DNA EXTRACTION AND OPTIMIZATION FROM FIBROUS LEAVES OF SOME DATE PALM CULTIVARS FROM PAKISTAN

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Abstract

Pure DNA is first and foremost requirement for PCR amplification and DNA fingerprinting analysis of plant species. Leaves of date palm are hard, fibrous and difficult to grind. An attempt was made to isolate the high molecular weight DNA from fibrous unopened pale yellow leaves of ten date palm cultivars. The leaf samples were collected from orchards of Khairpur district. DNA was extracted with CTAB method using liquid nitrogen and supernatant was kept for an overnight for precipitation of DNA at 4^0C. Quantification of extracted DNA was checked with spectrophotometer and on 0.8% agarose gel electrophoresis. DNA was further diluted for 10ng/µL and optimized for RAPD-PCR amplification.

Introduction

Date palm (*Phoenix dactylifera* L.) is the third important fruit crop in Pakistan after citrus and mango. Pakistan is the fifth largest dates producer in the world (Mirbahar *et al.,* 2014). Khairpur is a major dates producing district of Pakistan and plays an important role in revenue generation in agriculture sector of this district. The fruit of date palm is most nutritive and energy providing food. Dates are rich in certain nutrients and provide a good source of rapid energy due to high carbohydrate content. In addition to carbohydrates dates contain minerals, proteins, fats and vitamins etc. (Markhand *et al*., 2010). The date palm is a major fruit crop of North Africa and the Middle East. Date palm is also considered basic element of oasis and usually this tree grows in arid regions. Date palm grows in harsh environment which is not suitable for other crops. Dates are rich in nutrients and are a staple food for human being consumption in addition this is used as animal feed (Haymes *et al.,* 2004). The tremendous advantages of the tree are its resilience, its requirement for limited inputs, its long-term productivity and its multiple purposes attributes (Bekhet, 2013).

Various DNA fingerprinting techniques are commonly used for identification of date palm cultivars and biodiversity conservation (Arif *et al.,* 2010). Leaves of date palm are extremely fibrous to isolate good quality DNA. High quality DNA extraction is precondition for PCR amplification in DNA fingerprinting and DNA barcoding that have been developed recently. DNA isolation and purification are two important steps for molecular biology studies (Arif *et al.,* 2010). Like reagents, good quality DNA is an essential to achieve good results in experiments, especially in the Polymerase Chain Reaction (PCR), in which excess of cell debris and proteins may inhibit the amplification (Baise *et al.,* 2002). Many molecular biology techniques and investigations require genomic DNA as a starting material, which is especially true for forensic disciplines where the rapid and robust isolation of small quantities of DNA from tissues is critical (Vaseemuddin, 2010). Chemically plants contain thousands of primary and secondary metabolites, polyphenols and polysaccharides in comparison to other organisms. These compounds severely affect on DNA extraction and inhibit Polymerase Chain Reaction (PCR) by covalently binding to nucleotides and DNA oxidation. Similarly particular leaf textures and types can impede extraction of DNA. The major problematic groups of plants are succulents (e.g. Aloeaceae, Cactaceae, Crassulaceae), plant species having fibrous and hard leaves (e.g., Palms and Aquifoliaceae,), carnivorous plants containing resin or sap (e.g., Apocynaceae, Sapotaceae and Pinaceae). The aim of this study was to isolate highly pure genomic DNA from fibrous leaves of date palm and optimize the DNA concentration for RAPD-PCR.

Materials and Methods

Plant Material and DNA Isolation: Young date palm leaves were collected from date palm orchards of Khairpur distric. During collection leaf samples were properly labelled, kept in ice box and in laboratory kept in -20°C. Date palm leaves were washed with deionized water two to three times to remove dust and wax. About 1.5g samples of young pale yellow leaves were first cut into small pieces and ground to fine powder with liquid nitrogen in pre-chilled pestle and mortar. Total genomic DNA isolation was performed by classical cetyltrimethyl ammoniumbromide method (2% CTAB, 20 mM EDTA, 1.4 M NaCl, and 2% mercaptoethanol, 100 mMTris-HCl, pH 8.0) method described by Doyle and Doyle (1990). All the coded eppendorf tubes were kept in the freezer at -

20^0C, at Molecular Biology and Quality Control Laboratory, Plant Tissue Culture and Biotechnology Wing, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan. The remaining leaf samples were kept at -20^0C in freezer.

DNA Quantification: The nucleic acid concentration was determined using a spectrophotometer (Schimadzu, 2000). Silica (Quartz) Ultra Micro Spectrophotometer cuvette was used for holding the samples and TE buffer (10 mM TrisHcl (pH 7.5), 1 mM EDTA) solution was used as a standard/blank for calibrations of the spectrophotometer at 260 nm and 280 nm. The readings at 260 nm were used to calculate the DNA concentration in the samples by using the formula:

DNA concentration (µg/ml) = A260 x 50 x dilution factor (100)

Besides this DNA estimation was performed by electrophoresis on 0.8% agarose gel with λ DNA and stained with ethidium bromide. DNA samples were diluted in TE buffer to a working concentration of approximately 10 ng μl^{-1}.

Optimization of DNA Concentration for RAPD-PCR: An experiment was conducted to optimize DNA concentration through Polymerase Chain Reactions (PCR). Different concentrations of total cellular DNA were used such as, 05, 10, 20, 30, 40 and 50ng in a total volume of 25 µl PCR reaction mixture. Hi-Temp 96 wells Thermal cycler (Master Cycler, Eppendorf, Germany) was used for amplification under the following conditions: initial denaturation 94^0C for 05 min, 35 cycles (Denaturation 94^0C for 60 seconds, annealing 36^0C for 60 seconds and extension 72^0C for 120 seconds), final extension for 10 min. Electrophoresis was carried out to resolve the RAPD-PCR products on 1% agarose gel with 1x TBE buffer.

Results and Discussion

Manual extraction methods and commercial kits are used for DNA extraction from plant leaf samples. These protocols usually include liquid nitrogen for grinding the samples. In liquid nitrogen if any tissue immersed becomes fragile hard to facilitate crushing into powder, additional advantage of liquid nitrogen is to maintain low temperature of plant material. This step can be absconder for spongy and easy-to-grind material i.e. petals of flowers. Cellular DNA extraction from hard, fibrous leaves of date palm is very difficult in comparison to flower petals and soft leaves. The small laboratories of many developing countries are constantly facing the problem of unavailability of liquid nitrogen, its storage and maintenance is also difficult. Cetyltrimethyl ammonium bromide (CTAB) Sambrok *et al*., (1989) is a standard and highly versatile method for DNA extraction from a variety of plant materials. Generally three contaminants associated with plant DNA can interfere PCR reactions e.g. polyphenolic compounds, polysaccharides and RNA (Krishna *et al*., 2012). Phenolic compounds present in the leaves of date palm like quercetin, isorhamnetinhetersides, (+)-catechin, (-)-epicatechin, 5-caffeoylshikimic acid (dactyliferic acid) and its positional isomers (3-caffeoylshikimic acid and 4- caffeoylshikimic acid) can hamper the isolation of good quality DNA (Arif *et al.*, 2010). For molecular genetic analysis of crops availability of high-quality cellular DNA is an essential precondition. Extraction of intact, high molecular-weight DNA that can support PCR, genomic blot analysis, fingerprinting and other molecular analysis is not easy when the plant tissue is rich in polysaccharides, secondary metabolites or polyphenolics.

An optical density value 01 corresponds to approximately 50 µg/ml for double stranded DNA. The minimum O.D. 1.70 and maximum 1.82 was recorded (Table 1). Good quality DNA was extracted from all the leaf samples of date palm used in this study and amount of DNA isolated between minimum 379 µg/ml and maximum 549 µg/ml. Good quality DNA isolation is necessary which is comparatively free from several contaminants found in plant cells. Generally high amount of proteins is present in many plant species naturally (Angeles *et al*., 2005) and other substances are identified which can bind firmly to nucleic acids during extraction of DNA and can interfere in DNA amplification (Ribeiro and Lovato, 2007).

The present studies were conducted to optimize the concentration of total genomic DNA for the PCR (Polymerase Chain Reaction) by using RAPD primers. Initially six different concentrations of total genomic DNA were used for the RAPD- PCR amplifications like, 05, 10, 20, 30, 40 and 50 ng in 25 µl reaction volume. At the concentration of 05ng low molecular weight and light DNA bands were observed. Maximum bands were observed with 10 and 20 ng template DNA concentrations. As the concentrations increased from 20 to 50 ng the number of DNA bands decreased and lower bands showed extra low molecular weight bands likewise with 05 ng indicated with arrows in Figure 1. On the basis of above mentioned results, it was decided to use 25 ng template DNA which gave bright shiny bands; these results are in agreement with Aitchitt (1995) and Hussein *et al*., (2005). The present study is the first attempt/report to use RAPD marker for genetic diversity analysis among some Pakistani date palm cultivars.

Table 1. Date palm cultivars used for the DNA extraction.

Cultivar Name	Leaf samples in grams	O.D. Ratio	DNA µg/mL
Aseel	1.5	1.72	435
Asul Khurmo	1.5	1.75	517
Dedhi	1.5	1.74	439
Khar	1.5	1.74	464
Kupro	1.5	1.78	504
Nar Aseel	1.5	1.70	549
Noori	1.5	1.71	379
Otaquin	1.5	1.77	388
Halawee	1.5	1.82	460
Seedless	1.5	1.81	533

However different researchers have used different DNA concentrations in PCR such as, 20 ng (Yehya *et al*. 2012); 25 ng (Bader *et al*. 2007); 25 ng (Abdulla and Gamal. 2010); 30 ng (El-Rayes, 2009); 40 ng (Adawy *et al*. 2002); 50 ng (Eissa *et al*., 2009; Soliman *et al*., 2003); 100 ng (Kramer and Coen, 2001); 125 ng (Sijapati *et al.*, 2008). The reason for these differences might be the quality of extracted DNA, the accuracy of DNA quantification methods used to estimate the DNA concentration and the PCR conditions employed.

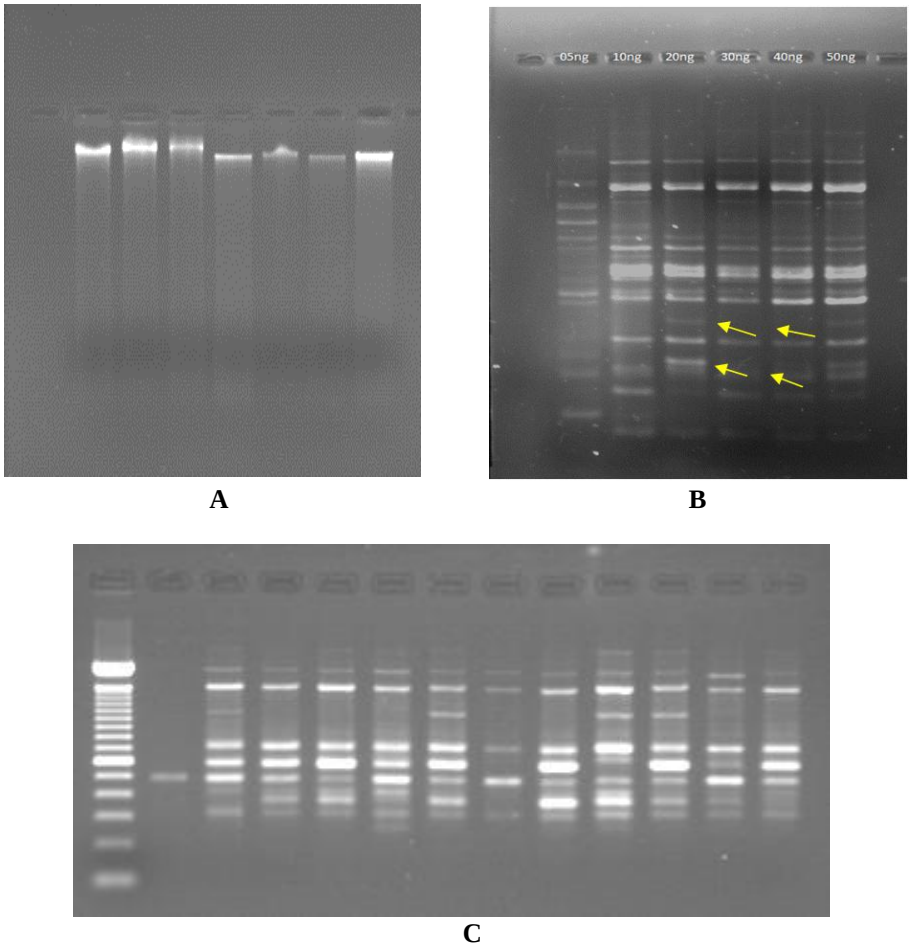

Fig. 1. (A) Quality of Extracted DNA (B) Optimization of DNA concentration for RAPD-PCR (C) RAPD-PCR results by using 25ng DNA template.

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