International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(4): 2011-2014

Received: 10-05-2019
Accepted: 12-06-2019

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Standardization of DNA extraction protocols from leaves of underexploited fruit crop carambola (*Averrhoa carambola* L.)

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Abstract
The objective of the study was to recommend the cheap, efficient time and genomic DNA extraction method for the underexploited fruit crop carambola (*Averrhoa carambola* L.). Based on the quality of genomic DNA determined by the ratio of A260/A280 and the amplifiable quality of DNA determined by the horizontal agarose gel electrophoresis using 1% agarose in TBE buffer at constant voltage of 60V, the modified CTAB methods yielded good quality DNA having OD (260/280) ratio of 1.52 having concentration of DNA 0.94 (µg/µl) as compared to Doyle & Doyle method OD (260/280) ratio of 1.45 having DNA concentration 0.68 (µg/µl) and the time requirement for this two DNA methods are almost same. Therefore, CTAB method could be recommended for the efficient DNA extraction method for this minor fruit crop for exploitation of superior genotype using molecular marker in the future.

Keywords: Starfruit, carambola, CTAB, Doyle & Doyle, genomic DNA methods

Introduction
Carambola (*Averrhoa carambola* L.), also called starfruit, is a curious attractive underutilized fruits of the family Oxalideaceae. The fruits of it are not taken as row because of high acidity but fruits of some genotypes are sweet in taste and taken afresh by making slices and mixing with sugar and salt (Rebica and Singh, 2017). However, the fruits have demand to produce a number of preserved products notably, pickles, Jam, Jelly, preserved, drink etc. and increased in popularity as a fresh fruit in tropical countries with large scale plantings in Malaysia as reported by Drew (1997) [4]. Arunachal Pradesh has a rich source of diversity of carambola and there is availability of different size and fruit quality having sour and sweet in taste. Still now this gaining popularity underutilized fruit crop has not taken research work for the selection of superior genotype which is available in this region which needs the exploration using molecular marker (Rebica and Singh, 2017). For molecular marker standardization of genomic DNA is very important without it molecular marker application for crop improvement is impossible.

Extraction of plant DNA in a relatively purified form is very important for further studies of plants which are based on the molecular biological for genetic diversity studies as reported by Lakmini and Kapilan (2015) [8]. DNA isolation from plant tissues / leaves is usually compromised by excessive contamination of secondary metabolites, polysaccharides and polyphenols which impede the extraction of high quality intact genomic nucleic acids (Hosseinpour *et al*. 2013) [6]. If these contaminants are not removed, it will affect further subsequent assays such as PCR (Tamari *et al*. 2013) [15]. The presence of polysaccharides in a DNA sample, form a highly viscous solution through the co-precipitation with extracted DNA as reported by Kumar *et al*. 2013 [7]. Besides, polysaccharides inhibit the activity of restriction enzyme and Taq DNA polymerase and the oxidized form of polyphenols bind with DNA covalently and give a brown colour and it is not suitable for further molecular studies.

Extraction of DNA with higher quality and quantity yield has lead to the development and introduction of new protocols; however the fundamentals of the extraction methods are similar (Tiwari *et al.* 2012) [16]. Many tree species require highly complex protocol than other annual plants because it is difficult to obtain DNA from trees than others annual plants (Kumar *et al*. 2013) [7]. Also a single isolation protocol is not successful for different plant species for getting high DNA yield (Padmalatha and Prasad, 2006) [10]. Many different methods were suggested for isolation genomic DNA from plant like CTAB method (Michiels *et al*. 2003) [9], original

hexadecyl trimethyl ammonium bromide (CTAB) method and Doyle and Doyle method (1987) are the important DNA extraction methods for a variety of plant tissues (Healey *et al*. 2014).

Disruption of plasma membrane and the nuclear membrane is occurred because of the protein digestion and by the action of ionic detergents (Tamari *et al*. 2013) [15]. Higher concentration of CTAB is important for the removal of polysaccharides. EDTA prevents the degradation of DNA by chelating the Mg^{2+} which is important for enzymes to DNA degradation (Tiwari *et al*. 2012) [16]. Contaminants are separated in the organic phase and the nucleic acids are separated in the aqueous phase by using chloroform: isoamyl alcohol mixture (Tamari *et al*. 2013) [15]. Besides, initial grinding of frozen plant tissue with liquid nitrogen (-196^{0C}) which would freeze the tissue to become fragile and make it to be a fine powder that increases the area of extraction Lakmini and Kapilan (2015) [8].

Plant research at molecular level is important for assessing the diversity of plants and for improving the medicinal and economical value of the traits through breeding (Kumar *et al*. 2013) [7]. DNA isolation can be done by using DNA isolation kits which are fast, simple, involves minimal handling by extraction DNA with sufficient quality. However, they are expensive and the chemicals used in the kits are mostly toxic and may lead to disease to human in long run (Cheng *et al*. 2003 and Ranganathan, 2015) [2, 11]. Looking at the importance of this underutilized fruit crop and high price value, the demand for its planting material is also increasing for the sweet genotype. However, no specific recommended variety is available, although a wide variability in fruit size and fruit taste exists throughout length and width of Arunachal Pradesh. Due to lack of suitable or recommended variety, the farmers have been planting seedling origin of unknown yield potential and fruit quality. Hence, there is a need to study and find out variation in physico-morphological characteristics of carambola fruits and tree to identify promising clone found in Arunachal Pradesh.

Therefore, it is essential to standardize the extraction of genomic DNA of this underexploited fruit crop carambola using diverse methods (modified CTAB and Doyle & Doyle method) to recommend the cheap and efficient DNA extraction method for this important underexploited fruit crop.

Materials and methods

Plant materials: Fresh young leaves of carambola were collected from different areas of East Siang district of Arunachal Pradesh and brought to the laboratory in ice box and stored at -20^{0}C freezer. Leaves were ground using motor and pestle until they become fine powder. Time to time the addition of liquid nitrogen facilated the grinding process of the samples as it help in removal of moisture. Resulted powder was stored in a sterile falcon tube at -20^{0}C until use for DNA extraction. The reagents use in DNA extraction and their functions use in the extraction for carambola are presented in Table 1. Similar reagents are also reported for the extraction of DNA for Jackfruit (Singh, 2008) [13].

Table 1: Reagents use in DNA extraction and their functions

Reagents	Functions
CTAB (Cetyl Trimethyl Ammonium Bromide)	It acts as a strong detergent and helps in rupturing of cell wall
Tris HCl	It helps in maintaining pH of buffer at 8.0
Sodium Chloride (NaCl)	Maintains the ionic balance and helps in separation of different dissolved organic debris
Poly Venyl Pyrothidone (PVP)	It helps in removal of phenolic compound
β-merceptaehanol	It helps in removal of protein
EDTA	Strong chelating agents which deplete Mg ions in solution, inhibit nuclease activity by that we can get nucleic acid
Chloroform	Removed proteins and help in separation of different particles in liquid and remove carbohydrates
Isoamyl alcohol	Removed proteins and avoids foaming
Iso-propanol	It help in precipitation of DNA
RNase	It help to removal RNA
Phenol	To help in removal of histone protein in DNA
Phenol : Chloroform	Removed traces of phenol
Sodium acetate:	Helped in removal of carbohydrates and proteins and also gives stabilization to DNA
Ethanol 70%	It absorbed moisture and salts in DNA pellet and acts on wetting agents and helps in better penetration in TE buffer
TE buffer	To dissolved the DNA pellet and maintain pH 8.0
Liquid nitrogen (-196^{0}C)	To help in removal the moisture from the leaf

(a) Isolation of DNA by modified CTAB method

Reagents for CTAB method:

i) CTAB buffer (100ml): 2.0g CTAB (Hexadecyl trimethyl-ammonium bromide), 10ml 1M Tris (pH 8.0), 4.0ml 0.5M EDTA (pH 8.0), 28.0ml 5M NaCl., 40.0 ml sterile distilled water and 1g PVP-40. Mix all the reagents and adjust the pH to 5.0 with HCL and make up to 100ml with distilled water.

Procedure

1. Grind 0.4-0.5g of plant tissue to a fine paste in approximately 400µl of CTAB buffer with the help of liquid Nitrogen. Transfer this CTAB/plant extract mixture to a micro-centrifuge tube. Add 75µl of SDS 10%.
2. Incubate the CTAB/plant extract mixture for 15 minutes at 65 ºC in a circulating water bath.
3. After incubation, spin the CTAB/plant extract mixture at 10000 rpm for 10 minutes to precipitate the cell debris. Transfer the supernatant to a clean micro-centrifuge tube.
4. To each tube add 250µl of Chloroform: Isoamyl Alcohol (24:1) and mix the solution by gentle inversion. After mixing, spin the tubes at 10000 rpm for 10min.
5. Transfer the upper aqueous phase (containing DNA) only to a clean micro-centrifuge tube.
6. To each tube, add 50µl of 7.5M Sodium acetate followed by 500µl of ice-cold isopropanol.
7. Invert the tubes gently several times to precipitate the DNA. Alternatively the tubes can be placed for 1 hour at

-20 ºC after the addition of Isopropanol to precipitate the DNA.
8. Following precipitation, precipitate can be isolated by spinning the tube at 12000rpm for 5 minutes to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice-cold 70% ethanol.
9. After the wash, allow the DNA pellet to dry. Do not allow the DNA to over-dry or it will become hard to re-dissolve.
10. Resuspend the DNA in sterile DNase-free water (50-100µl sterile HPLC grade water depending on how much DNA is isolated)
11. RNase A (10µg/ml) is added prior to dissolving the DNA to remove any RNA in the preparation.
12. After resuspension, incubate the DNA at 65 ºC for 20 min to destroy any DNases that may be present and store at -20 ºC.
13. Agarose gel electrophoresis of the DNA will show the integrity of the DNA.

(b) Isolation of DNA by Doyle and Doyle method

Reagents

1) Extraction buffer (4x): For 1000 ml: 250 g Sorbitol, 48 g Tris (0.4 M), 7.4 g EDTA-sodium salt (20mM) and 80 ml distil water. Before use convert 4X into X by dissolving 25ml extraction buffer into 75 ml distil water.
2) Lysis buffer: For 1000 ml: 200 ml of 1M Tris pH 8.0 (maintain pH), 200ml of 250 mM EDTA (1000mM=1M), 200ml distil water, 20g CTAB and 400ml of 5M NaCl
3) T.E buffer: 10mM Tris pH 8.0, 1mM EDTA and dissolve in 100ml of distil water. For this buffer, dissolve Tris first in 100ml of distil water and maintain pH to 8.0 and then add EDTA.
4) Sarcosine 5%
5) Isopropanol
6) Chloroform:Isomyl alcohol mixture (24:1)
7) Ethanol 70% and 100%

Procedures

1. Grind 1g fresh leaf material in liquid nitrogen to fine powder using pre-chilled mortar and pestle in presence of 25µl Beta-mercaptoethanol and a pinch of sodium metabisulphite. Add 3ml of pre-warmed (at 65 ºC) 1X extraction buffer and grind well.
2. Transfer the homogenate into a 50ml polypropylene centrifuge tube containing 4ml lysis buffer. Mix by inversions.
3. Add 1ml of 5% Sarcosine and mix well by inversion.
4. Incubate samples at 65 ºC for 10min with occasional mixing by inversion.
5. Add equal amount of Chloroform: Isoamyl alcohol and mix by inversion to emulsify. Spin at 10,000 rpm for 20 min at 4 ºC.
6. Take the supernatant in a clean tube and 0.6 volume of chilled isopropanol (measure the volume of supernatant in measuring cylinder and multiply it with 0.6. this multiplied volume of chilled isopropanol is added to tube containing supernatant) and mix well by quick inversion till the DNA precipitate. Keep at -20 ºC for an hour.
7. Centrifuge at 10,000 rpm for 20 mins and pour off the supernatant and drain well
8. Wash the DNA pallet in 70% ethanol followed by 100% ethanol.
9. Spin for 5 mins at 10,000 rpm and decant ethanol.
10. Air dry the pallet, dissolve in 50µl of T.E buffer plus 500µl of molecular grade water and store at -20 ºC.

Estimation of DNA quality: The ratio of absorbance at 260 and 280 ŋm of spectrophotometer is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA and a ratio of ~2.0 is generally accepted as pure RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorbed strongly at or near 280 ŋm. The quality of the DNA was also checked using 1% agarose gel electrophorosis.

Results and discussion

Among the DNA extraction methods from the fresh leaves of starfruit (*Averrhoa carambola* L.), the quality of DNA from the CTAB methods was found better quality as compared with the Doyle and Doyle methods. DNA of highest quality with the mean absorbance ratio (A260:280) of 1.52 was obtained from CTAB methods. Through the modified Doyle and Doyle methods also resulted in good quality of DNA all over with the absorbance ratio of 1.45. Gel running of samples from the fruit samples of Carrabolla using the two methods of DNA extraction showed that there was variation in the time required for different DNA extraction methods in which modified Doyle and Dolye methods several time consuming extraction steps and more time to finish the entire processes. Besides, among the two DNA extraction methods, Doyle and Doyle methods failure for observing clear band on the gel from the samples of starfruit may be explained by the low purity ratio of these DNA samples indicating protein co-precipitation of extracted genomic DNA. DNA quality was examined by the absorbance of DNA at 260 and 280 nm and computing A260:A280 ratio. A260:A280 ratio of more than 1.8 confirms the extraction of very good quality genomic DNA whereas values less than 1.8 indicate contamination of the genomic DNA by proteins and the values more than 2.0 indicate the presence of alcohol or acetone in the DNA preparation (Ranganathan Kapilan, 2015, Webb and Knapp, 1990) [11, 17].

DNA extraction methods and the plant species were significant sources of variation for the quality of DNA extracted (Smith *et al*., 2011 and Webb and Knapp, 1990) [14, 17]. The DNA extracted from Doyle and Doyle methods has low purity ratio it is possible that trace levels of co-precipitation of phenols or other secondary metabolites which could not removed by this methods. Quality and quantity of DNA are critical factors in molecular marker studies. Variation among extraction methods may be due to different composition of extraction buffers, different components for precipitation and purification of DNA and the time duration to complete the procedure (Weising *et al*., 1995). Besides, variation in the quality of DNA can be due to the genetical, structural and biochemical variation among leaf samples, variation in composition of the buffers used for extraction and the differences in the chemicals, their exposure time to plant tissue and concentration of chemicals (Arumuganathan *et al*. 1991) [1].

This study recommends the need for selection of appropriate DNA extraction technique for carambola fruit crops. Single extraction methods may not be suitable to extract DNA with suitable quantity and quality for this important underutilized fruit crop. The important properties of genomic DNA such as quantity, quality, suitability for amplification and the total time required for extraction, among the two extraction methods investigated, the modified CTAB and the modified

Doyle and Doyle method, CTAB was the best methods for this underexploited fruit crop carambola, which can be use for the application of molecular maker. Considerably high quantity and quality of DNA extracted using this methods and it took similar time to complete the entire procedure. This method does not required environmentally hazardous reagents and expensive equipments and it could be performed even in low technology laboratories for the crop improvement work for this in the future.

Acknowledgement
We are obliged to the Department of Fruit Science, College of Horticulture & Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India for the cooperation during the research work for this underutilized fruit crop.

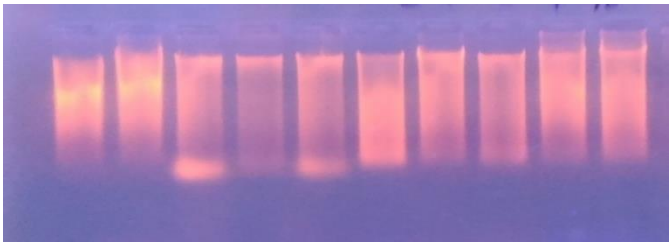

Fig 1: Gel of CTAB methods for DNA extraction for carambola

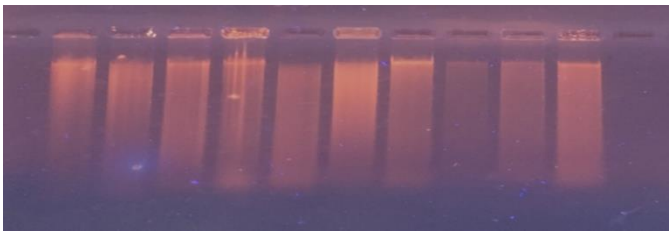

Fig 2: Gel of Doyle and Doyle methods for DNA extraction for carambola

Fig 3: Genetic diversity of carambola (*Averrhoa carambola*) – found in Arunachal Pradesh

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