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DEVELOPMENT, IMPLEMENTATION OF AN EFFICIENT AND CHEAP METHOD FOR DNA EXTRACTION FROM DRY LEAVES OF DATE PALM WITHOUT USING LIQUID NITROGEN

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) is the most significant economic fruit tree planted in Iraq. DNA isolation is one of the most important steps in the application of molecular biology, especially dependent on PCR and other techniques. The difficulty of DNA extracting from palm tissue could be because the solidity and high content of polyphenolic and polysaccharides compounds, that consequently obstruct DNA extraction and have prompte researchers to find the efficient methods for the isolation. This study describeds an effictive method for DNA extraction by using CTAB (Cetyl Trimethyl Ammonium Bromide) without liquid nitrogen. We have developed a simple and reliable method for DNA isolation from the leaves for six of date palm cultivars, the obtained results recorded significant concentration and purity of isolated DNA which, were (567.6 -1047.1) ng/µL, and (1.82-2.00) nm respectively. DNA product was tested by four SSR-PCR markers, to produce 61 clear bands, consequently, our result confirm that a modified method is perfect to DNA extraction from plant tissue without liquid nitrogen.

Keywords: Date palm; CTAB; liquid nitrogen; SSR.

INTRODUCTION

The date palm is dioecious plant that began to be cultivated in Mesopotamia (Iraq) around 4000 BC [1]. It is considered one of the most important ancient fruit crops [2], and considered one of the sacred trees in the Islamic heritage because of its historical and social extension in people's lives.

The plant leaf especially date palm is characterized by the presence of fibers and hard textures which making the extraction of DNA difficult, in addition to a highly concentration of secondary metabolites, such as polysaccharides, proteins, polyphenols and tannins, These component, when present in the leaf it will interfere with the extraction of DNA, and gives

low quality and quantity of DNA, Therefore many of researcher have worked on this point to find a suitable way to extract a good quality and quantity of DNA [3].

With the development of different molecular markers based on PCR technology, such as RAPD, SSR, ISSR, STR, AFLP, and RFLP, and their extensive use in molecular biology to detect genetic relationships, differences between varieties and sex determination in the early stages, especially in the date palm, the process of DNA extraction are very important to obtain good and effective results to achieve the desired goal [4]. In most of DNA extraction methods, some dangerous chemicals such as liquid nitrogen are used to grind plant tissues in order to break down the cell walls of plants [5]. In addition, storing liquid nitrogen in the laboratory is expensive and difficult, the process of transporting and using during tissue grinding is cumbersome and may cause some healthy risk, therefore, developing an extraction method without liquid nitrogen will be beneficial to researchers. Here we describe a simple and inexpensive protocol to isolate DNA from dry leaf of date palm which can be used widely in molecular applications.

MATERIALS AND METHODS

Collecting and Preparing Samples

Plant samples were collected from six date palm cultivars grown in orchards in the southern region (Basra) of Iraq in the agricultural season 2019-2020, Fresh white leaves that chosen are near apical shoot and free from disease infections, The samples were wrapped with aluminum foil and transferred in the icebox to the laboratory. Work was done in the Genetic Engineering Laboratory - Department of Horticulture and landscape- College of Agriculture- University of Basra.

It washed the leaves with distilled water several times, to clean them from dust, then wiped with wet medical cotton with 70% ethanol for sterilizing purpose, they were cut into small pieces (1 cm^2) using sharp clean sterile scissors, after aerial drying sample It was crushed using mechanical mill to a fine powder, then kept in the refrigerator for later DNA extraction.

DNA Isolation and Purifications

DNA was extracted using the CTAB method with some deeply affect chemical for DNA extract which chosen carefully as described following –

1 – Put 100 mg of the ground sample into Eppendorf tube (2 ml).
2- Add 800 µl of CTAB-PVP buffer (2.0 % CTAB, 100 m M Tris-Hcl pH 8.0, 20mM EDTA, 1.4 M NaCl, PVP 2% then immediately add β –mercaptoethanol (0.3% v/v) .
3- The CTAB/plant extract mixture incubate at 65 °C for about 30-45 min in a water bath (micro-tube were inverted every 10 min)
4- To each tube add 600 µl of Chloroform: Iso Amyl Alcohol (24:1), vortex (for 5 min). After homogenate, centrifuge the tubes at 14000 rpm for 5 min.
5- Transfer supernatant layer to a new Eppendorf tube (contains DNA) (2 ml).
6- Is added 800 µl of cold isopropanol (stored at -20°C). Add 300 µl of 5M NaCl. Mix by vortex then cooling at -20°C for 20 min.
7- Spin at 14000 rpm for 10 min at 4°C, supernatant liquid was discarded carefully then the precipitate belt was dried aerial for 15 min.
8- For cleaning, added the 100 µl of 5.2 M Ammonium Acetate then 700 µl of cold absolute ethanol.
9- Reverse the tubes slowly several times to precipitate the DNA. Generally, the DNA can be seen as precipitated layer. Transfer for 20 min at -20°C after the addition of ethanol to precipitate the DNA.
10 - Spin the tubes at 14000 rpm for 10 min at 4°C.
11- Delete the supernatant and wash DNA pellet by adding 600µl cold 70% ethanol with inversion for several times.
12- After the wash, centrifuged it at 6000rpm for 5 min then remove upper phase and allow the DNA pellet to airly drying (approximately for 15 min). Do not allow the DNA to over dry; it will be hard to re-dissolve.
13- Addition 100 µl of TE buffer solution to each sample and then stored in refrigerating at ˚ 4°C until use.

Table 1. SSR primers equipped from BIONEER

Primers	Sequence	Annealing temp (°C)	GC %	Source
PDCAT6	F : AATCAGGGAAACCACAGCCA R : GTTTAAAGCCTTCTCAAGATAGCCTCAG	53	46	Akkak et al. [6]
PDCAT 18	F : CCTAAACCTGAATGAATCAAAGCA R : ACTAACATAAGGACAGTGCTATGTGATTG	54	38	Akkak et al. [6]
MPDCIR78	F : CCCCTCATTAGGATTCTAC R : GCACGAGAAGGCTTATAGT	49.3	47	Billotte et al. [7]
DP175	F : ACACACACACACACACACACC R : GTGGCTTCTTTTTGGCTGTC	57.6	51	Elmeer et al. [8]

Estimation of DNA Quality and Quantity

Two DNA quantification and qualification methods were used. The first method was performed in a Nano Drop ND-2000 spectrophotometer (THERMO SCIENTIFIC, USA) at 260nm and the purity was checked depending on A260/A280 ratio. The second method was DNA pattern by agarose gels at 1% in TBE buffer Electrophoresis. Gels were stained with Diamond Nucleic Acid Dye, the bands intensity of PCR products was determined under UV light.

PCR Amplification

After testing the quality of DNA PCR amplification was applied by using simple sequence repeat (SSR), four primers were used for this technique produced by BIONEER company. Table 1 show the some primer characteristics, sequences, sources annealing temperature. The SSR-PCR reactions was performed in 25 µl a final volume containing 2 µl (50 ng) genomic DNA, 12.5 µl Go Taq® Green Master Mix (Promega, USA), 1 µl of forward and reverse primers, 8.5 µl Sterile distilled water.

PCR sitting program consisted of 35cycles, denaturation at 95ºC for 10min then denaturation at 95 ºC for 30 seconds, annealing at 49.3 – 57.6 ºC for 45 seconds and extension at 72ºC for 1 min and a final delay phase at 72°C for 7 min [8].

The PCR products were separated by gel electrophoresis on 2% agarose gel in 1 X TBE buffer, stained with Diamond Nucleic Acid Dye. Use the volumetric guide 100 bp DNA Ladder from Promega . Then the poles of the relay were connected to the power supply with a voltage of 65V and current of 80 mA for two hours. After making sure that the semantic dye reached the end of the gel, it was removed from the transfer basin, then carefully transferred to the Gel Documentation, and photographed using a high-resolution digital camera.

RESULTS AND DISCUSSION

Results from shown the quantity and quality of genomic DNA as described at Table 2 without using liquid nitrogen or phenol, because of this chemical is dander and it must be take care when used DNA genome without using liquid nitrogen and phenol [9]. AS described in Table 2, the highest concentration of DNA reached to (1047.1 ng / µL) at Nbiti, while the lowest concentration was 567.6 ng / µL for Sakri and the other DNA concentrations of rest the cultivars ranged between these two concentrations. The quality test was done at A260/A280 Fig. 1, the ranged recorded between 1.82 for Abd-alhadi for 2.00 at Habsi cultivars. A high quality of dna must be between 1.8-2, the number upper or down this range refer to contamination by protein or polysecried [10].

Table 2. Quantity and quality of isolated genomic DNA of six Date palm cultivars

Cultivare	DNA yield (ng/µl)	A260/280 ratio	A260/230 ratio
Swadani	852.4	1.9	1.89
Sakri	567.6	1.92	1.86
Abd-Alhadi	709.1	1.82	1.79
Habsi	667	2	1.90
Nbaiti	1047.1	1.89	1.85
Ashger	855.7	1.86	1.83

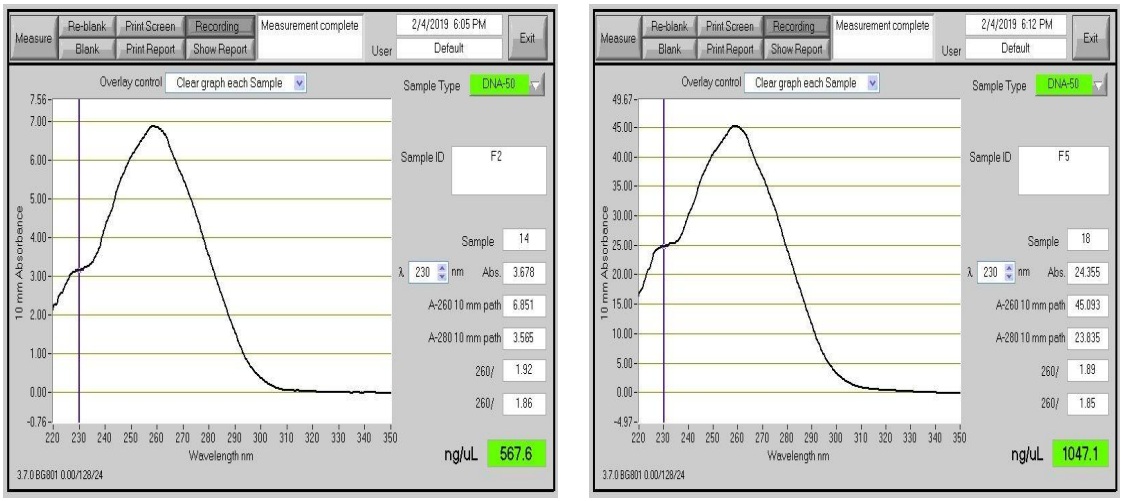

Fig. 1. DNA concentration according to the Nano drop

The quality of the DNA was also confirmed by using electrophoresis on an agarose gel at a concentration of 1%. (Fig. 2), that obtained a good image of extracted DNA for PCR application.

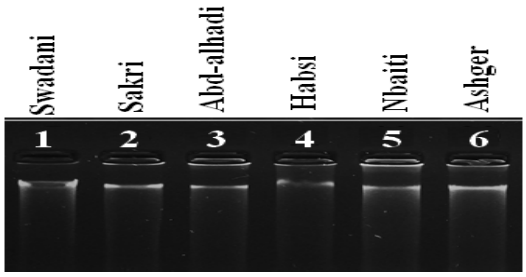

Fig. 2. Agarose gel electrophoresis of total DNA image. A representative 1% agarose gel showing DNA extracted from the six date palm cultivars

It is clear from the results that our method recorded a good concentration of DNA, which is considered high compared to the traditional extraction method with liquid nitrification, and this may be due to the fact that the process of grinding the tissues into a fine powder led to the ease of cell wall decomposition as PVP forms complex hydrogen bonds with phenols that are deposited with Cell debris upon cell lysis [11].

As it is known, phenols and secondary compounds cause contamination and damage DNA [12].

PVP-Phenolic complexes can be removed between the aqueous and organic phases to isolate the DNA. In addition, high concentrations of β-mrcaptoethanol helped reduce brown coloration in DNA resulting from phenolate oxidation [13].

The results of our method also showed that the value of A260/A280 ratio was very acceptable (1.82-2.00), which indicates the extracted DNA was free from contaminants such as polyphenols, proteins and polysaccharides [14].

In the process of common plant DNA extraction, some chemicals such as liquid nitrogen and phenol are used, which considered risk chemicals during handling as well as require preventive measures that may not be available in regular laboratories. On another hand, the process of collecting samples from date palm trees requires time and efforts that may cause Delaying the delivery of samples in fresh form to the laboratory, affecting's the process of grinding the tissues with liquid nitrogen, Numerous efforts have been made by researchers to isolate the DNA from fresh tissues without using liquid nitrogen or phenol or both [15], In current study, the genomic DNA of date palms was extracted from dry tissue without liquid nitrogen or phenol, use which obtained an excellent results for quality and quantity of extracted DNA.

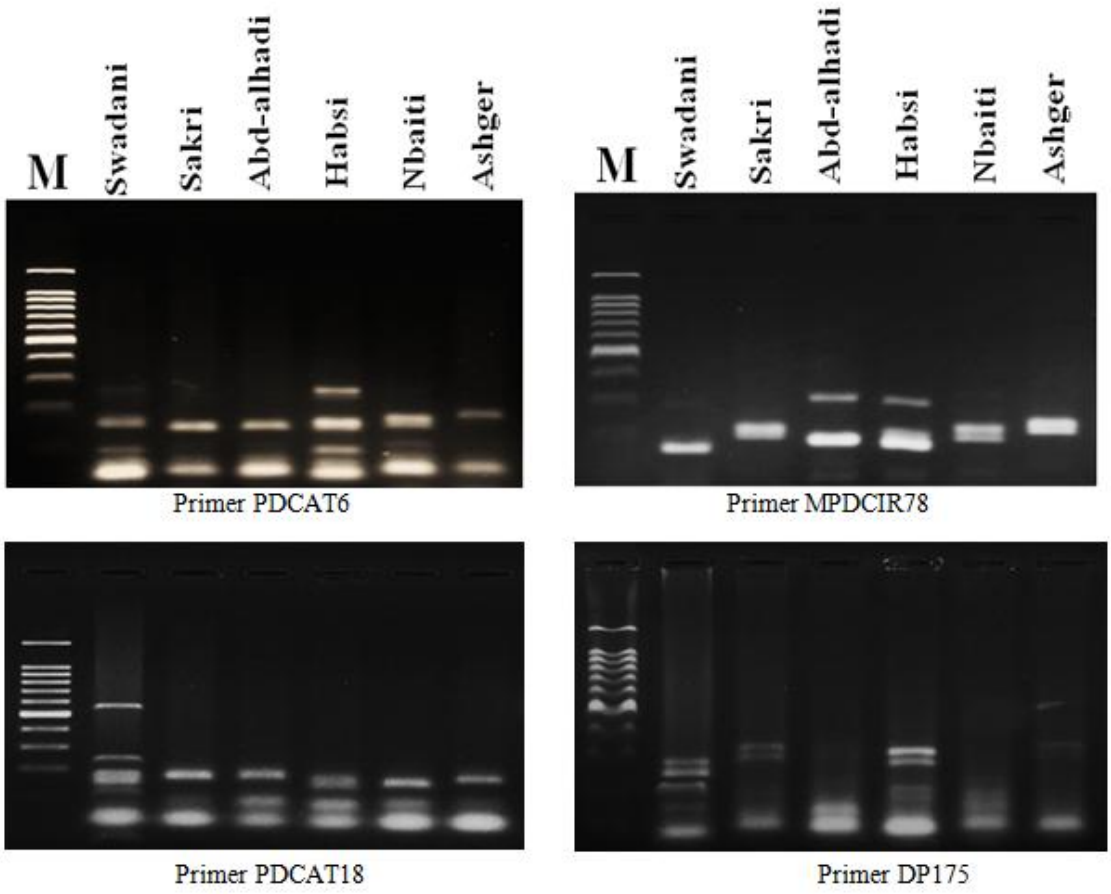

Fig. 3. Agarose gel electrophoresis of SSR-PCR experiments showing amplification with primers that gave marker bands with most cultivars. . The name of the cultivar is indicated above each lane and the primer number is indicated below each plate. M is a 100 bp DNA ladder from Promega

To investigate the efficiency of DNA extraction method in this study, the extracted DNA was examined by PCR amplification procedure using four SSR primers, which gave clear pattern ranges (Fig. 3), SSRs are fragments of repeated DNA sequences in the higher eukaryote genome. They can be used as genetic markers that are rich in information [16]. Also it could be used in plant diversity analysis because they are common PCR-based dominant markers that exhibit high allelic diversity at different sites [8]. Simple sequence repeats are abundant in the genomes of higher eukaryotes and are more polymorphic than other genetic markers. These are useful for taxon identification, lineage analysis, germplasm characterization and gene mapping studies [7].

CONCLUSION

The previous results show that our protocol is useful due to its simplicity, lowering cost and safety, in addition to achieving high concentration and purity of genetic extracted DNA, Moreover the isolated DNA is capable used in PCR applications including the detection of genetic variances between varieties sex determintione, and next generation of DNA sequences in the plants generally and date palm specially.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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