Comparison of Three DNA Extraction Methods from the Leaves of *Capsicum annuum* L. (Nga-yoke)

Yoon Myat Noe[1], Swe Swe Aye[2]

Abstract

This study is focused on comparison of plant genomic DNA extraction from *Capsicum annuum L.* The molecular technique is another highly reliable alternative for plant species identification besides phytochemical profiling. The DNA were extracted from the tender leaves of *C. annuum* L. by using three different methods such as CTAB, Dellaporta and SDS methods to determine the most cost-effective and time efficient method to extract DNA. Then, the quality of extracted genomic DNA was amplified Polymerase Chain Reaction (PCR) and checked by gel electrophoresis and NanoDrop spectrophotometer.The result of NanoDrop spectrophotometer revealed that DNA from modified CTAB method yielded 841.7 ng/µL concentration with A_{260}/A_{280} ratio of 1.99 and with A_{260}/A_{230} ratio of 1.34. That isolated DNA has no protein although a little others impurities contaminations. The gel electrophoresis of modified CTAB method gave the intact DNA band and PCR had the expected band (~500 bp). Among three extraction methods, the CTAB method with little modifications was superior in the amount and purity of DNA compared to the Dellaporta and SDS methods.

Keywords: DNA, PCR, NanoDrop spectrophotometer, CTAB, *Capiscum annuum* L.

Introduction

DNA extraction is the first fundamental step for many molecular biology applications. Grooms (2015) reported that usable DNA from plant materials is difficult to extract for many reasons including the fibrous, polysaccharides and polyphenols. Moeller *et al*. (2014) highlighted that these compounds can interfere with DNA extraction and downstream processes such as Polymerase Chain Reaction (PCR), sequencing, cloning, etc. Numerous DNA extraction protocols use phenol to separate cellular molecules and debris from the DNA which is toxic, hazardous, expensive, and requires special containment facilities. DNA extraction from young leaves is easier than from mature leaves. Sahu *et al*. (2012) highlighted that the mature leaves have high levels of polysaccharides, polyphenols, lipids, and other secondary metabolites so that to detergent these substances requires a long time. There are many genomic DNA extraction methods such as Sodium Dodecyl Sulfate (SDS), Cetyltrimethylammonium Bromide (CTAB) and the use of extraction kits (www. https://international.neb.com).

The quality and concentration of extracted genomic DNA can be determined by using the NanoDrop spectrophotometer, gel electrophoresis and PCR. Nga-yoke belongs to the family Solanaceae and sub-family Solanoideae, popularly known as sweet pepper, bell pepper. Capsicum is a high value solanaceous vegetable crop grown extensively in Yangon, Bago, Magway and Sagaing Regions in Myanmar. Therefore, the present investigation was to establish an efficient extraction method using inexpensive and un-harmful chemicals, to safely and reduce the time-consuming protocol for DNA extraction from plant parts, to obtain good quality DNA in a relatively purified form which can be used for further investigations.

Materials and Methods

Collection of Samples

Capsicum annuum L. is one of the Capsicum species used for all three different genomic DNA extraction protocols. The samples were collected from the Vegetable and Fruit Research and Development Center, Hlegu Township, Yangon Division.

[1] Demonstrator, Department of Botany, University of Yangon
[2] Associate Professor, Department of Botany, Taunggoke University

Extraction Methods

The three genomic DNA Extraction methods were the modified CTAB method (Roomi *et al*., 2013), Dellaporta method (Doyle and Doyle, 1987), SDS method (Akaneme *et al*., 2014) which were slightly modified and used.

Extraction of genomic DNA

Apparatus

1.5 ml microcentrifuge tube, Pipette, Mortar and pestle, Vortex, Refrigerated Centrifuge, Microcentrifuge, Beaker, Water bath, Freezer and Refrigerator.

Required chemicals

SDS, NaCl, Tris, EDTA, KOAc, PVP, Sodium bi-sulphite.

Modified CTAB method (Roomi *et al*., 2013)

Reagents and solutions

CTAB extraction buffer: (0.1 M Tris-Cl (pH 9.5), 20 mM EDTA (pH 8),1.4 M NaCl, 2% CTAB), pure cold (-20°C) isopropanol, Cold 75% ethanol, Phenol: Chloroform: isoamyl alcohol (25:24:1, v/v), RNase A, TE buffer (10 mM Tris,1 mM EDTA; pH 8.0).

Procedure

The sample 0.13 g of leaves stored at -20°C were ground using a pestle and mortar with the addition of 500 µL of DNA extraction buffer and added powder of PVP. The mixture was transferred into the microcentrifuge tube and vortexed for 60s. The tube was incubated in a water bath at 65°C for 20 mins for cell lysis. The tube was cooled for 5 mins at a room temperature. After adding 400 µL phenol: chloroform: isoamyl alcohol (25:24:1) solution, it was gently shaken by hand and centrifuged at 12,000 rpm for 5 mins at 27°C. The supernatant was transferred to a new tube and 500 µL of cold isopropanol was added and inverted 5-10 times. The tube was kept at -20˚C for 20 min and centrifuged at 12,000 rpm at 8°C for 5 mins. The pellet was seen on the bottom of the tube. After removing the supernatant, the pellet was washed with 500 µL of 75% ethanol. The tube was inverted 5-10 times and then centrifuged at 12,000 rpm at 8°C for 5 mins and followed by ethanol. DNA pellet was air-dried for about 5-10 mins (The pellet dried for a long time causing it to not completely dissolve the pellet in TE buffer) and was dissolved in 50-100 µLTE buffer and 0.5 µL RNase A was added, and it was kept in the tube at 37˚C for 1 hr. Finally, the DNA sample was stored at -20°C.

Dellaporta method (Doyle and Doyle, 1987)

Reagents and solutions

Extraction buffer: 1.25 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, 2% CTAB, β-mercaptoethanol, Chloroform, 70% Ethanol, Cold isopropanol, RNase A (10 mg/ml), TE buffer: 10 mM Tris -HCl, 1 mM EDTA.

Procedure

The samples were stored at -20°C and ground with the help of mortar and pestle by adding 300 µL CTAB extraction buffer with 0.2% β- mercaptoethanol. The homogenate was transferred to a microcentrifuge tube and incubated in water bath at 60°C for 40 mins with occasional and proper mixing after every 5-10 mins, not to form any clumps at the bottom. The tube was removed from the water bath and then cooled at room temperature. An equal volume of chloroform was added to the tube and mixed thoroughly by gentle inversion for 5-10 times and incubated for 5 mins at a room temperature. After that the tube was centrifuged at 12,000

rpm for 12 mins at 4˚C temperature and to attain clear separation of three layers. The clear aqueous phase (supernatant) was carefully pipetted out into a new tube. The chloroform treatment was repeated twice to remove the organic contaminants in the supernatant. The amount of 500 µL of cold isopropanol was added to the tube and mixed gently by inversion and centrifuged at 12,000 rpm for 12 mins at 4˚C temperature to pellet out DNA. The supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 5 mins at 4˚C and repeated twice with ethanol. The tube was allowed to air dry for about 5-10 mins until the pellet was completely dried. The pellet was dissolved in 50-100 µL TE buffer and 0.5 µL of RNase A was added and then incubated at 37°C for 1 hr. Finally, the DNA sample was stored at -20˚C for further use.

SDS method (Akaneme *et al*., 2014)

Reagents and solutions

CTAB extraction buffer: (10 mM Tris-HCl (pH 9.5), 2 mM EDTA (pH 8),150 mM NaCl, 0.5% SDS), Chloroform: isoamyl alcohol (24:1, v/v), cold absolute ethanol, RNase A, TE buffer.

Procedure

The sample was ground with 200 µL of extraction buffer. The homogenates were transferred into the new tube. The tube was incubated in a water bath at 65°C for 35 min and inverted the tube once after 15 min for cell lysis. The tube was cooled on ice for about 51 mins. The tube was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was transferred to a new tube. And amount of 40 µL of Chloroform: isoamyl alcohol (24:1) was added to the tube and inverted gently. (Care was taken not to mix two liquids. The denatured protein formed a white layer on top of the chloroform: the isoamyl layer. The tube was incubated for about 10 mins at a room temperature. The supernatant was transferred into new tube. The chloroform: isoamyl alcohol (24:1) treatment was repeated twice. The tube containing supernatant was cool on ice for 10 mins and 400 µL of cold absolute ethanol was added into the tube (Slowly pour down side of the tube). The tube was inverted by hand. A few minutes later, a white stringy precipitate was formed on top of the ethanol. The tube was centrifuged at 12,000 rpm for 8 mins at 4°C and repeated with ethanol. The supernatant was discarded and the pellet was air-dried. The pellet was suspended in 50-100 µL of TE buffer and added 0.5 µL of RNase A into the tube and then incubated 1hr at 37°C. Finally, the DNA pellet was incubated at -20°C for further use.

Analysis of DNA using NanoDrop spectrophotometer

Thermo ScientificTM NanoDropTM One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) was used to access quality and quantity of extracted DNA. The amount of 1µL of DNA sample from extraction was used to measure the nucleic acid concentration (Quantity) and DNA quality by means of measuring the absorbance ratio of A260/A280 for protein contamination and that of A260/A230 for other impurities involved in the sample. For DNA, the range of A260/A280 must be between 1.8- 2.0 and that of A260/A230 must be greater than 2.

Preparation for Polymerase Chain Reaction (PCR)

EmeraldAmp® Max PCR Master Mix was used to isolate part of the psbA-trnH gene (~400 bp) for amplification. The sample of DNA from extraction was diluted to 5 ng/ µL. The PCR buffer was prepared by adding 9.5 µL of dH2O, 1 µL of each forward primer (psbA-F (5' to 3'): GTTATGCATGAACGTAATGCTC) and reverse primer (trnH-R (3' to 5'): CGCGCATGGTGGATTCACAATCC) and 12.5 µL of enzyme mixture. The amount of 24 µL

of this PCR buffer was needed for 1 µL of DNA Template for PCR amplification. As a PCR thermal cycler, a TechneTM PCRmax Alpha Cycler 1 PCR Machine (PCR max, UK) was used. PCR condition must be at 95°C for 5 minutes for initial temperature, at 95°C for 30s for denaturing, at 55°C for 30s for annealing Temperature, at 72°C for 40s for extension, at 72°C for 10 minutes for final extension and for hold (10°C for infinity). The reaction was repeatedly cycled for 35 times. The psbA (forward primer) and trnH (reversed primer) were intergenic spacer regions and also universal primers (~400bp). These primers possess conserved flanking sites, short sequences and discriminate between species. So, it was suitable for running of PCR. (Kress and Erickson, 2012)

Preparation of TAE Buffer for Gel Electrophoresis

Firstly, a 50X concentration of TAE stock solution was made. For this, 242 g of Tris was dissolved in 500 mL of distilled water. The amount of 18.61 g of EDTA (pH- 8) was weighed and dissolved in 100 mL of distilled water and stirred with a magnetic stirrer and then added some NaOH up to pH- 8. These two solutions were combined and 57.1 mL of acetic acid was added to it. Then distilled water was added to bring the final volume to 1 L. To make 1X TAE buffer, 20 mL of stock solution was diluted with 980 mL of distilled water. To make 0.5X TAE buffer 10 mL of stock solution was diluted with 990 mL of distilled water.

Preparation for Gel electrophoresis

Amount 0.17 g of agarose gel (1% agarose) for a small casting tray was weighed and put in a 200 mL beaker and then 17 mL of TAE buffer was added and then the dissolved in the microwave for about 5 min until the agarose powder completely dissolved. After that, 0.6 µL of SYBR Safe DNA gel stain (Thermo Fisher Scientific, USA) was added and slowly shook the beaker. Then the gel solution was poured into the gel tray and the comb was put and then waited for about 30 min. The comb was then removed and the prepared gel tray was placed on the gel bed of the migration tank containing the 1X TAE buffer. For genomic DNA, 2.5 µL of DNA sample with 1 µL of loading dye was loaded into the wells for protocols. For PCR products, each 2.5 µL of PCR products was loaded into the well of an agarose gel containing SYBR stain. Then, the 100bp marker (Thermo Fisher Scientific, USA) was placed into the well for comparing with the size of the samples and run for 15 min at 100 voltages. And then, the gel was placed in the Omni Doc Gel Documentation System which is already connected with the computer. The exposure was adjusted to 3 and switched to UV light then took the photo of the gel.

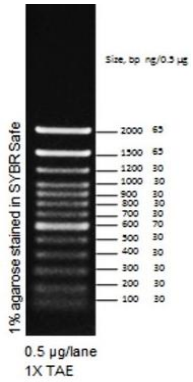

Figure 1. 100 bp ladder (Invitrogen, Thermo Fisher Scientific)

Results

The species *Capsicum annuum* (cv) was collected from the Vegetable and Fruit Research and Development Center, Hlegu Township, Yangon Division during the flowering period of June - August, 2019.

Outstanding Characters of *C. annuum* (cv)

Annual or perennial herbs; pubescence of simple hairs. Stems branched. Leaves solitary or paired, petiolate; leaf blade simple, entire or sinuate. Inflorescences solitary; peduncle absent. Flowers nodding or erect, actinomorphic. Pedicle erect or nodding. Calyx broadly campanulate to cup-shaped, denticulate, sometimes slightly enlarged. Corolla white, campanulate or rotate, divided halfway or more. Stamens inserted near the distal end of the corolla tube; filaments slender; anthers yellow or purplish, ovoid, and dehisce longitudinally. Ovary 2- (-3)- locular; ovules numerous. Style slender; stigma small, capitate. Fruit is a moist berry, sometimes large, erect, nodding or reflexed. Seeds yellowish, discoid; embryo coiled, subperipheral.

Figure 3. Habit of *Capsicum annuum* (cv)

Genomic DNA

i. Quality and Quantity Assessment Using NanoDrop Spectrophotometer

The genomic DNA of tender leaves of *C. annuum* L. was extracted by using three different methods in plant analytical laboratory. The quality and quantity assessment of extracted DNA was evaluated by the NanoDrop spectrophotometer, gel electrophoresis and PCR.

According to the result of the NanoDrop spectrophotometer, DNA from the modified CTAB method yielded an 841.7 ng/µL concentration with an A_{260}/A_{280} ratio of 1.99 and an A_{260}/A_{230} ratio of 1.34. The DNA has no protein but a little other impurity contamination.

DNA yielded from the Dellaporta method yielded 63.6 ng/µL with a 1.5 ratio of A_{260}/A_{280} and a 0.38 ratio of A_{260}/A_{230}. The DNA sample of the Dellaporta method was contaminated with protein and other impurities.

DNA given from the SDS method gave 304.3 ng/µL with a little protein contamination (1.76 ratio of A_{260}/A_{280}). The SDS method was better than the Dellaporta method even though it had an impurity presence of 0.99 ratio of A_{260}/A_{230}. All the NanoDrop results of the method are shown in Table 1.

Table 1. Genomic DNA Concentration and Quality Using NanoDrop Spectrophotometer

Method	DNA Concentration (ng/µL)	Indication Ratio for Protein Contamination (A_{260}/A_{280})	Indication Ratio for Impurities Contamination (A_{260}/A_{230})
CTAB	841.7	1.99	1.34
Dellaporta	63.6	1.5	0.38
SDS	304.3	1.76	0.99

(All DNA concentration, ratios for protein contamination and impurities contamination were average values.)

ii. Quality and Quantity Assessment Using Gel Electrophoresis and PCR

According to the results of gel electrophoresis, the whole genomic DNA of *Capsicum* sp. might be between 3-3.5 Gb. The result of the modified CTAB method showed that the genomic DNA was free from protein contamination and a little degraded. The genomic DNA of the Dellaporta and the SDS method were contaminated with protein and other impurities In summary, the modified CTAB method is the best quality of DNA to carry out for further investigations. The Gel electrophoresis results are shown in Figure 4.

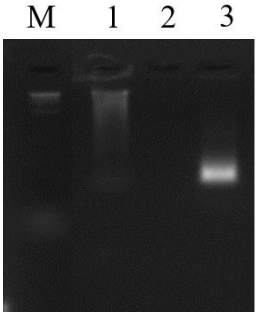

Figure 4. Checking Genomic DNA Quantity by using Gel Electrophoresis. M (λ DNA/*Hind* III Fragments), Lane 1 (modified CTAB method), Lane 2 (Dellaporta method), Lane 3 (SDS method). DNA sample was loaded 2.5 µL per lane.

In addition, the results of PCR products demonstrated that the DNA concentration diluted to 50 ng/µL had an expected band (~500 bp) were shown in Figure 5. The results of the PCR product from the modified CTAB method by NanoDrop spectrophotometer was 1156.43 ng/µL concentration with A 260/280 ratio of 1.88 and with A_{260}/A_{230} ratio 1.01.

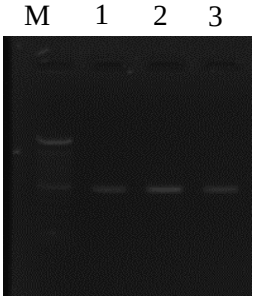

Figure 5. Checking of PCR products of genomic DNA quantity by using PCR. M (100bp ladder), Lane 1 (modified CTAB method), Lane 2 (Dellaporta method), Lane 3 (SDS method) DNA sample was loaded 5 ng per lane.

The estimated cost of genomic DNA extraction of *C. annuum* (cv) was described in Table 2.

Table 2. Comparison of genomic DNA with different methods

Method	Hazardous chemicals	Time	Quantity (ng/µL)	Quality (A_{260}/A_{280})	Quality (A_{260}/A_{230})	Estimated Cost (MMK)
CTAB	Used	2:20 hr	841.7	1.99	1.34	95
Dellaporta	Used	2:30 hr	63.6	1.5	0.38	103
SDS	Used	3:30 hr	304.3	1.76	0.99	54

Discussion and Conclusion

This research was conducted with the objective of developing a simple and rapid method to extract DNA from the leaves of *C. annuum* (cv). It was also intended to provide information for the best DNA extraction method from plants, which was inexpensive, safe and reducing time consuming. The quality of the extracted DNA sample from the modified CTAB method was verified spectrophotometrically using a NanoDrop instrument, gel electrophoresis and amplified by PCR. The NanoDrop absorbance profile was useful for the detection of contaminants such as proteins, salts and polysaccharides, which can inhabit and interfere in DNA purification. The A_{260}/A_{280} nm ratio of 1.99 and the A_{260}/A_{230} nm of 1.34 indicated that extracted DNA had high purity.

The Dellaporta method produced an A_{260}/A_{280} nm ratio of 1.5 and the A_{260}/A_{230} nm of 0.38 due to the presence of protein contamination whereas the SDS method was 1.76 due to the precence of a little protein contamination. The purity ratio of SDS method was the A_{260}/A_{280} nm ratio of 1.76 and the A_{260}/A_{230} nm ratio of 0.99 which indicates that presence of impurities. Numerous variations and modifications of extraction techniques have been reported on *C. annuum* L. (Sharma *et. al.*, 2000; Ahmadikhan, 2009; Roomi *et al*., 2013; Doyle and Doyle, 1987; Akaneme *et al.,* 2014). The results of gel electrophoresis in the modified CTAB method was free from contamination but a little degraded. Also the DNA bands of Dellaporta and SDS methods were degraded and contaminated with protein and other impurities. The PCR products demonstrated that the DNA had the expected band (~500 bp). The DNA extracted with the optimized protocol presented a reduced degradation and excellent overall quality. The developed procedure was fast and reproducible.

In conclusion, three DNA extraction methods were compared in this study. The results showed the CTAB method with little modification to be the most cost effective and time-efficient technique to isolate genomic DNA from *Capsicum annuum* (cv).

Acknowledgements

I would like to express my deep and sincere gratitude to Dr. Thidar Oo, Professor and Head, Department of Botany, University of Yangon. And also thanks to Professors from Department of Botany, University of Yangon for their kind permission, warm encouragement and kind support for all the needs for research. Also my deepest thanks to the Department of Zoology, University of Yangon, for granting me permission to use the NanoDrop Spectrophotometer for my research. I have no words to express my deepest and respectful gratitude to my supervisor. I have no valuable words to express my thanks, but my heart is still full of the favours received from every person.

References

Ahmadikhan, A. 2009. **A rapid mini-prep DNA extraction method in rice (*Oryza sativa*)**. African Journal oF Biotechnology. **8** (2):234-238.

Akaneme, F. I., I. C. Odo and L. A. Okafor. 2014. **DNA extraction protocols for *Citrullus lanatus* and *Capsicum frutescens*: Effects of incubation temperatures and ethanol concentrations on DNA purity and quantity.** Academic journals **13** (5):634-639, 29.

Doyle, J. J and J. L. Doyle. 1987. **A rapid DNA isolation procedure for small quantities of fresh leaf tissue.** Phytochemical Bulletin. **19**: 11–5.

Grooms, K. 2015. **DNA Purification from Plants: Not all methods are equal.** Proemga.com.

Kress, W. J. and D. L. Erickson. 2012. **DNA Barcodes: Methods and Protocols.** Methods in Molecular Biology, vol. 858.

Moeller, J.R. *et al.* 2014. **Paramagnetic cellulose DNA isolation improves DNA yield and quality among diverse plant taxa**. Appl. Plant. Sci. 2 (10).

Roomi, S., B. Sabiha, M. A. Zia, A. Iqbal, T. Kumar, M. Z. Ahmad, S. H. Shah, F. Rashid, A. Ghafoor, N. Ali and M. Suleman. 2013. **Protocol optimization for deoxyribonucleic acid (DNA) extraction from dried, fresh leaves, and seeds of groundnut (*Arachis hypogaea* L.).** African Journal of Biotechnology. Vol. **12** (51), pp. 7070-7073.

Sahu, S. K., M. Thangaraj, and K. Kathiresan. 2012. **DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol,** International Scholarly Research Notices, vol., Article ID 205049, 6 pages.

Sharma, K. K., M. Lavanya and V. Anjaia. 2000. **A Method for Isolation and Purification of Peanut Genomic DNA Suitable for Analytical Applications.** Plant Mol. Biol. Rep. **18**: 393a–393h.

From websites,

1) **Genomic DNA Extraction & Purification**. New England BioLabs Inc.

https://international.neb.com/products/nucleic-acid-purification/genomic-dna-extraction-and-purification/genomic-dna-extraction-and-purification