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Evaluating the efficiency of Chelex 100 for DNA extraction in pomegranate (*Punica granatum*)

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ABSTRACT

Pomegranate is a widely distributed species in many tropical and subtropical regions. Pomegranate is consumed as fresh fruit or raw material to produce various products such as juice, syrup, jams, and wine. DNA extraction is a critical way to study the genetic diversity and phylogeny of the pomegranate species. Hence, chelex is an accessible and safe method to extract the DNA of pomegranate. So far, chelex was not used for DNA extraction in this plant and therefore it has been evaluated for DNA isolation in South African pomegranate for the firs time. This study was carried out at Genetic lab at the University of Limpopo during 2021to compare the two different extraction DNA methods from pomegranate (*P. granatum*). The DNA from the pomegranate was extracted using Chelex 100 "method 1" (whole night incubation at 56°C) and "method 2" (Ten minutes incubation at 95°C) using the fresh leaves of the pomegranate. For this purpose, four samples of *P. granatum* from northen parts of South Africa were studied. Quantitative and qualitative parameters were assessed using a spectrophotometer. The PCR reaction with primers for 28S were used on all samples to confirm and evaluate the extracted DNA. The results for the spectrophotometer indicated that the highest quality of extracted DNA was in "method 2" (1.45-1.69). However, the protein (1.41-1.52 mg/mL) was detected through "method 1" in the tested samples. The qualitative and quantitative tests for PCR processing realised that the total DNA extracted using "method 2" had improved quality than "method 1". Amplification of samples using 28S rDNA primer showed a higher concentration and purity of DNA extracted using "method 2". In conclusion, both methods yield enough DNA, however, "method 2" yielded higher quality DNA in a less time.

Key words : DNA extraction, pomegranate, *Punica granatum*, spectrophotometer

INTRODUCTION

Most studies based on plants are performed at the molecular level, which needs reliable, quick DNA extraction protocols. DNA extraction with high quality is a foundation for further study in the molecular field (Kumar *et al.*, 2018). The various methods for genomic DNA created several DNA extraction methods (Abdel-Latif and Osman, 2017). However, some methods, such as chloroform-based DNA extraction, is not safe for human. Additionally, chloroform-based DNA extraction needs the use of toxic chemicals, magnetic separation, and silica-based DNA extraction inclined to be expensive (Kumar *et al.*, 2018).

Pure and rapid extraction of DNA is a prerequisite for most advanced techniques such as genetic mapping, fingerprinting, and marker-assisted selection. However, the extraction of high-quality DNA can be time-consuming, arduous, and costly due to multiple steps. The Chelex method has proven efficient in extracting DNA in PCR analyses in a wide range of experiments. Chelex procedures are simple, rapid, do not involve harmful organic solvents, and do not require multiple transfers between tubes for most types of samples (Kumar *et al.*, 2018).

The quantity and quality of the extracted DNA are particularly sample-dependent. Furthermore, the chemical-physical composition affects DNA extraction. Several plant species, including the Solanaceae family, produce secondary metabolites like phenolic compounds, tannins, flavonoids, and alkaloids, which present in the extract solution can interfere with DNA analysis and inhibit the PCR processing (Turci *et al.*, 2010).

Pomegranate (*Punica granatum* L.) is widely distributed in many tropical and subtropical regions. It is the well-established fruit of a shrub (*P. granatum* L.) that is mainly cultivated in west Asia and in the area around the Mediterranean, as well as other parts of the world, including America, where the climate is suitable for its growth (Pagliarulo *et al.*, 2016). The shrub typically grows up to 5 m, but in some cases, it may reach a morphology of a tree that is as tall as 10 m, except for dwarf cultivars that grow up to 1–2 m (Russo *et al.*, 2018). Climates that simulate the Mediterranean (with high sunlight-exposed mild winters and dry summers) seem ideal for pomegranate growth. Most varieties are deciduous, although there have been reports of evergreen and conditionally deciduous pomegranates, depending on the altitude and temperature of the zone. The fruit is categorized as a fleshy berry (Kandylis and Kokkinomagoulos, 2020). Pomegranate is consumed as fresh fruit or raw material to produce various products such as juice, syrup, jams, and wine (Drinic *et al.*, 2020). As a result, pomegranate seeds, which make up about 3% of the weight of the fruit (Russo *et al.*, 2018), are a waste product of the food industry.

Hence, this study aimed to evaluate the Chelex efficiency for DNA extraction in pomegranate with an overnight incubation at 56 ° C, and the Chelex efficiency for DNA extraction in pomegranate with ten minutes incubation at 56 ° C.

MATERIALS AND METHODS

Sample Collection

Four samples of *P. granatum* were collected from the commercial Floradade seedling in Polokwane (23°52’24.695" S, 29°30’44.294" E) in 2021. All samples were transferred to the molecular lab for DNA extraction and molecular analysis.

Molecular Analysis

In “method 1”, DNA extraction was done using the Chelex method (Shokoohi, 2021a). Specimens of *P. granatum* were hand-picked and transferred to a 1.5 ml Eppendorf tube containing 20 µL double distilled water. The pomegranate leaves in the tube were crushed with the tip of a fine needle and vortexed. Sixty microliters of 20% Chelex® were added to each microcentrifuge tube containing the crushed pomegranates and mixed. The tubes were incubated at 56°C overnight for 12 hrs. Finally, it spun for 2 min at 16000 rpm (Aminisarteshnizi, 2021a). The PCR product was stored at –20 °C.

The “method2” for DNA extraction was done using the modified Chelex method (Liu *et al.*, 2015). Specimens of *P. granatum* were hand-picked and transferred to a 1.5 mL Eppendorf tube containing 20 µL double distilled water. The pomegranate leaves in the tube were crushed with the tip of a fine needle and vortexed. Sixty microliters of 20% Chelex-100® were added to each microcentrifuge tube containing the crushed pomegranates and mixed. The tubes were incubated at 95°C for 10 minutes. The mixture was vortexed for 10-30s. Then the tubes were centrifuged at 16000 rpm for 1 minute, and the supernatant was used as a template for PCR. Each method was repeated with four replicates. Thus, the experiments were performed twice.

Spectrophotometric analyses of DNA

Thermo Scientific NanoDrop™ One Spectrophotometer (Thermo Scientific, Germany) was used to determine sample concentration, purity, and absorbance ratio at 260–280 nm (A260/A230 ratio). These were measured using one µL of each sample. Thus, each sample’s measurements were repeated three times.

PCR Amplification

The 28S ribosomal DNA is a commonly used DNA marker in DNA barcoding analysis recommended in plant DNA barcodes. For polymerase chain reaction (PCR) analysis, the forward and reverse primers, D2A (5'-ACAAGTACCGTGAGGGAAAGTTG–3'), D3B (5'-TCGGAAGGAACCAGCTACTA–3') (Shokoohi, 2021b), was used for partial Amplification of the 28S rDNA. PCR was conducted with five µL of the DNA template, 12.5 µL of 2X PCR Master Mix Red (Promega, USA) for the South African specimens, one µL of each primer (10 pmol/µL), and ddH_2O for a final volume of 30 µL. The

PCR processing was done using an Eppendorf master cycler gradient (Eppendorf, Hamburg, Germany), with the following program: 94°C for 3 min at, next 37 cycles of denaturation for 45 s at 94°C; 56 °C annealing temperatures for 28S rDNA; extension for 45 seconds to 1 min at 72°C, and finally an extension step of 6 min at 72°C followed by a temperature on hold at four °C. After DNA amplification, 4 µL of product from each tube was loaded on a 1% agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and one mM EDTA) to evaluate the DNA bands. The PCR products were assessed using RedGeldye and visualized and photographed using a UV transilluminator (Koohkan *et al.*, 2014; Koohkan *et al.*, 2015).

RESULTS AND DISCUSSION

DNA Quality and Quantity Assessment

The quality of extracted DNA sample was evaluated using a NanoDrop instrument. We observed a high-quality DNA ranging between 1.8 and 2.0 at A260/280. In this study ratio of 260/280 was found to be in a range of 1.03–1.69. The DNA extracted must be free of contaminating substances, such as polysaccharides and phenols. The extraction and purification of high-quality DNA are generally tricky. The presence of these compounds affects the quality and quantity of isolated DNA, rendering the sample non-amplifiable (Aminisarteshnizi, 2021b). The "method 2" produced DNA samples with purity ratios in a range of 1.45–1.69, whereas the purity ratio of samples extracted by "method 1" was between 1.03-1.37 (Table 1).

Approximately 100 ng/uL of DNA concentration is necessary for the polymerase chain reactions to see the PCR product after 30 cycles. The DNA concentration for "method 2" was found to be in a range of 196-701 ng/uL, whereas for "method 1", it was in a range of 379-533 ng/uL.

The NanoDrop device measured the amount of protein extracted from the samples (Fig.1). The results showed that the amount of protein in "method 1" and "method 2" was different. Furthermore, we observed a higher total protein in "method 1" compared with "method 2". These results could explain the reason for the low quality of the "method 1".

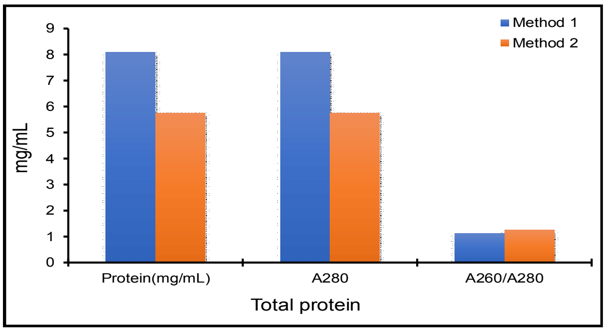

Fig. 1. Total protein obtained for all pomegranate sample extracts using Methods 1 and 2.

PCR Amplification for DNA Detection

The extracted DNA of methods one and two were used for DNA extraction of a fresh sample of *P. granatum* detected using the 28S rDNA primer. The rDNA-28S fragment was produced by DNA amplification following "method 1" and "method 2". The fragment size was approximately 680 base pairs (Fig. 2), consistent with the expected result. The result indicated a good band in the PCR products. The amplified bands obtained from "method 2" extraction of pomegranate samples were neat and clear, compere to "method 1".

The NanoDrop absorbance profile is helpful for the detection of contaminants such

Table 1. Mean DNA obtained for all sample extracts using method one and two (Mean±SE).

Sample	Nucleic acid (ng/uL)	260/280	260/230	A260	A280
M1R1	379 ± 22	1.16 ± 0.01	0.81 ± 0.02	7.59	6.54
M1R2	533 ± 15	1.03 ± 0.02	0.76 ± 0.01	10.66	10.29
M1R3	456 ± 19	1.37 ± 0.01	0.75 ± 0.01	9.92	7.2
M1R4	412 ± 21	1.29 ± 0.03	0.80± 0.02	8.53	6.62
M2R1	701 ± 14	1.56 ± 0.01	0.87 ± 0.02	14.02	12.66
M2R2	196 ± 46	1.45 ± 0.02	0.80 ± 0.01	3.92	2.69
M2R3	448 ± 25	1.69 ± 0.01	0.75 ± 0.02	8.3	4.9
M2R4	512 ± 24	1.64 ± 0.02	0.62 ± 0.01	10.19	6.2

M1: Method 1; M2: Method 2.

as polysaccharides, salts, and proteins, which can interfere with and inhibit DNA sequencing. The ratio of 1.8 in 260/280 nm indicated that the extracted DNA had high quality with the absence of proteins and phenols. A purity ratio higher than 1.9 indicates the presence of RNA in the extracted DNA sample. However, we did not have more than 1.9. The ratio of <1.7 in some samples of DNA extracted by the “method 1” suggests the presence of higher total proteins in those samples. These differences could be explained by the ability of some of the procedures to eliminate contaminating molecules. Liu *et al.* (2015) reported that DNA quality was evaluated by polymerase chain reaction. The results showed that genomic DNA extracted using the Chelex-100 method was better than using the CTAB method.

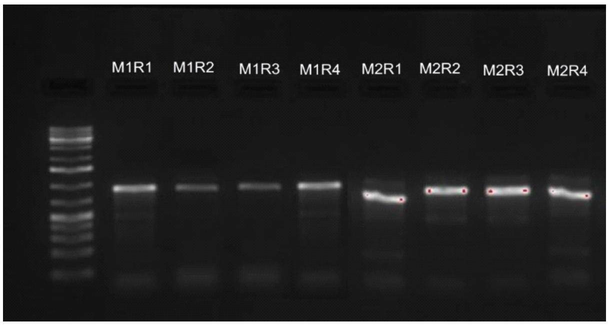

Fig. 2. Agarose gel electrophoresis of rDNA-28S primers in "method 1" (M1R1-M1R4) and "method 2" (M2R1-M2R4) for pomegranate collected in Limpopo province, South Africa.

Chelex has been investigated on the nematodes with high-quality PCR products (Shokoohi *et al.*, 2013; Shokoohi *et al.*, 2014; Shokoohi *et al.*, 2016). However, DNA extraction needs no proteinase K in plants, which is an advantage. Turan *et al.* (2015) used the Chelex method for extracting DNA for *Venturia inaequalis* spores. Chelex resin was evaluated and compared with a well-established DNA extraction method utilizing CTAB. They reported that DNA samples isolated using the Chelex method were better than those extracted using CTAB. HwangBo *et al.* (2010) used Chelex for extracting DNA in some plants such as tomatoes. The PCR analysis showed successfully amplified transgenes. The result obtained in this study agrees with the result obtained by several studies in this field. Singh *et al.* (2018) studied a method for improving the quality of genomic DNA obtained from minute quantities of tissue and blood samples using Chelex 100 resin. They found the Chelex method was non-toxic, easily available, and inexpensive reagents, as well as minimal amounts of blood or tissue samples for the DNA extraction process. Sajiba *et al.* (2017) studied a simple, efficient, and rapid Chelex method for good quality DNA extract Sajiba from rice grains. They reported this method reproducibly extracts DNA with good purity indices and requires only a few steps. Therefore, we tried to provide a better protocol for DNA extraction by the Chelex method in this study. Generally, in different DNA extraction protocols, polyphenolic residues mainly inhibit DNA polymerase activity during PCR analyses (Shokoohi *et al.*, 2015). In the present study, only Chelex was used to extract DNA. The PCR analyses showed that the DNA samples prepared by both methods could be successfully used for PCR amplification with an rDNA primer. Thus, these methods could be used for genetic diversity and phylogenetic purposes.

CONCLUSION

The use of Chelex to extract DNA is prevalent among researchers. In this study, we had two methods for extracting DNA from *P. granatum.* Both methods worked, but the quality of “method 2” was much better than the “method1”. High quality of DNA is required for PCR and sequencing. Therefore, it must be free of protein, RNA, or polysaccharides contamination. Furthermore, among the two methods, “method 1” needs more time (12 hours) than “method 2” (10 minutes), which causes extraction of the DNA with higher total protein. Additionally, the low cost of Chelex, which allows us to use it for many DNA extractions, creates an excellent option for molecular research in plant genetics and phylogenetic studies. Therefore, Chelex is highly recommended for DNA extraction from plant sources, especially in the family Solanaceae.

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