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Research Article

Comparative Assessment of Quick and Cost-efficient Quality DNA Extraction Methods from Rice Leaves

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ABSTRACT

DNA extraction from rice leaves was developed earlier but a quick and cost-efficient DNA extraction method is still in search to conduct molecular research swiftly. Four DNA extraction methods i.e., extraction buffer (E1), K-acetate (E2) and two modified CTAB (E3 and E4) based protocols were tested for DNA concentration, purity of the extracted DNA, time and cost required per sample. Moreover, a Mixer mill (Mi; electrical method) and a Mortar and pestle (M; manual method) were used to test the performance of four methods. DNA concentrations are insignificant among the methods MiE1 (266.61 ng/µL), MiE3 (260.58 ng/µL) and MiE4 (245.49 ng/µL) but these are all significantly different from the method MiE2 (137.13 ng/µL). ME2 required the maximum time (59 minutes), followed by ME1 (55 minutes), ME3 (40 minutes), and ME4 (21 minutes). In terms of cost, MiE3 (CTAB-1) and MiE4 (CTAB-2) required the highest (356.39 taka (4.19 dollars)/100 samples) amount of cost, where the lowest price (101.81 taka (1.20 dollars)/100 samples) was required in MiE2 (K-acetate). For Purity-1 (260/280) (PU-1), no significant difference was found among the methods, but for Purity-2 (260/230) (PU-2), MiE2 significantly differed from the others methods. Through the mortar and pestle, the highest DNA concentration was found in ME1 (382.68 ng/µL) which significantly differed from ME2, ME3 and ME4. Highest times required in ME2 (59 min) followed by ME1 (55 min), ME3 (40 min) and ME4 (21 min) methods. The highest costs (356.39 taka (4.19 dollars)/100 samples) were required for ME3 (CTAB-1) and ME4 (CTAB-2) methods followed by ME2 (121.83 taka (1.43 dollars)/100 samples) and ME1 (101.81 taka (1.20 dollars)/100 samples). No significant difference was found among the methods for PU-1 but for PU-2, ME2 significantly differed from other methods. CTAB based methods are best and quick for DNA extraction but these methods are costly, whereas the other two methods are cheaper but they are time consuming compared to CTAB.

Introduction

Discovery of the DNA structure in early 1950's has led to remarkable developments in the field of modern biology. With present knowledge, target genes can be isolated and inserted in another DNA molecule at a desired position by the action of restriction enzymes, crossing and through many other modern biological techniques. After structure discovery and extraction of DNA, genome sequencing, molecular marker, gene identification, organism detection etc. are possible with high accuracy. Climate is changing and changes climate are responsible for the emergence of drought, salinity, cold, flood, disease and insect-pest which causes a significant crop loss (Aziz et al., 2022; Mamun et al., 2018; Mezanur-Rahman et al., 2016; Rahman et al., 2021). To sustain the crop under climate change, deployment of molecular strategies like marker assisted selection breeding for stress tolerant variety development, molecular markers use for novel stress tolerant gene identification, rapid pathogen or insect detection etc. are important to face off the climate change. Molecular markers are now widely used to develop biotic and abiotic stress tolerant varieties, resistant gene detection, identification of germplasm and pathogens, diversity analysis etc. (Akter et al., 2022; Anik et al., 2022; Latif et al., 2022; Nihad et al., 2022, 2021; Rashid et al., 2021; Xin et al., 2003). Development of polymerase chain reaction (PCR) technique and molecular markers made a breakthrough in the history of biotechnological research (Asem et al.,

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2015). And to adopt any molecular techniques, first and foremost condition is to extract quality DNA with less time and consumables.

Extraction of quality DNA is the first condition for successful PCR and others molecular biology research (Aliyu et al., 2017). Too many methods have been developed for the DNA extraction from plant tissue but all are not suitable in case of rice. Problems in DNA precipitation (Hayashi et al., 2004), low PCR success rate (＜95%) from extracted DNA (Xin et al., 2003), poor quality of DNA (Wang et al., 1993), sensitivity to conditions (Kasajima et al., 2012) are the major causes of avoidance of DNA extraction methods. An effective DNA extractions method should be simple, cost effective and reliable to extracts DNA from many samples (Ahmadikhah, 2010; Aliyu et al., 2017). Though different methods are now available for DNA extraction but for the first time DNA was extracted by Friedrich Miescher in 1869 (Dahm, 2005). DNA extraction mainly divided into two sub-parts: first one to disrupt the cell and availability of the cell in extraction solution, second one is to removal of contaminants (chemicals, protein, RNA etc.) from the DNA, albeit these steps are depends on methods of extraction, use of chemicals, time and availability of facilities (Boom et al., 1990; Carpi et al., 2011).

In plants, a breakthrough in DNA extraction came in 1980 with the development of the Cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980). CTAB methods combining with Polyvinylpyrrolidone (PVP) and salt solutions are the cheapest methods compared to commercial kits. Use of toxic chemicals in CTAB method raised a question on its competence. However, search for a cheaper but effective genomic DNA extraction protocols is still in progress.

Rice is the staple of Bangladesh and here rice security is equivalent to food security (Mamun et al., 2021). Bangladesh Rice Research Institute (BRRI) is the leading rice research institute in Bangladesh where stress tolerant breeding, QTL mapping, genome editing, new gene identification, pathogens detection, genome sequencing etc. are the examples of ongoing research works. Along with CTAB method, many other protocols are using in different laboratory of BRRI for the extraction of DNA from rice leaves. But yet, no comparative study has been done for the identification of which method is simple and best in terms of time, concentration, purity, DNA band visualization and cost expense per sample. Not only that, in context of Bangladesh, no comparative study has been found among different DNA extraction protocols of rice along with cost analysis. So, herein, we have aimed to decipher the best, time efficient and cost-effective protocols for DNA extraction from rice leaves which will be helpful for rice scientist as well as for other molecular researchers.

Materials and Methods

Plant sample collection and Crushing method

Rice leaves samples were collected from BRRI dhan71 at 21 days after transplanting. Leaves were taken from the tip portion of the young leaf and instantly preserve in icebox. After that, samples were kept in -20°C freeze for further use. Leaf crushing was done through both mixer mill (Retsch Mixer Mill MM 400) and mortar and pestle to compare the effect of two crushing method on DNA extraction.

Reagent preparation

Chemicals list of each DNA extraction method are given in Table 1. To extract DNA, different solutions are prepared as per the following description.

Table 1. Number and types of chemicals required for different methods

Chemicals	Protocol-1 (Extraction buffer, Miniprep)	Protocol-2 (K-acetate)	Protocol-3 (CTAB-1)	Protocol-4 (CTAB-2)
PVP	-	-	+	+
CTAB	-	-	+	+
EDTA (0.5M)	+	+	+	+
TRIS Base	+	+	+	+
SDS	+	+	-	-
Potassium Acetate (5 M)	-	+	-	-
NaCl	+	+	+	+
Chloroform	+	+	+	+
Isoamyl alcohol	+	+	+	+
Phenol	-	-	+	+
Sodium Bisulfite	+	-	-	-
Isopropanol	+	+	+	+
70% Ethanol	+	+	+	+
100% Ethanol	+	+	-	-
Total chemical numbers	10	10	10	10

Note. '+' sign indicates required; '-' sign indicates not required; PVP, polyvinylpyrrolidone; CTAB, cetyl trimethyl ammonium bromide; EDTA, Ethylenediaminetetraacetic acid; Tris base; tris(hydroxymethyl) aminomethane; SDS, Sodium Dodecyl Sulfate; NaCl, Sodium chloride;

Cetyltrimethylammonium bromide (CTAB) buffer preparation (100 mL)

For 100 mL CTAB buffer preparation, 10 mL 1M Tris base (pH-8.0) were taken in 250 mL conical flask followed by 4.0 mL 0.5 M EDTA (Ethylenediaminetetraacetic acid) (PH-8.0), 28.0 mL 5M NaCl, 1g PVP, 2.0g CTAB and added required amount of double distilled water to make the final volume 100 mL. Solution mixtures was dissolved by using hot plate magnetic stirrer.

Chloroform: Isoamyl alcohol: Phenol (24:1+5%) (CIP) solution preparation

Chloroform: Isoamyl alcohol: Phenol (24:1+5%) solution was prepared by taking 5 mL phenol in a 250 mL beaker followed by 91.2 mL chloroform, 3.8 mL isoamyl alcohol and required amount of distilled water to make the final volume 100 mL. Solvent was dissolved by using hot plate magnetic stirrer.

Tris -Borate-EDTA (TBE) buffer preparation (1000 mL)

TBE buffer was prepared by taking 108g Tris base in 1000 mL beaker followed by 55g boric acid, 9.3g EDTA and required amount of distilled water to make the volume 1000 mL (1 litre) using hot plate magnetic stirrer.

Ethanol solution (70%) preparation (100 mL)

To make 70% ethanol, 70 mL ethanol and 30 mL distilled water were mixed properly in a measuring cylinder.

DNA extraction methods

Use of mixer mill for DNA extraction (electrical method)

Method-1 (MiE1)

Method-1 was carried out by following the miniprep method of DNA extraction (Edwards et al., 1991) with slight adjustment. Concisely, 10 mg leaves were taken and added 300 µL DNA extraction buffer. Crushing was done through a mixer mill and placed at 65°C heat block for 10 minutes. Afterward, 300 µL Chloroform:Isoamyl alcohol (24:1) was mixed followed by two minutes invert shaking. After, centrifugation at 13000 rpm for 10 minutes, 200 µL supernatant was taken and added 400 µL ice cold 100% ethanol. After mixing, again centrifugation was done at 13000 rpm for 5 minutes. DNA pellet was precipitated at the bottom of the centrifuge tube. Then centrifugation was done at 13000 rpm for 3 minutes followed by adding 400 µL ice cold 70% ethanol. DNA pellet was dried at room temperature and added 100 µL Tris EDTA (TE) buffer for further use.

Method-2 (MiE2)

DNA was extracted according to description of Khan et al. (Khan et al., 2017); with slight modification. Shortly, 10 mg rice leaf sample was taken and crushed for 2 minutes through a mixer mill after adding 300 µL DNA extraction buffer. After that, crushing sample placed at 65°C heat block for 10 minutes. Potassium acetate (5 m) was added and after mixing well, incubation was done in ice for 5 minutes. A total of 100 µL supernatant was taken followed by centrifugation at 13000 rpm for 10 minute and added 100 µL isopropanol. After 15 minutes incubation, centrifugation was done at 13000rpm for 10 minutes and DNA pellet was found at the bottom of the tube. Then, 300 µL 70% ethanol was added and centrifuge was done at 13000 rpm for 3 minutes for washing purpose. After drying, 100 µL TE buffer was added and stored in -20°C for further use.

Method-3 (MiE3)

DNA was isolated according to the method of Ferdous et al. (Jannatul Ferdous, 2012); with minor modification. Briefly, around 10 mg rice leaves were grinded in a 2 mL tube followed by adding 300µL 2X CTAB (cetyltrimethylammonium bromide) solution. A mixer mill (Retsch MM400) was used for grinding for 2 minutes. After crushing, added 300µL chloroform: isoamyl alcohol: phenol (24:1:5%) in the crushing solutions and mixed by invert shaking. The solution mixtures were centrifuged for 10 minutes at 13000 rpm. Supernatant (200µL) was taken in a new 1.5 mL centrifuge tube and added 200µL ice cold Isopropanol. After 10 minutes incubation at room temperature, centrifugation was done at 13000 rpm for 10 minutes to precipitate the DNA pellet. Then discarded the solution and added 400 µL ice-cold 70% ethanol followed by centrifugation at 13000rpm for 3 minutes to wash the DNA pellet. DNA pellet was dried at room temperature and added 100 µL TE buffer for re-suspension and kept in -20°C for further use.

Method-4 (MiE4)

Method four is the modification of method-3 with purpose of time reduction. All procedure was maintained according to the description of method-3 except reduction of centrifuge time by 5 minutes with no incubation period for 10 minutes.

Use of mortar and pestle for extraction of DNA (manual method)

Above four methods were also tested by using mortar and pestle for crushing the leaves for DNA extraction. Others all steps were followed by instructions of method-1, method-2, method-3 and method-4. However, above four methods by using mortar and pestle designated as ME1, ME2, ME3 and ME4.

DNA purity measurement

Purity of the extracted DNA was performed through a Nanodrop spectrophotometer by calculating 260/280

and 260/230 absorbance ratio. A 260/280 ratio between 1.8 and 2.2 indicative of pure DNA, while a ratio below 1.8 indicates contamination by proteins and a ratio above 2.2 indicates phenol contamination. However, 260/230 was used as a secondary way to measure the nucleic acid purity. The 260/230 ratio of good quality DNA are generally ranged from 2.0-2.2. Ratio below 2.0-2.2 indicates the presence of chemical contaminates such as carbohydrate, phenol etc.

Cost analysis

Cost analysis was done based on the required chemical for each solution and enlisted price of each chemical by the supplying companies (Table 2). Cost of each chemical and hundred samples were calculated by unitary rules (general calculation) of mathematics. For instance, to isolate DNA of one sample 500 µL 70 % ethanol required for washing and so for 100 samples it requires 5000 µL or 50 mL 70% ethanol. In 50 mL 70% ethanol, 35 mL is pure ethanol and other 15 mL is water. In market 2500 mL molecular grade ethanol price is 10,000 taka and so 35 mL ethanol price is 140 taka. So, for 100 leaves DNA isolation, 140taka (1.4 taka per sample) ethanol requires to wash the DNA. By this way other chemicals price is calculated by considering molarity (M, solution concentration) and amount of chemicals required for each sample as well as for 100 leaves DNA isolation.

Table 2. Price list of chemicals

Sl. No.	Chemicals Name	Amount	Cost (in taka)
1.	Chloroform, Molecular Grade HPLC, Merck, Germany.	2.5L	5,000.00
2.	Ethanol, Molecular Grade HPLC, Merck, Germany	2.5L	10,000.00
3.	Isopropanol, Molecular Grade HPLC, Merck, Germany	2.5L	5,000.00
4.	Phenol, Merck, Germany	1kg	10,625.00
5.	Isoamyl Alcohol, Merck, Germany	2.5L	6,900.00
6.	Tris Base, Promega, USA	500g	7,500.00
7.	Sodium Chloride, Promega, USA	500g	3,958.00
8.	EDTA Disodium Salt, Promega, USA	500g	11,063.00
9.	PVP (Wako, Japan)	500g	10,000.00
10.	CTAB (Sigma)	500g	25,000.00
11.	SDS (Promega, USA)	500g	15,200.00
12.	Potassium Acetate (Wako Japan)	500g	4,000.00

Note. Cost was considered according price quotation of 2020 provided by Invent Corporation, Biotech Concern and Sigma company of Bangladesh. Here, PVP, polyvinylpyrrolidone; CTAB, cetyltrimethylammonium bromide; EDTA, Ethylenediaminetetraacetic acid.

Table 3. DNA concentration, purity, time and cost of four DNA methods

Treat.	DNA (ng/µL)	PU-1 (260/280)	PU-2 (260/230)	Time (Minute)	Cost/Sample (taka)	Cost/100 samples (taka)	Cost/100 samples (US dollar)
MiE1	266.61±21.34	2.09±0.01	1.91±0.07	51	1.02	101.81	1.20
MiE2	137.13±11.98	2.07±0.01	1.17±0.08	55	1.22	121.83	1.43
MiE3	260.58±14.76	2.08±0.01	1.99±0.07	36	3.56	356.39	4.19
MiE4	245.49±5.79	2.08±0.01	1.99±0.05	17	3.56	356.39	4.19
ME1	382.68±20.75	2.09±0.01	2.01±0.04	55	1.02	101.81	1.20
ME2	272.53±16.50	2.09±0.01	1.61±0.08	59	1.22	121.83	1.43
ME3	317.91±6.73	2.09±0.01	2.01±0.04	40	3.56	356.39	4.19
ME4	251.58±9.91	2.08±0.01	2.01±0.06	21	3.56	356.39	4.19

Note. Treat., Treatment; PU, Purity; 1 dollar=85 taka (as per dollar rate during 2021), as time and cost are equal for every replication, so SE of time and cost is 0, Mi; Mixer mill; M, Mortar and pestle; E1 (Miniprep extraction buffer), E2 (K-acetate based), E3 (CTAB-1), E4 (CTAB-2) indicates the four methods.

Polymerase chain reaction (PCR)

To conduct PCR, SSR primer RM336 (Forward-CTTACAGAGAAACGGCATCG, Reverse- GCTGGTTTGTTT CAGGTTCG) was used to visualize the DNA band of BRRI dhan71 (Hore et al., 2022). For single sample PCR reaction, 10 µL solution was prepared by mixing 3 µL DNA, 1 µL primers (0.5 µL of forward and reverse primer each), 5 µL G2 Go Taq Flexi DNA master mix (Promega, USA), 1 µL nuclease free water and the PCR was done by alpha PCR thermocycler machine. The PCR profile was as follows: pre-denaturation: 95°C for 5 minutes, 35 cycles of denaturation: 95°C for 1 min, annealing: 55°C for 30 sec, extension: 72°C for 30 sec and 7 min at 72°C for the final extension.

Gel preparation and gel electrophoresis

Agarose gel (2%) was prepared by adding 5.5 g agarose in 220 mL 1X TBE buffer followed by heating in micro-oven for 3 minutes. After gel preparation, 6 µL PCR product of each sample was loaded in each well of the gel and run the gel at 100 Volt for 100 minutes to complete the gel electrophoresis. After gel electrophoresis ethidium bromide was used to staining the gel for gel documentation through Biometra gel documentation system.

Statistical analysis

Statistical analysis for complete randomized design was performed through STAR software as well as through R software. ANOVA and mean separation test i.e., Least significant difference (LSD) test were done for parameters of respective method to find significant (p<0.005) difference among the methods. Cost analysis of 100 samples was performed on the basis of price quotation of chemicals supplied from the renowned company.

Results

DNA extraction methods performance by adopting mixer mill

Fig. 1a depicted the gel image of extracted DNA by using four methods and mixer mill. Fig. 2 and Table 3 depicted the DNA concentration, time and cost per 100 sample of different DNA extraction method by using mixer mill. DNA concentration are insignificant in the MiE1 (266.61 ng/µL**)**, MiE3 (260.58 ng/µL) and MiE4 (245.49 ng/µL) but these are all significantly differed from method MiE2 (137.13 ng/µL) (Fig. 2a). In case of times required for DNA extraction was significantly differed from each other. MiE2 (55 min) required highest amount of time followed by MiE1 (51 min), MiE3 (36 min) and MiE4 (17 min) (Fig. 2b). In term of cost, MiE3 and MiE4 required the highest amount of cost 356.39 taka for 100 samples extraction. Conversely, MiE1 required the lowest price 101.81 taka/100 samples where through MiE2 121.83 taka is required for 100 samples extraction. Moreover, in case of Purity-1 (260/280) the value was 2.09, 2.07, 2.08 and 2.08 for MiE1, MiE2, MiE3 and MiE4, respectively (Fig. 2b). There is no significant difference was found among the DNA extraction methods for Purity-1. For Purity-2 (260/230), no significant difference was found among MiE1 (1.91), MiE3 (1.99) and MiE4 (1.99) but they all are significantly varied from the Purity-2 value of MiE2 (1.17).

DNA extraction methods performance by adopting mortar and pestle

The gel image of extracted DNA by using four methods and mortar and pestle are illustrated in Figure 1b. Through mortar pestle procedure, the highest DNA concentration was found in ME1 (382.68 ng/µL) and the lowest was found in ME4 (251.58 ng/µL) (Fig. 2c). Moreover, 272.53 ng/µL and 317.91 ng/µL DNA concentration were found in ME2 and ME3, respectively. DNA concentration of ME1 significantly differed from other methods where no significant difference was found between ME2 and ME3 and ME2 and ME4. But significant difference was found between ME3 and ME4. In case of time, significant difference was found among the four methods. The highest times required in ME2 (59 min) followed by ME1 (55 min), ME3 (40 min) and ME4 (21 min) methods (Fig. 2c). In terms of cost, highest cost required for ME3 (356.39 taka/100 samples) and ME4 (356.39 taka/100 samples) methods followed by ME2 (121.83 taka/100 samples) and ME1 (101.81 taka/100 samples) (Fig. 2c). No significant difference was found between ME3 and ME4 but they both are significantly differed from ME1 and ME2. Moreover, significant difference was found between ME1 and ME2. In case of Purity-1 (260/280), 2.09 was found in ME1, ME2 and ME3 and 2.08 was found in ME4 (Figure 2d). No significance difference was found among the methods for PU-1. Moreover, in case of PU-2 (260/230), no significant difference was found among ME1 (2.01), ME3 (2.01) and ME4 (2.01) but they significantly differed from method ME2 (1.61).

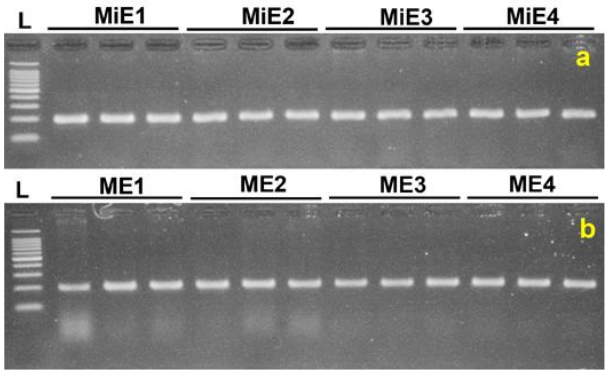

Figure 1. Gel image depicted the DNA band quality of the extracted DNA of four methods. Here, ME indicates that DNA was isolated by using Mortar and pestle and Mi indicates the use of Mixer mill for leaf crushing. E1 (Extraction buffer, Miniprep), E2 (K-acetate based), E3 (CTAB-1), E4 (CTAB-2) indicates the four methods. L means 100bp Ladder.

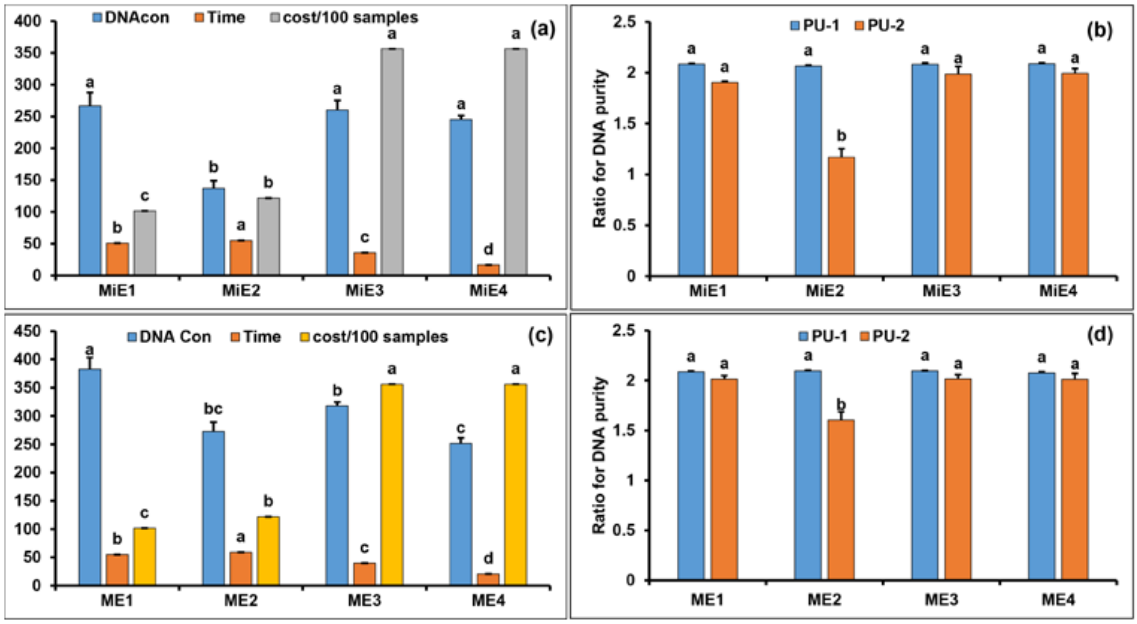

Figure 2. Quantitative comparison of DNA concentration, purity, cost and time requirement of four DNA extraction methods. Here, Mi; Mixer mill; M, Mortar and pestle; E1 (Extraction buffer, Miniprep), E2 (K-acetate based), E3 (CTAB-1), E4 (CTAB-2) indicates the four methods. L means 100bp Ladder.

Discussion

DNA extraction is the first step in the molecular characterization of a species or genes, and it is an essential step for ensuring the success of the downstream enzymatic reactions (Latif et al., 2022). Obtaining high-quality genetic material from a given DNA extraction method is essential for successful PCR amplification (Lucena-Aguilar et al., 2016). Samples must contain minimal amounts of impurities to prevent inhibition of the enzymatic reactions or interference with the gel migration patterns (Adamska et al., 2012).

DNA concentration was almost similar in the MiE1, MiE3 and MiE4 methods but compared to them, the lowest was found in MiE2 method. Moreover, PU-1 and PU-2 almost similar among the three methods except MiE2. Chemical composition of these four methods except method MiE3 and MiE4 are different which might be the cause of DNA concentration and purity variation among the methods. Moreover, the highest cost was needed for MiE3 and MiE4 followed by MiE2 and MiE1. Both ME1 and ME2 used same chemicals except Na and that's why they significantly differed from each other. In case of MiE3 and MiE4 methods, the highest cost required because of used of expensive chemicals CTAB, PVP and Phenol. But due to use of CTAB, PVP and Phenol, handling of supernatant is easy compared to other two methods. These chemicals specifically phenol, solidify the leaf debris at the bottom and tiny shake does not affect the solidity of the debris which make it easy to transfer the supernatant. In case of time, the highest time was required in MiE2 followed by MiE1, MiE3 and MiE4. MiE1 and MiE2 required highest amount of time because of ice and hot water incubation period increase the time requirement than the other methods. Moreover, MiE3 and MiE4 did not require such kind of incubation except a 10 minutes room temperature incubation and so these two methods are very much time efficient method for DNA extraction from rice leaves. Though the highest time required for MiE2 but the yield of DNA concentration is lower compared to others and it might be due to the chemical composition of that method. Conversely, the lowest time (17 min) was required for MiE4 method but DNA concentration almost similar to the MiE1 and MiE3 methods. Moreover, in case of mortal and pestle methods, four methods performance was almost similar to the methods of Mixer mill procedure. But through mortar and pestle 6 minutes required for each leaf crushing where through mixer mill method 2 minutes required for each sample. Not only that, through mixer mill method 20 leaves sample crushing required 2 minutes only but in case of mortal and pastel, 20 samples DNA extraction requires 120 minutes (2 hours).

Choosing an extraction method often involves a trade-off between cost (materials and labor), the optimal yield of DNA and the removal of substances that could interfere with the PCR reaction (Sánchez-Hernández and Gaytán-Oyarzún, 2010). A Clear understanding of the chemistry and function of different reagents and buffers helped researchers to construct alternative

methodologies for genomic DNA extraction from different sample sources. While designing the protocol for DNA extraction, the compositions of the reagents were determined based on the chemical effects of each reagent on various cellular organelles.

The Phenol-chloroform method that is usually used has advantages and disadvantages. DNA yield and purity are high in Phenol-chloroform method compared to other methods. The salting out methods for DNA isolation involves the addition of salts then precipitation of DNA from the protein in a subsequent step with isopropanol or ethanol and pelleting by spinning with a centrifuge with the supernatant removed. Usually, the salting out methods are laborious and time consuming but this method is safer than the phenol-chloroform method and cheaper than commercial kits (Carpi et al., 2011; Chacon-Cortes and Griffiths, 2014; Miller and Martin, 1988). In addition, the phenol-chloroform method is not safe due to the use of phenol and chloroform (toxic materials) to remove the proteins from DNA (Falcon, 1982).

The cetyltrimethylammonium bromide (CTAB) method is one of the most popular protocols for rice DNA isolation, including other plants, bacteria (Caccavo et al., 1994), fungi (Thuan et al., 2006) and animals (Shahjahan et al., 1995). A number of modifications have been made to the CTAB method to extract the DNA (Allen et al., 2006). Some methods have been reported to minimize the DNA extraction steps, but they need a large amount of plant tissue and liquid nitrogen (Tapia-Tussell et al., 2005). CTAB is a cationic surfactant that helps to lyse the cell membrane. It may also help to precipitate and remove all the unnecessary junk materials such as membrane debris, denatured proteins, polysaccharides etc. However, due to its positive charge, CTAB may form a complex with DNA and precipitate it. This is undesirable, and therefore NaCl is added, which provides Na^+ ions into the reaction. These Na^+ ions neutralize the negative charges on the phosphates of DNA by forming ionic bonds that otherwise would cause the DNA molecules to repel each other. Moreover, when present at a higher ionic strength, NaCl disturbs the formation of the CTAB- DNA complex and helps to keep the DNA in solution. NaCl together with CTAB is known to remove polysaccharides that interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases (Arif et al., 2010). However, the suitable NaCl concentration mentioned in literature varies between 0.02 M and 6M (Ghosh et al., 2009). β-mercaptoethanol and PVP are thought to forms complex hydrogen bonds with polyphenolic compounds and help their precipitation and separation from DNA by centrifugation (Wang and Stegemann, 2010). On the other hand, a low concentration of Tris Buffer would speed up the lysis process and help to maintain the pH of the buffer at a steady state. In the case of DNA extraction buffer, Tris buffer was used at a higher concentration. This was followed by the addition of EDTA to the extraction buffer, which binds divalent cations such as calcium and magnesium. These ions help to maintain the membrane integrity and their binding with EDTA destabilizes the membrane and helps the DNA to come out to the buffer solution.

Use of sodium dodecyl sulfate (SDS) along with the extraction buffer acted as strong anionic detergent that can solubilize the proteins and lipids of the membranes. These chemicals combination break the cell membranes and nuclear envelopes and expose the chromosomes that contain the DNA. In addition to removing the membrane barriers, SDS may also be useful in releasing the DNA from histones and other DNA binding proteins by denaturing them. During the SDS lyses phase, proteins and polysaccharides become trapped in large complexes that are coated with dodecyl sulfate. These complexes are precipitated when sodium ions are replaced by potassium ions (Dellaporta et al., 1983). Potassium acetate is generally used in laboratory routines. It can be used as a salt for ethanol precipitation of DNA and in molecular biology applications, potassium acetate precipitates SDS and SDS-bound proteins to allow their removal from DNA.

Chloroform: Isoamyl alcohol helps in binding and precipitation of protein and lipids of cell membrane. This step resulted in the formation of an aqueous phase containing DNA and a non- aqueous phase containing lipids and proteins. At this stage, DNA molecules are surrounded by water molecules forming the shell of hydration. Therefore, isopropanol is added at this stage as it may act as a dehydrating agent and disrupts the hydration shell resulting in precipitation of the DNA, which can then be separated, from the remaining soluble components through centrifugation.

Therefore, the higher the purity of the extracted samples, the better the results of the PCR produced. Choosing the best extraction protocol can be based on two parameters: (1) the integrity of the DNA when analyzed using an agarose gel; and (2) the 260/280 absorbance ratio as measured using spectrophotometry. A 260/280 ratio between 1.8 and 2.0 is indicative of pure DNA, while a ratio below 1.8 indicates contamination by proteins and a ratio above 2.0 indicates phenol contamination (Lucena-Aguilar et al., 2016). The 260/230 ratio of good quality DNA are generally ranged from 2.0-2.2. Ratio below 2.0-2.2 indicates the presence of chemical contaminates such as carbohydrate, phenol etc. (Lucena-Aguilar et al.,

2016). Impure DNA can cause false result and blur gel band after gel electrophoresis and gel documentation. So, quality of the DNA is essential for clear band as well as for DNA sequencing.

Conclusion

CTAB based methods are best for DNA extraction but have to remind in that these procedures are costly. Conversely, others two methods used in this study are cheaper compared to CTAB but these are time consuming. However, we are concluded that, four methods of our study are suitable for gene detection and among them CTAB is best for DNA isolation due to its less time requirement. Conversely, K-acetate or DNA extraction buffer-based methods are cheaper but time consuming. On the other hand, mixer mill-based DNA crushing and extraction is best for quick extraction of DNA compared to mortar and pestle. The lab which has sufficient budget for research and wants to harvest DNA from a large number of samples then they can use CTAB based methods of our study by using mixer mill for leaf crushing.

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