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Standardization of Protocol for Isolation of High Quality Genomic DNA from Polyphenol and Polysaccharide Containing *Pyrus* Species

Z. A. Bhat[1*], W. S. Dhillon[2], T. R. Rather[1], Rizwan Rashid[3], H. U. Rehman[3], W. Shafi[3], M. Salmani[3] and A. H. Mir[3]

[1]Ambri Apple Research Center, Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST-K), India.
[2]Department of Horticulture, Punjab Agricultural University (PAU), Ludhiana, 141004, India.
[3]Central Institute of Temperate Horticulture, Rangreth, Srinagar, Kashmir, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author ZAB designed the study, performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Author WSD performed the final draft. Authors TRR, RR and HUR managed the analyses of the study. Author WS, MS and AHM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

High quality DNA is a prerequisite for several molecular biology applications such as PCR, genomic library construction, endonuclease restriction digestion etc. For plant species like pear, having high polysaccharide and polyphenol, the availabble DNA extraction protocols do not yield good quality DNA. Moreover presence of polysaccharides and polyphenols causes browning of tissues and supernatant even after PVP supplementation. For isolating high quality DNA we optimized a simple and rapid protocol by modifying CTAB procedure through inclusion of 0.1N copper acetate in addition to PVP and several other modifications at different steps during extraction. The isolated DNA was free from polysaccharides, polyphenols, RNA and other contaminants. DNA was easily digestible with endonucleases, suitable for SSR based genetic diversity analysis and long term

**Corresponding author: E-mail: zahoornano@gmail.com;*

storage. The average yield of DNA ranged from 0.5 to 1 mg/g of sample. In addition to extraction of DNA, the DNA purification was also accompolished as a part of the protocol and not as a separate one, thus saving the time and labour.

Keywords: Copper acetate; genomic DNA; polysaccharide; Pyrus; PVP.

1. INTRODUCTION

Isolating high quality DNA is pre requisite for all DNA manipulations including molecular research. At present preparation of long and pure DNA is the major concern because the forces that are required to break the cell walls can also shear the DNA. Young leaves are the best source for good quality DNA [1]. Polysaccharide contamination interferes with extraction of high quality DNA and renders it unsuitable for molecular studies and for long time storage in most woody plants [2]. Pear and related species have been used for genetic studies during last two decades due to its importance worldwide [3-5]. Contaminants such as tannins and phenolic glucosides like arbutin [6,7] have also been reported to cause difficulty in DNA extraction and purification in other plant species polysaccharides [8]; polyphenols [9,10,2]; and sticky and resinous materials [11]. These contaminants make DNA resistant to endonuclease digestion [12] and unamplifiable in PCR analysis. In pear polyphenols are released from vacuoles during homogenization and react rapidly with enzymes in cytoplasm [13,14] and in their oxidized form bind covalently to proteins and DNA, making it brown and useless for most molecular studies [15].

PCR and restriction based techniques such as RAPD, microsatellite, RFLP and amplified fragment length polymorphism (AFLP), genome mapping and DNA fingerprinting require high quality and/ or purity DNA. Hence there is a need for rapid and efficient procedure for DNA extraction in woody plants including pear.

Several plant genomic DNA extraction protocols for removing polysaccharides in perennial fruit crops have been reported but they often produce unsatisfactory yields and/or quality [4]. Few modifications were made in the CTAB protocol to remove phenols and polysaccharides and DNA was precipitated selectively in the presence of high salt. This method is suitable for rapid isolation of genomic DNA from woody fruit crops like pear, apple, walnut, cherries etc.with maximum recovery and higher molecular weight.

2. MATERIALS AND METHODS

2.1 Plant Material

Young leaves from different *Pyrus* species/ varieties were used for DNA extraction. Leaf samples were collected in butter papers and were placed in ice containers while transferring from field to laboratory. DNA extraction procedure as proposed by Doyle and Doyle [16,17] with few modifications like treatment with copper acetate and other modifications was adopted so as to remove the polyphenols thereby preventing their interaction with DNA and yielding high quality DNA.

2.2 Equipments and Reagents

Various equipments and reagents that were used during the experiment are listed as follows

- Mortar and Pestle
- Water bath
- Centrifuge
- Gel electrophoresis
- Liquid Nitrogen
- Extraction buffer (Table 1)

Table 1. Composition of extraction buffer

Reagent	Concentration (working)	Molecular weight(g)	g/ liter
Tris base	100 mM	121.14	12.141
EDTA	20 mM	372.24	7.44
NaCl	1.4 M	58.5	81.9
CTAB	2%	364.5	20.0

All chemicals were mixed in distilled water at magnetic hot plate and stirred for one hour. CTAB and other reagents were dissolved by heating to 65℃. β-mercaptoethanol (0.2%) and 2% (w/v) PVP(polyvinylpyrrolidone) were added just before use.

- Chloroform-isoamyl alcohol (24:1)
- 1xTE buffer [10 mM Tris-HCl (121 mg/ 100 ml), 1 mM EDTA (37 mg/ 100 ml)]:

Mixed and made volume to 80 ml and adjusted pH to 8.0 and the final volume (100 ml) was made with distilled water.

- 10 mg/ml RNase A (free of DNase)
- Ethanol (99.8%)
- 100% isopropanol
- 0.1 N Cu- acetate
- 70% ethanol
- Agarose

2.3 DNA Extraction Protocol

Different steps that were followed are discussed as under:

- Collect unexpanded young leaves in liquid nitrogen or on ice and store at or below -80℃ until used and grind 1 g of leaves. Transfer the powder into a 50 ml tube.
- Add 15 ml of CTAB buffer to the ground leaves and shake vigorously and incubate at 65℃ for one hour in a water bath. Occasional mixing should be performed during this period.
- Add 15 ml of chloroform: isoamyl alcohol (24:1) and invert tubes for 5 minute for proper mixing. Alternatively mechanical shaking can be performed for further mixing for a period of 30 minutes.
- Centrifuge samples for 15 minutes at 10,000 rpm so as to separate the phases.
- Transfer the supernatant to a 50 ml Falcon tube.
- Add 30 µl of copper acetate (0.1 N) and mix thoroughly and centrifuge at 12000 rpm for about 3 minutes.
- Chilled isopropanol (10 ml) should be added to precipitate the DNA and kept in refrigerator for 15 minutes so as to separate the DNA.
- Hook out the DNA and discard the supernatant and transfer to an Eppendorf tube.
- Centrifuge the Eppendorf tube containing DNA pellet at 10,000 rpm for 7 minutes so as to precipitate the DNA at the bottom
- Wash the pellet twice with 70% ethanol.
- Centrifuge again at 3000 rpm/ 5000 rpm for 3 minutes each
- Dry the DNA pellet by leaving the tubes uncovered at 37℃ for 20 to 30 minutes to remove ethanol.
- Dissolve in 200 to 300 µL TE buffer.
- Treat with 1µL RNAase A / 100 µL DNA solution and incubate at 37℃ in water bath for one hour.
- Quantify DNA in a spectrophotometer at A_{260}/ A_{280} or Gel electrophoresis.
- Keep DNA at -80℃ for long term storage.

3. RESULTS AND DISCUSSION

DNA yields from various *Pyrus* species and varieties by the above procedure are presented in Table 2. DNA was extracted by modifying CTAB method with Copper acetate used to remove polysaccharides and PVP to purge polyphenols. The procedure is fast and simple and 40 to 50 DNA samples can be processed in a single day. Too young or too old leaf tissues has resulted in poor yields, thus selection of proper tissue for extraction is an important step. Young leaves that have partially opened are the best material as DNA obtained from fully expanded leaves was low and incompletely digestible. With the addition of PVP to the extraction buffer the DNA so obtained was of good quantity and quality even from fully expanded leaves. Polyphenols from mature, damaged and improperly stored leaf tissues is removed by the addition of PVP [9] as PVP forms complex hydrogen bonds with polyphenolic compounds which can be separated from DNA by centrifugation [18]. Freezing the plant material before extraction and by using PVP in the DNA extraction procedure the concentration of polyphenols is reduced to a greater extent. The optimal time for leaf collection was during the period of active shoot elongation following bud break (February- March in our case). However, with increase in leaf age and temperature the DNA extraction was difficult and the quality and quantity of DNA was poor and unstable for long term storage even at freezing temperature.

Restriction endonucleases were used for completely digesting the DNA and was amplified in PCR indicating the absence of polysaccharides. The DNA becomes sticky, viscous in TE buffer due to presence of polysachrides and thereby interferes with several biological enzymes viz. polymerases, ligases and restriction endonucleases [19,20,21].DNA amplification was not observed untill removal of polysaccharides were not removed. Further the addition of 100 µLCu-acetate was effective in removing the polysaccharides by increasing their solubility in ethanol and thus preventing their co-precipitation with DNA.

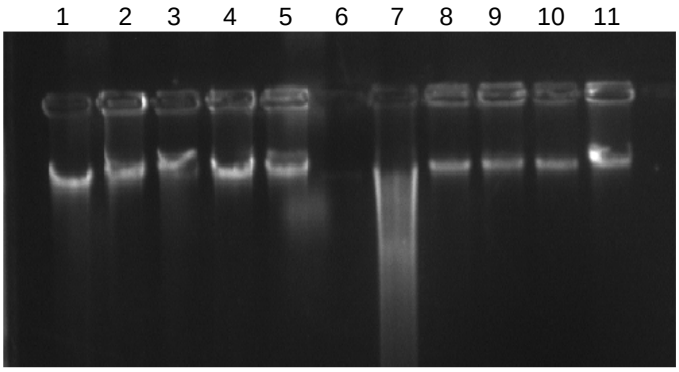

Fig. 1. Quantification of DNA: 1- *Pyrus serotina,* 2-Punjab Beauty, 3-Shinsheiki, 4-Yali, 5-Smith, 6-*Pyrus pashia, 7*-Keifer, 8-Bagugosha, 9-*Pyrus calleryana,* 10-Pathernakh, 11- Leconte

Table 2. DNA yield of various Pyrus species and varieties

Pyrus species/ Variety	DNA yield (µg/ g leaf tissue)
Pathernakh	700
Pyrus pashia	500
Pyrus serotina	1000
Punjab Beauty	1000
Shinsheiki	1200
Yali	1000
Smith	1000
LeConte	1200
Keifer	1000
Bagugosha	1000
Pyrus calleryana	1000

The DNA can be easily be extracted from plant species having high polyphenol and polysaccharide content like *Pyrus* species, and others such as apple, peach, plum, almond etc. Moreover, DNA yield is also higher compared with other procedures used for DNA extraction from Pyrus species. A high quality DNA can be obtained by our modification of Doyle and Doyle [10] DNA extraction procedure that will remain usable more than two years when stored at -20℃.

4. CONCLUSION

It could be concluded that the simplicity of the procedure makes it very practical for DNA extraction especially from *Pyrus* species, and generally from other plant having high polyphenol and polysaccharide content, besides, higher DNA yields compared with other procedures used for DNA extraction from *Pyrus* species. Our modification of Doyle and Doyle [16] DNA extraction procedure produces high quality DNA which remains usable for at least two years when stored at -20℃.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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