupham et al. (2007). According to Xia et al. (2019), the study investigated four procedures (SDS, CTAB, DP305, and DNeasy Plant Mini Kit) to find the best efficient DNA extraction method. Leza et al. (2017) backed up my DNA comparative analysis experiment.

PCR AMPLIFICATION RESULT

Nanodrop had a range of 1000 g/ml to 3000 g/ml DNA. As a result, we dilute between 3 and 20 times. We performed PCR and gel runs after dilution, as well as seeing Fig. 5. As a result, we've finished optimising PCR amplification for Scot1 markers (CAACAATGGCTACCACCA).

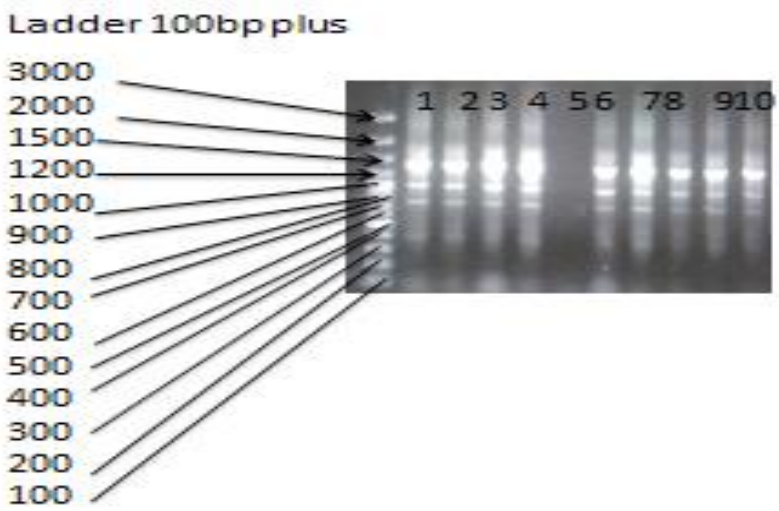

Fig5.Scot1: DNA extraction was performed using a standardised CTAB technique with 10 Finger millet samples, and isolate DNA performance was optimised and monitored using PCR product amplification.

We've shown an amplified PCR result of extracted DNA in this. The amplifying band was in good shape and scoreable, but no polymorphism was demonstrated. In a second experiment, we decided that the first protocol, the modified Steps CTAB approach, was the best and isolated gDNA from additional 10 finger millet samples. As a result, the performance of isolate gDNA was assessed using NanoDrop and PCR amplification products, as well as in fig.5, SCoT 1, which did not demonstrate polymorphism.

According to Gupta and Ranjekar, the CTAB approach was the optimum protocol for gDNA extraction for analysis of DNA sequence, trait identification, and stress resistance gene for finger millet (1981). The tetraploid genome of finger millet necessitates a more informative DNA marker for genetics and plant breeding, according to Pandian et al. (2018), therefore fewer simple sequence repeat (SSR) markers are required. DNA extraction from many plants is problematic, according to Zidani et al. (2005), due to metabolites that interfere with DNA.

As a result, we changed the CTAB (2%) procedure (1.4 M NaCl, 1% polyvinylpyrrolidone (PVP), 1% -Mercaptoethanol, and 100% ethanol) and shortened the centrifugation duration during DNA

extraction. The CTAB (Cetyl Trimethyl Ammonium Bromide) method and the Dodecyl Trimethyl Ammonium Bromide (DTAB) method were both reported by Leza et al. (2017). Due to good intensity of DNA bands based on gel electrophoresis and optical density, the CTAB approach outperformed the DTAB method. Brhana et al. (2017) employed a modified version of Borsch et al. (2003)'s CTAB approach.

In comparison to another methodology in Fig.2, Fig.3, and Fig.4, joshi et al.(2020) validated my experimental results of DNA extraction (in Fig. 1), which are outstanding. The CTAB method for DNA extraction with 10% SDS modification was published by Umar et al. (2016). (in Fig.4). DNA extraction using this procedure, according to Umar et al. (2016), is not superior than Joshi et al (2020).

Conclusion: We developed four types of CTAB modification approaches to use in future investigations. 1. We used NanoDrop, electrophoresis gels, and PCR to assess band quality. There are four different modified protocols available, but we only allowed the one that produces the best DNA band (36 and 6). First, we determined that the DNA band was acceptable according to protocol. Nanodrops, electrophoresis gel runs, and PCR amplification were used to assess band quality. Protocol-I was found to be the most effective when compared to other protocols (Fig.2, Fig.3, Fig.4) and is given in the table. 1. As a result, the first DNA extraction methodology is suitable for finger millet DNA extraction and is simple to use. In the near future, we can go on to the next experiment, such as marker assistant, sequencing, genomic library, and so on.

Acknowledgments: We thank the U.P. Council of Agricultural Research in Lucknow, as well as the genetics and plant breeding department at Banda University of Agriculture and Technology, for assisting us with this endeavour and providing resources such as the molecular lab, chemicals, and appropriate environment.

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