Effect of Weight and Incubation Time in DNA Quality of Kepel (Stelechocarpus burahol) Leaves

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Abstract. Kepel (Stelechocarpus burahol) is a rare fruit plant which becomes the identity plant of the Yogyakarta Region. Kepel plants need to be conserved so they don’t become extinct. Germplasm of kepel must be collected to get the genetic diversity of Kepel. Research on the genetic diversity of Kepel plants has never been done, so it needs the right method to obtain good quality DNA for molecular studies for future research. This study aims to obtain the best sample weight and incubation time to produce high-quality DNA for future research. This study used samples from young green leaves of kepel. DNA was extracted and purified from the leaves using a modified CTAB method to obtain DNA of kepel with high-purity and high-concentration. The result of DNA isolation and purification was analyzed by spectrophotometer and agarose gel electrophoresis method. The yield of DNA isolation on young kepel leaves had higher quality and quantity than DNA isolation on mature kepel leaves. The results demonstrated that DNA isolation from young green leaves of kepel had a purity level of 1.64-2.01. In addition, it also showed that DNA isolation from young green leaves in weight 0.2 g with 30 minutes incubation gave the highest DNA concentration (880 ng/µl).

Keywords: DNA isolation, kepel, cetyl trimethyl ammonium bromide, conservation

1. Introduction
Kepel (Stelechocarpus burahol) is a fruit plant that becomes one of the identical plants of the Special Region of Yogyakarta [1]. Kepel fruit plants resemble meaningful fists as a symbol of unity and mental and physical wholeness. Kepel plants are included in the List of Rare Plants because they are difficult to find [2-3]. The factors that cause kepel plants to become rare in DIY due to the establishment of opinion that this plant should only be planted around the palace, so there is a lack of people to cultivate this plant.

The problem with kepel plants is the lack of information and research about kepel plants, so there needs to be efforts to preserve the kepel plant. The genetic conservation measures that can be taken on kepel plants are through the study of genetic diversity, exploration, ex situ genetic conservation, characterization and evaluation [4]. Identification and characterization of kepel diversity vegetative
character of Kepel have conducted in Kulon Progo [5], however, the characterization information of kepel plant using molecular marker is very limited. DNA-based molecular markers are able to detect genetic diversity at a higher resolution level than morphological markers, although morphological markers are still needed for early identification.

Molecular analysis of plants depends on the number and purity of DNA samples [6]. Storage at certain temperatures aims to allow extracted DNA samples to be stored for weeks. Success in DNA isolation can be seen from the results of high quality and quantity of DNA. It can be attempted by optimizing DNA influenced by several factors including sample weight and incubation time [7].

To obtain optimality in DNA isolation, there are several factors that will affect the process, namely: (1) the isolation technique used, (2) the type of plant, (3) the type of explant, (4) the life of the explant, (5) the number/weight of the extracted explant, (6) the formulation of chemicals, and (7) the tools used. The explant age factor in the DNA isolation of the kepel plant is classified into mature leaves and young leaves. To establish the DNA concentrations of leaves samples, many methods with varied concept should be exploited [8].

DNA isolation using CTAB buffer (cethyltrimethyl ammonium bromide) has high accuracy because it is able to separate DNA from polysaccharides and polyphenol compounds (Handayani, 2008). Therefore, this research aims to obtain the best sample weight and incubation time to produce high-quality DNA for molecular analysis. The results of this study are expected to provide basic information as well as improve methods on DNA isolation of kepel genomes to obtain DNA with high purity so that it can be used for the biotechnology development of kepel plants.

2. Material and Methods

This research has been conducted at the In Vitro Culture Laboratory of the Faculty of Agriculture of Muhammadiyah University of Yogyakarta and the Integrated Research and Testing Laboratory of Gadjah Mada University in January – April 2020.

Samples used for DNA isolation include young leaves and kepel mature leaves (figure 1). Micalia material for isolation, purification, and DNA analysis, namely CTAB (cethyltrimethyl ammonium bromide), PVP (Polyvinylpyrrolidone), buffer Tris-Cl, EDTA, NaCl, Chlorophorus, Isopropanol, Ethanol 70%, Tris-Cl EDTA (TE), Liquid Nitrogen, Na-Acetate, β-merchaptoethanol, absolute Ethanol, and CI solutions, DNA Loading, Ethidium Bromide and marker λ styl. The CTAB extraction buffer solution used is the extraction buffer according to Orozco-Castillo methods [10].

Experiments conducted in the Laboratory include: DNA isolation of young and mature kepel leaves, DNA purification, spectrophotometer, and electrophoresis. Tried treatment i.e. the sample weight of young leaves and mature leaves as much as 0.1 g and 0.2 g with an incubation length of 30 minutes and 60 minutes. In total there are 8 units of treatment, and every treatment was repeated 3 times.

Figure 1. Young kepel leaf (a), and mature kepel leaf (b) used as samples in DNA isolation
2.1 DNA Isolation and Purification

The leaf sample is separated from the leaf bone and weighed 0.1 g and 0.2 g according to treatment, then the leaves are cut into small pieces, wrapped in aluminum foil and incorporated into liquid nitrogen. Extraction buffer solution is prepared, consisting of CTAB solution (2%) as much as 600 μL, PVP 100 μL and plus β-mercaptoethanol 1%. The extraction buffer solution was incubated at 65°C for 2 minutes before use. Mortars that have been cooled in -20°C for 12 hours are prepared, then the leaf samples are crushed in freeze conditions (using liquid nitrogen) until crushed into fine powder. The leaf powder is inserted in a tube of 1.5 mL then added well-mixed extraction buffer solution. The sample is incubated in water bath at 65°C for 30 minutes and 60 minutes according to treatment. The sample was centrifuge at 11,000 rpm for 10 minutes. The supernatant was taken and transferred to the new tube and added a CI (Chloroform: Isoamyl alcohol) (24:1) with a 1:1 ratio between supernatant and CI. Next it was mixed and centrifuge at 11,000 rpm for 10 minutes. This was repeated 2 to 3 times until a clear supernatant was obtained. Supernatant was taken and then added cold isopropanol (4°C) with a ratio of 1:1 and stored at a temperature of 4°C for 30 minutes. The result of further isolation is centrifuge at 11,000 rpm for 5 minutes. Discarded supernatant, the pellets added 100 μL TE buffers, 10 μL Na acetate, and 250 μL absolute ethanol. The sample was incubated at -20°C for 1 hour. After 1 hour of incubation, the sample was centrifuge at 12,000 rpm at 4°C for 10 minutes. The pellets obtained added 100 μL ethanol 70% and centrifuge at 10,000 rpm for 5 minutes. The acquired pellets are then added 50 μL TE solution. The isolated DNA is ready to be analyzed using spectrophotometers and electrophoresis.

2.2 DNA Isolation Results Analysis

Analysis of DNA isolation results is done quantitatively and qualitatively. Quantitative Analysis includes the purity and concentration of DNA. DNA concentration is measured using the Tecan Spark 20M spectrophotometer. DNA concentrations are calculated based on the results of optical density absorbance (OD) at wavelengths of 260. DNA purity is measured by calculating the comparison of absorbance values in wave of 260 nm and 280 (Å260/ Å280). Qualitative analysis of DNA is done by visually measuring the intensity of DNA molecules in gel electrophoresis. A total of 5 μL of each DNA sample is inserted into the well. Electrophoresis is run at a voltage of 80 volts for 25 minutes.

3. Result and Discussion

3.1 Quantitative Analysis

DNA isolation from kepel leaves in this study using a modified CTAB Orozco-Castillo extraction buffer solution [10]. Modifications made are by using liquid nitrogen during extraction with the addition of PVP and β-mercaptoethanol. The addition of the three ingredients for the modification of kepel leaf DNA isolation is due to preliminary research, in the absence of the addition of these substances, the resulting DNA is brown. In addition, the quality of the resulting DNA is very low (unpublished). Using PVP in the DNA isolation will increase the DNA purity and DNA concentration and also to suppress polyphenol oxidation [11]. The addition of liquid nitrogen also to prevent thawing during extraction and facilitate the grinding of samples of kepel leaves into powder [12]. The use of PVP and β-mercaptoethanol is intended to help to kepel leaf cells lysis that have a fairly high content of polysaccharides and polyphenols [13].

Based on table 1., it can be known that the purity of the DNA of the young kepel leaf sample ranges from 1.64 to 2.01, while the purity of DNA from mature leaves ranges from 0.06 to 0.78. DNA isolation results are pure when they have a purity value of 1.8 – 2. When the purity level is higher than 2, it indicates a high enough protein content, whereas if the purity value is less than 1.8 indicates that there is still a lot of RNA and polysaccharides [14]. The results of DNA isolation on young kepel leaves at the sample weight and different incubation times have a fairly high purity level
with an average purity of 1.80 to 1.97. Meanwhile, DNA isolation results in mature kepel leaves show very low purity, well below the set DNA purity value. Mature kepel leaves are dark green leaves, thick and rigid enough to inhibit the process of lysis or destruction of cell walls when DNA isolation is performed. In addition, mature kepel leaves have a very high content of phenolic, lignin, and polysaccharides (data published later). This causes the grinding of leaf samples to take longer and allows the compounds to oxidize and there is less thawing. In addition, the purity of DNA from RNA is also somewhat inhibited due to the difficulty of the extraction process due to leaves that are too hard to affect the purity of DNA [15].

Table 1. DNA purity levels result from isolation of young and mature kepel leaves in some sample weight and incubation temperature.

| Treatments | Young leaves of Kepel | Mature leaves of Kepel |
|------------|-----------------------|------------------------|
|            | Rep.*  | DNA Purity | Rate of DNA purity | Rep.  | DNA Purity | Rate of DNA purity |
| 0.1 g 30 min | 1  | 1.73       | 1.84                  | 1  | 0.10       | 0.34                  |
|            | 2  | 2.00       |                       | 2  | 0.44       | 0.78                  |
|            | 3  | 1.80       |                       | 3  | 0.48       |                       |
| 0.1 g 60 min | 1  | 1.83       |                       | 1  | 0.83       |                       |
|            | 2  | 1.95       | 1.80                  | 2  | 0          | 0.78                  |
|            | 3  | 1.64       |                       | 3  | 1.52       |                       |
| 0.2 g 30 min | 1  | 2.01       |                       | 1  | 0.83       |                       |
|            | 2  | 1.85       | 1.97                  | 2  | 0          | 0.06                  |
|            | 3  | 2.05       |                       | 3  | 0.09       |                       |
| 0.2 g 60 min | 1  | 1.91       |                       | 1  | 0          |                       |
|            | 2  | 2.01       | 1.95                  | 2  | 0          | 0.09                  |
|            | 3  | 1.94       |                       | 3  | 0.29       |                       |

*Rep: Repetition.

Table 2 shows the concentration of DNA resulting from the isolation of the DNA of young and mature kepel leaves using the modified CTAB Orozco-Castillo reaction buffer [10]. DNA isolated from samples of young kepel leaves showed a fairly high average concentration. In the sample treatment 0.2 g produced DNA concentrations ranging from 358.50 ng/μL to 880.00 ng/μL, while the treatment of samples weighing 0.1 g resulted in DNA concentrations ranging from 0 ng/μL to 73.00 ng/μL. This is likely because a larger number of samples will produce more DNA as well. The weight of the larger leaf samples means that the number of cells that undergo lysis also results in DNA being isolated as well. However, in DNA isolation using a tube of 1.5 mL does not mean that the more leaf samples used, the more DNA produced. In preliminary research using a sample of 0.3 g of young kepel leaves with three replays resulted in low DNA concentrations of 108 ng/μL, 182 ng/μL, and 201 ng/μL. Isolation of 0.3 g mature kepel leaves using micro tube 1.5 mL causes the sample inside the tube to be full enough, therefore the CTAB reaction buffer cannot lysis the entire cell and only part of the cells have lysis.

The result of DNA isolation in mature kepel leaves results in very low concentrations. The low concentration range in mature leaves is thought to be because mature leaves have thicker leaves resulting in difficulty breaking down cell walls in the extraction process. In addition, outside contamination during grinding and mature leaves have high levels of phenolic and polysaccharides. Other factors are also influenced by the addition of sample weight which will complicate buffer performance and result in low resulting DNA concentrations. The data in Table 1. and Table 2., it can be known that the DNA isolation of mature kepel leaves has not worked well, so for qualitative tests only carried out on DNA results in DNA isolation of young kepel leaves. DNA isolation using CTAB from leaves with high contains of polysaccharide and polyphenol compounds resulted low
concentration and purity of DNA [16].

Table 2. DNA concentrations resulting from isolation of young and mature kepel leaves in some sample weight and incubation temperature.

| Treatments       | Young leaves of KePel | Mature leaves of KePel |
|------------------|-----------------------|------------------------|
|                  | Rep. | DNA Concentration (ng/µL) | Rate of DNA Concentration | Rep. | DNA Concentration (ng/µL) | Rate of DNA Concentration |
| 0.1 g 30 min     | 1    | 35.00                       | 108.00                  | 1    | 51.50                       | 61.83                   |
|                  | 2    | 219.00                      |                         | 2    | 61.50                       |                         |
|                  | 3    | 70.00                       |                         | 3    | 72.50                       |                         |
| 0.1 g 60 min     | 1    | 36.00                       |                         | 1    | 17.00                       |                         |
|                  | 2    | 217.00                      | 98.30                   | 2    | 0                           | 10.33                   |
|                  | 3    | 42.00                       |                         | 3    | 14.02                       |                         |
| 0.2 g 30 min     | 1    | 880.00                      | 669.20                  | 1    | 8.00                        | 27                      |
|                  | 2    | 563.50                      |                         | 2    | 0                           |                         |
|                  | 3    | 564.00                      |                         | 3    | 73.00                       |                         |
| 0.2 g 60 min     | 1    | 358.50                      |                         | 1    | 0                           |                         |
|                  | 2    | 429.50                      | 437.70                  | 2    | 0                           | 0                      |
|                  | 3    | 525.00                      |                         | 3    | 0                           |                         |

*Rep: Repetitions

3.2 Qualitative Analysis

The analysis of the cauldron was carried out by analysis of DNA fragments using an electrophoresis method based on the intensity of staining DNA fragments with ethidium bromide on agarose gel 0.8% which can be observed using ultraviolet (UV) light. The results of the DNA isolation electrophoresis of young kepel leaves are listed in figure 2.

Figure 2. Electrophoresis DNA analysis results of young kepel leaves at various leaf weights and incubation temperatures.
Based on figure 2, it can be known that there are still a lot of polish under the DNA fragments especially in the C and G samples (2 g leaf samples for 30 minutes replays 1 and 2); sample D and H (leaf samples 2 g for 60 minutes replays 1 and 2); and sample I (leaf sample 1 g for 30 minutes replay 3). This suggests that based on electrophoresis, the resulting DNA is likely to be cut into small fragments, so when electrophoresis there is a lot of polish on the gel agarose. In the electrophoresis images it can also be known that there is still RNA in almost all samples, except samples A, B, F, and J. RNA contamination in this DNA isolation is shown by the high color intensity at the bottom of the gel agarose, although the DNA produced based on the spectrophotometer results has shown a high degree of purity. This is likely because this study did not use RNase, a chemical that serves to degrade the remaining RNA during DNA isolation.

Electrophoresis results in figure 2. It also showed that sample F (leaf sample 0.1 g for 60 minutes replay 2) and sample J (0.1 g leaf sample for 60 minutes replay 3) did not notice the absence of DNA tape. Based on the results of the DNA spectrophotometer produced in both samples, 217. 00 ng/μL and 42.00 ng/μL. It is likely that 0.1 g leaf samples are too little to produce genome DNA, to know if both samples (F and J) contain DNA, DNA amplification is necessary. Therefore, in the next study it is necessary to amplify DNA using PCR with primary random to prove whether there is actually DNA in both samples.

4. Conclusion
DNA isolation results from young kepel leaves produce better quality and quantity than DNA isolation from mature kepel leaves. The results demonstrated that DNA isolation from young leaves of kepel had an average of purity level of 1. 80 – 1.97. In addition, it also showed that DNA isolation from young leaves in weight of 0.2 g with 30 minutes incubation gave the highest DNA concentration (880 ng/μl).

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