

Full Length Research Paper

The effects of three different grinding methods in DNA extraction of cowpea (*Vigna unguiculata* L. Walp)

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Rapid DNA extraction is a prerequisite for molecular studies. Generally, plant tissue is ground in liquid nitrogen to isolate DNA; but, liquid nitrogen is dangerous and volatile. Besides, liquid nitrogen is not always available in many developing countries. To investigate if high quality DNA could be obtained for downstream PCR analysis without liquid nitrogen, the cowpea DNA was extracted by Hexadecyl trimethyl ammonium bromide cetyl trimethylammonium bromide (CTAB) method and sodium dodecyl sulphate (SDS) method, respectively, each with three different grinding methods, including ground in liquid nitrogen, in preheated mortar and in non-preheated mortar. The DNA was compared according to their yield, purity, integrity and functionality. The results showed that high quality DNA could be obtained by three grinding methods both in CTAB method and SDS method. Without liquid nitrogen, grinding plant tissue in preheated or non-preheated mortar with extraction buffer to extract DNA is feasible.

Key words: Cowpea (*Vigna unguiculata*), grinding method, liquid nitrogen, DNA extraction.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, Leguminosae ($2n = 2x = 22$), is an essential food crop in developing countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia, and Central and South America (Singh et al., 1997), with an annual production of more than five million metric tons world-wide (FAO, 2010). Cowpea is a most versatile African crop, it feeds people, their livestock and because of its ability in nitrogen fixation, it improves soil fertility, and consequently helps to increase the yields of cereal crops when grown in rotation and contributes to the sustainability of cropping systems (Agbicodo et al., 2009). As new tools that dramatically enhanced the efficiency of plant breeding, DNA based molecular markers have been widely used in cowpea genetic analysis (Zannou et al., 2008; Malviya et al., 2012; Badiane et al., 2012), genetic map construction (Muchero et al., 2009; Xu et al.,

2011; Ouedraogo et al., 2002), QTL analysis (Muchero et al., 2010; Andargie et al., 2011; Kongjaimun et al., 2012) etc.

Rapid DNA extraction with expectable quality is the prerequisite for molecular studies. If intact and high molecular weight DNA was not acquired, the PCR downstream analysis would face some problems. Generally, the procedure is to grind plant tissue in liquid nitrogen and transfer it to a preheated extraction buffer (Dellaporta et al., 1983; Saghai-Marooof et al., 1984).

The liquid nitrogen is used to freeze the plant tissue and blend it to powder, and minimize DNA degradation when grinding samples. However, liquid nitrogen is not only dangerous that needs particular attention when using it but also volatile, which will add the cost of experiment. What's more, continuous liquid nitrogen supply is a problem in many developing countries. If plant tissue could

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be ground in room temperature instead of in liquid nitrogen, then the DNA could be extracted in a shorter time at a lower cost, and molecular studies could be performed as usual even when liquid nitrogen is unavailable. Therefore, many researchers have reported some DNA isolation methods without liquid nitrogen. Ouenzar et al. (1998) eliminated the use of liquid nitrogen by grinding the 0°C precooled plant tissue in -20°C precooled mortar and pestle with precooled extraction buffer. Biswas et al. (2011) ground precooled finely chopped plant tissue and dried tissue in precooled mortar and pestle. Ferdous et al. (2012) ground Rice leaf and seed tissues with 600 µL extraction buffer by mortar and pestle in room temperature. Sharma et al. (2003) developed a protocol in which leaves were fixed in alcohol before CTAB DNA extraction, making liquid nitrogen unnecessary. Rajendrakumar et al. (2011) soaked the dehusked rice seed or grain in 600 µl extraction buffer for 30 to 40 min at 37°C in a sterile 1.5 ml microcentrifuge tube and ground the sample using a sterile micro pestle till the tissue disintegrates.

According to Doyle et al. (1987), plant DNA could be extracted by grinding 0.5 to 1.0 g fresh leaf tissue in 60°C CTAB isolation buffer in a preheated mortar. In this research, the effects of three different grinding methods (including ground in liquid nitrogen, in preheated mortar with extraction buffer and in non-preheated mortar with extraction buffer) applied in CTAB method described by Doyle et al. (1987) and SDS method described by Dellaporta et al. (1983), respectively, were compared. The aim was to see if high quality DNA could be acquired by directly grinding plant tissue in extraction buffer without liquid nitrogen.

MATERIALS AND METHODS

Plant material

The cowpea cultivar (Cheng-jiang 7), which belonged to *Vigna unguiculata ssp. sesquipedalis*, was provided by the Chengdu Academy of Agriculture and Forestry Sciences, China. Seeds were grown under greenhouse conditions and leaves were harvested from two-week-old seedlings for DNA isolation.

DNA extraction

DNA was extracted using CTAB method and SDS method, respectively. 100 mg of the leaf tissues were weighed in an electronic balance.

CTAB method

Firstly, the leaf tissue were ground into a fine powder in liquid nitrogen by a pestle and mortar, transferred into a 1.5 ml microfuge tube, 500 µl CTAB extraction buffer was added (100 mM Tris-HCl, pH 8.0; 1.4 mM NaCl; 20 mM EDTA; 2% CTAB; 0.2% 2-mercaptoethanol), and incubated in 60°C for 30 min. Secondly, the leaf tissue was put in 60°C preheated mortar and pestle, 500 µl.

CTAB extraction buffer was added, and ground for 30 s, transferred into a 1.5 ml microfuge tube, and incubated in 60°C for 30 min.

Thirdly, the leaf tissue was ground in non-preheated mortar and pestle (room temperature) with 500 µl CTAB extraction buffer for 30 s, transferred into a 1.5 ml microfuge tube, and incubated in 60°C for 30 min. The next steps were completely followed by Doyle et al. (1987). Each grinding method was replicated ten times.

SDS method

The grinding methods were the same as for CTAB method described above, but with 500 µl SDS extraction buffer (100 mM Tris, pH 8; 50 mM EDTA, pH 8.0; 500 mM NaCl, 10 mM mercaptoethanol). After the grinding, the mixture was transferred into a 1.5 ml microfuge tube, 50 µl 20% SDS was added and incubated in 65°C for 10 min. The next steps were completely followed by Dellaporta et al. (1983). Each grinding method was replicated ten times.

RNAse treatment

After DNA was dissolved in 100 µl TE buffer (10 mM Tris-cl pH 7.4, 1 mM EDTA, pH 8.0), RNAse treatment was carried out according to the method of Doyle et al. (1987).

DNA concentration and purity

The concentration of the extracted DNA was determined spectrophotometrically with an Eppendorf Biophotometer. 5 µl of the DNA was diluted 1:10 in Tris-EDTA buffer (pH 8.0). The DNA concentration and OD260/280 ratio were given by the machine. OD260/280 readings ratio were taken as an indicator of DNA purity (Sambrook et al., 1989).

Restriction analysis

DNA was restricted by 15 units of Hind III using approximately 1 µg of DNA. The reaction mixture was incubated at 37°C for 2 h. Afterwards, undigested and digested DNA were separated on 0.8% agarose gel at 90V for 100 min, then stained with ethidium bromide for 20 min, and photographed in SYNGENE-GeneGenius.

PCR amplification

Functionality of DNA extractions were tested with two types of PCR. First, a 195 bp sequence containing SSR derived from EST sequence of *V. unguiculata* (downloaded from NCBI EST database, <http://www.ncbi.nlm.nih.gov/nucest>) was amplified. Primers were designed by Primer3 software with Forward sequence: 5'-CCTTGAAGGCTTTGCAACC-3', reverse sequence: 5'-ATTCTCTGCCTGCTCCATG-3'. The primers were synthesized by Life Technologies (AB & Invitrogen). PCR (50 µl volumes) contained approximately 100 ng of genomic DNA, 0.25 µl Taq (5U/µl, Fermentas), 5 µl 10× PCR reaction buffer, 3 µl MgCl₂ (25 mM), 4 µl dNTP mixture (2.5 mM), 1 µl each primer (20 µM), and dH₂O up to 50 µl. The amplification was performed in a Peltier Thermal Cycler (BIO-RAD DNAEngine). Cycling conditions consisted of 5 min initial denaturation at 95°C, followed by 1 min denaturing at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C repeated for 40 cycles and 5 min extension at 72°C. PCR products were subsequently separated with 2% agarose gel electrophoresis at 80 V for 90 min, then stained and photographed as described above.

Secondly, a 773 bp fragment from the chloroplast gene for the photosystem II protein D1 (psbA) of *V. unguiculata* was also amplified. Primers were designed from the psbA photosystem II protein D1 sequence of *V. unguiculata* taken from Genbank database (GeneID:

Table 1. DNA yields and OD 260/280 ratios obtained from different grinding methods in CTAB and SDS methods.

Extraction method	Grinding method	Yield (ug)	OD260/280
CTAB	Liquid nitrogen	4.70±0.13	1.80±0.03
	Room temp mortar	11.53±1.28	1.83±0.04
	Preheat mortar	13.80±1.13	1.84±0.02
SDS	Liquid nitrogen	4.20±0.20	1.77±0.02
	Room temp mortar	14.17±3.03	1.76±0.06
	Preheat mortar	14.86±3.97	1.73±0.03

Values are mean ± SE.

13080510, <http://www.ncbi.nlm.nih.gov/gene/13080510>) using Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>): forward primer PD1_F with sequence 5'-GGCCAAGCAGCTAGGAAGAA-3', reverse primer PD1_R with sequence 5'-ACCAGCACCGAAAATCGTCT-3'. The primers were synthesized by Life Technologies (AB & Invitrogen). PCR (50 ul volumes) contained the same mixture and was performed with the same PCR machine as used for SSR amplification using the same settings. PCR products were subjected to 1.5% agarose gel electrophoresis at 75 V for 80 min, then stained and photographed as described above.

Data analysis

The data analysis was followed by Chen et al. (2010). The general linear model (GLM) was applied to test the effects of grinding methods and extraction methods on the DNA yield and on the OD260/280 ratio. Tukey's pairwise comparisons with the confidence interval of 95% were used to compare the yield or the ratios between the grinding methods and extraction methods. The statistical analyses were accomplished by using the MINITAB® software Release 14.20 (www.minitab.com).

RESULTS

Yield of the DNA extracted by the CTAB method and SDS method with three different grinding methods are listed in Table 1. With the same grinding method, for example, ground in liquid nitrogen, the DNA yield had no significant difference between CTAB and SDS extraction methods ($F = 4.26$, $df = 1$, $P = 0.066$). The same results were obtained with preheated mortar ($F = 0.30$, $df = 1$, $P = 0.595$) and non-preheated mortar ($F = 1.40$, $df = 1$, $P = 0.264$).

In CTAB method, the DNA yield had no significant difference between grinding the leaf tissue in preheated mortar and non-preheated mortar with 500 ul extraction buffer; both of which obtained significantly higher DNA yield than grinding in liquid nitrogen (Turkey's, 95%). In SDS method, the results were the same.

The assessment of the purity of a nucleic acid sample is often performed by a procedure commonly referred to as the OD260/280 ratio. A pure sample of DNA has the ratio at 1.8 (Chen et al., 2010). The mean OD260/280 ratios of DNA isolated by the three different grinding methods with both CATB and SDS methods had no significant

difference (Turkey's, 95%). They were all close to 1.8, which indicated that all the DNA extractions were pure. This was further proved by agarose gel electrophoresis (Figure 1).

The integrity, that is presence of high molecular genomic DNA, was determined by electrophoresis on a 0.8% agarose gel. High molecular DNA bands with no smear were obtained from both CTAB method and SDS method by ground in liquid nitrogen (Figure 1; lanes 1 and 7), indicating that the DNA were intact and pure while DNA isolated by grinding the plant tissue in preheated and non-preheated mortar with extraction buffer in both CTAB and SDS methods showed high molecular DNA bands with little smear (Figure 1; lanes 3, 5, 9 and 11), demonstrating that the DNA were intact but there existed some degraded DNA.

To test the DNA function, the DNA was digested with restriction enzyme Hind III, and amplified in two PCR reactions. As shown in Figure 1, the DNA was completely digested with Hind III restriction enzyme, as evidenced by the characteristic "smearing" and the absence of the high molecular weight bands seen in the adjacent lane of undigested DNA.

Besides, the SSR (Figure 2) and the target fragment from the psbA photosystem II protein D1 sequence of *V. unguiculata* were successfully amplified with respect to all the DNA extractions (Figure 3).

DISCUSSION

From the data in Table 1 and agarose gel in Figure 1, DNA isolated by grinding plant tissue in liquid nitrogen was intact and pure while the DNA extracted by grinding leaf tissue in preheated and non-preheated mortar with extraction buffer degraded a little. However, as the DNA yield of ground in preheated and non-preheated mortar were twice as many as ground in liquid nitrogen, there exists considerable intact and high quality DNA which could be used in downstream PCR analysis. When grinding plant tissue in preheated or non-preheated mortar and pestle, the CATB or SDS extraction buffer could provide some protection to the plant tissue, and this is theoretically feasible. Tris maintains the pH of the solution

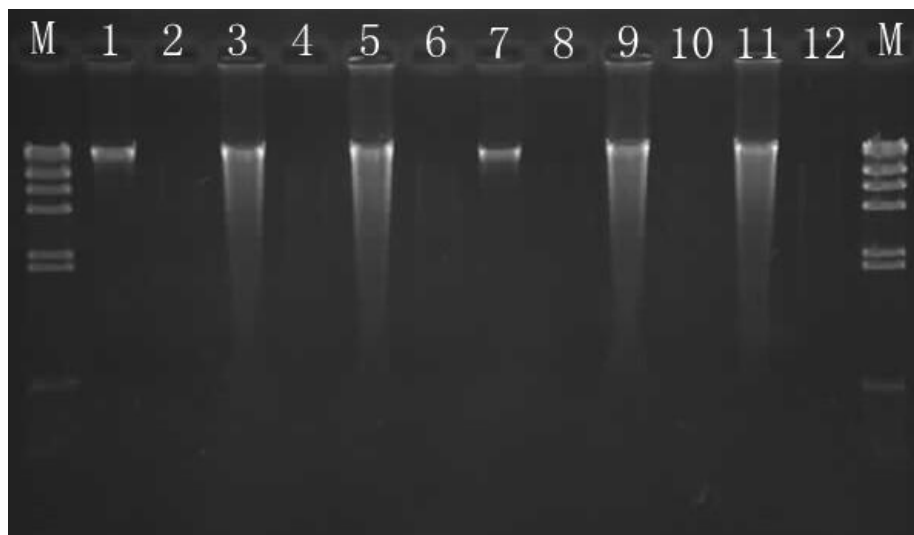


Figure 1. Agarose gel of undigested and digested cowpea DNA. The isolated DNA was digested by the restriction enzyme Hind III. Lanes designated (M) are lambda/Hind III molecular weight markers (Fermentas). Lanes 1-6, the DNA isolated by CTAB method in the order ground in liquid nitrogen, non-preheated mortar with extraction buffer, preheated mortar with extraction buffer; lanes 7-12, the DNA isolated by SDS method in the same grinding order as described above, alternating undigested and digested DNA.

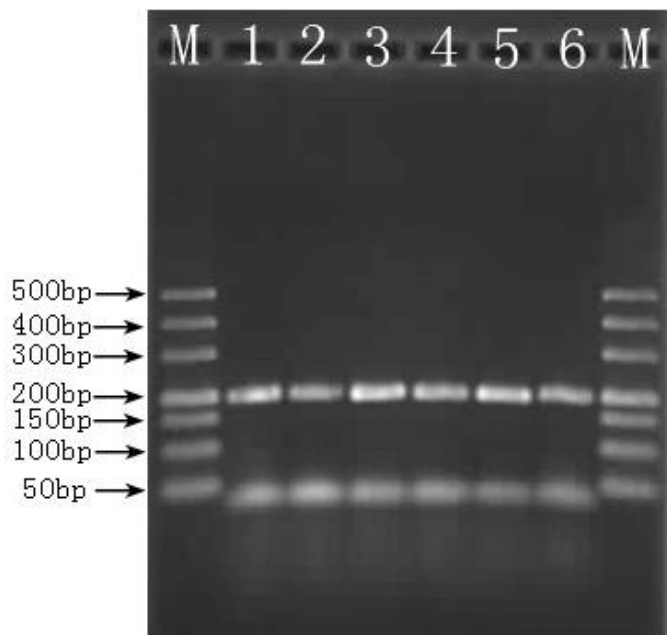


Figure 2. PCR amplification of the SSR from the cowpea DNA. Lanes marked (M) are 500 bp molecular weight markers (Fermentas). Lanes 1-6 are the SSR amplified from DNA isolated using CTAB + liquid nitrogen, CTAB + non-preheated mortar, CTAB + preheated mortar, SDS + liquid nitrogen, SDS + non-preheated mortar, SDS +preheated mortar, respectively.

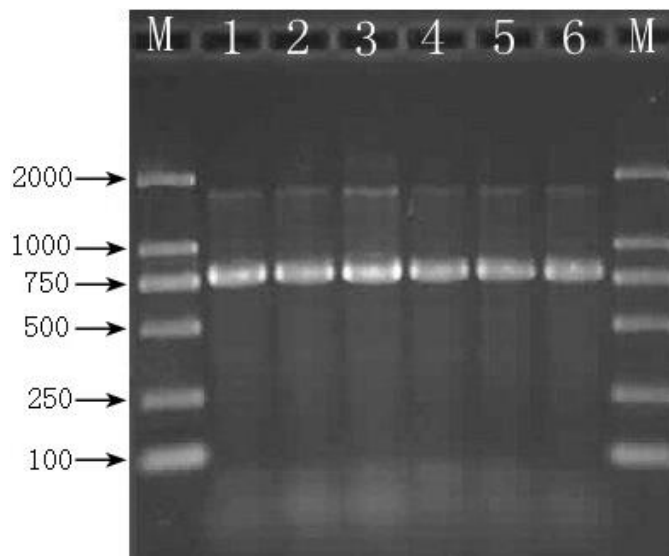


Figure 3. PCR amplification of the partial *psbA* gene from cowpea DNA. Lanes marked (M) are 2000 bp molecular weight markers (Fermentas). Lanes 1-6 are the *psbA* gene fragment amplified from DNA isolated using CTAB + liquid nitrogen, CTAB + non-preheated mortar, CTAB + preheated mortar, SDS + liquid nitrogen, SDS + non-preheated mortar, SDS + preheated mortar, respectively.

and interacts with the lipopolysaccharides present on the outer membrane which helps to permeabilize the membrane. NaCl provides Na⁺ ions that will block negative

charge from phosphates on DNA, thus contribute to the precipitation of DNA. EDTA binds with Mg-ion and nullifies the action of DNase. CTAB could facilitate in the lysis of cells so DNA can be released into the bulk of the solution. SDS is a strong anionic detergent that can solubilize

the proteins and lipids that form the membranes. The extraction buffer was essential. The authors have tried grinding plant tissue in preheated or non-preheated mortar and pestle without any buffer or liquid nitrogen, as a result, negligible DNA was obtained. The reason maybe DNA was cut by the DNase or destroyed by the oxidized polyphenol, but when grinding plant tissue in mortar with extraction buffer, no matter what extraction protocol to choose, CTAB or SDS, there always exists some degraded DNA. This was consistent with the study of Rajendrakumar et al. (2011), who observed that grinding of seed/grain in extraction buffer results in distinct DNA degradation. Maybe during the grinding process, few plant tissue was exposed to the air and polyphenol was oxidized to quinone, or the extraction buffer was too less to cover the entire plant tissue, which means that the EDTA was not able to inhibit the activity of DNase. Although some modified methods obtained high quality genomic DNA without liquid nitrogen (Ouenzar et al., 1998; Biswas et al., 2011; Sharma et al., 2003), they added some other steps, spent more time or money. According to Doyle et al. (1987) and Zheng et al. (1995), fresh leaf tissue can be ground directly in extraction buffer. In this research, complete digestion with restriction endonuclease and successful amplification in PCR indicated that all the cowpea DNA extractions were all successful, which proved that grinding leaf tissue in mortar with extraction buffer to isolate DNA from cowpea is practicable. The mortar and pestle could be preheated or not. Add preheated extraction buffer to the mortar and grind plant tissue directly are able to obtain high quality DNA from cowpea which could be used in molecular analysis.

In this research, only cowpea was studied. Whether the grinding method can be successfully applied in other plant species or not needs to be confirmed by further experiment. It may not be able to obtain ideal results in some species. This may be due to the differences in levels of polysaccharides, fibers and associated anti-quality factors in these plant species (Sharma et al., 2010).

Conclusion

This research proved that grinding cowpea leaf tissue in preheated mortar with extraction buffer was able to obtain high quality DNA which was suitable for molecular study, thus eliminating the use of liquid nitrogen. In addition, after comparative analysis, grinding in non-preheated (room temperature) mortar was also practicable. This grinding method could be tried in other plant species, but whether it will work or not requires confirmation by further experiment.

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