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STANDARDIZATION OF DIFFERENT PROTOCOLS FOR GENOMIC DNA ISOLATION FROM *PHOENIX DACTYLIFERA* L.

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Abstract

DNA fingerprinting, gene sequencing, genetic diversity studies and other valuable applications in molecular biology require highly purified DNA isolation for further use. DNA isolation is quite difficult from the hard and fibrous leaves of date palm. Tissues also contain high concentration of polyphenolics and polysaccharides linked with genomic DNA that may interfere with PCR amplification. This study was aimed at selection of the most appropriate method for DNA isolation from fibrous tissues of date palm and finding the alternative ways of grinding leaf samples to evade the problems linked with availability and usage of liquid nitrogen in date palm growing areas. DNA extraction from leaves of five date palm varieties was accomplished using different protocols including modified DNAzol method, cetyltrimethylammonium bromide (CTAB) method: (a) using liquid nitrogen for sample grinding and (b) grinding of leaf samples using sterile sand. Three different lysis buffers with different constituents were used. Study was carried out to observe the sodium chloride (NaCl) effects on produce and pureness of DNA individually and in combination with polyvinylpyrrolidone, (PVP) and lithium chloride (LiCl). It was concluded that DNA extracted using DNAzol was best in purity and yield. It was observed that crushing the leaf samples of date palm using sterile sand along with addition of lysis buffer containing NaCl (1.4 M) without addition of liquid nitrogen, or PVP and LiCl, gave adequate DNA yield and satisfactory purity.

Key words: DNA Isolation, Date palm, DNAzol, Lysis buffer, Sterile sand.

Introduction

Date palm (*Phoenix dactylifera* L.) belonging to Arecaceae family, is a dioecious monocotyledon and has a significant socioeconomic impact. Isolation of DNA from the tough date palm leaves is time consuming and difficult (Al-Amiry, 2018). The stiff, leathery and fibrous leaves are notoriously difficult for grinding. In addition high content of polyphenolic compounds and polysaccharides in the tissue intersect with DNA extraction. In this era of plant genomics, systems comprising of molecular markers are extensively used to study DNA polymorphisms, evaluate the genetic diversity and conduct phylogenetic studies (Chiong *et al*., 2017). These investigates can be performed on a large number of germplasm and it becomes crucial to lessen the time consuming steps. In addition, it must be under consideration that the concentration and the quality of DNA should be suitable for further use in PCR (Jamil *et al*., 2021) Methods of extracting DNA using commercial kits often require crushing of leaf material in liquid nitrogen. Plant tissues become brittle solid when exposed to liquid nitrogen and then it becomes fine powder on crushing. Use of acid-washed sand or glass powder for crushing of the date palm may help in avoiding the problems associated with use and storage of liquid nitrogen (Ibrahim *et al*., 2010).

Extreme difficult isolation of genomic DNA of appropriate quality is attributed to the higher proportion of polyphenolic compounds and polysaccharides in plant tissues. Three main impurities related to plant isolated DNA that may cause significant problems when accompanying PCR experiments are polyphenolic compounds, polysaccharides and RNA (Sahu *et al*., 2012). Occurrence of phenolic pool like quercetin, isorhamnetinheterosides,,(+)-catechin,'(−)-epicatechin, 5-caffeoylshikimic'acid (dactylifric'acid) and its positional isomers (3-caffeoylshikimic;acid and 4-caffeoy lshikimic;acid) in plant tissues may restrict the effective isolation of DNA appropriate for further use in PCR (Mirbahar *et al*., 2014; Alturki, 2017). Some DNA isolation protocols for high quality DNA from date palm leaves were optimized and presented in this paper. The effects of grinding of samples in liquid nitrogen, sand grinding, individual as well as mutual effects of NaCl,"PVP" and LiCl (Lysis,buffer) in combination with the modified CTAB,protocol were examined in this study. Another method using DNAzol, a harmless and ready-to-use reagent (Invitrogen, USA) was also used for extraction of PCR amplifiable DNA. The main principle behind using this reagent is the activity of new guanidine-detergent"lysing solution, which hydrolyzes RNA and permits the settling of DNA selectively from the lysate (Chomczynski *et al*., 1997; Sajid *et al.*, 2015).

The study was conducted with the objective of optimizing an efficient, simple, low-cost and quick procedure for DNA isolation from hard and fibrous leaf samples of date palm without ignoring the product yield and purity.

Material and Methods

DNA isolation protocols

Liquid nitrogen grinding followed by CTAB method: Plant leaf sample (2g) was taken and ground in liquid

nitrogen. Added 4ml of preheated CTAB buffer (1% mercaptoethanol) and placed in water bath for 45 minutes for incubation at 65℃. Addition of equal volume of chloroform: isoamyl alcohol (24:1) was followed by centrifugation at 4,000 rpm for about 10 minutes. Supernatant was collected in a new falcon tube (15ml) and equal volume of chilled isopropanol was added into the tube and it was placed in freezer (-40℃) for 15 minutes. The supernatant obtained by centrifugation of the tubes at 4,000 rpm at 4℃ for 10 minutes was removed. 70% ethanol was used for washing the pellet thrice and left the washed pellets overnight for drying. Pellet was dissolved with 200µl nuclease free d_3H_20 followed by addition of 4µl RNAase and incubation at 37℃ for 1h. Chloroform: isoamyl alcohol (24:1) was added in equal volume and tubes were centrifuged at 9,000 rpm for 10 minutes and supernatant was collected in a new eppendorf tube.3M NaCl ($1/10^{th}$ of total volume of supernatant) and absolute ethanol (two volumes of total) were added followed by centrifugation in (4℃) for approximately 15 minutes at 11,000 rpm. Finally 70% ethanol was used for washing of final pellets thrice after discarding the supernatant. Pellet was air dried overnight again and dissolved in 200µl nuclease free d_3H_20.

Using sand grinding and different lysis buffer: Five varieties of date palm were used for separation of genomic DNA. Fresh leaf sample (2g), sterile sand (100mg) and 1ml lysis buffer (A-D) (Table 1) were poured in the same mortar. Pestle was used for fine crushing of leaf samples. The crushed sample was placed at 25°C for almost 5 min. Ground leaf sample (2g) was shifted to a 2 mL eppendorf tube followed by addition of the same lysis buffer (2ml) used during crushing the leaf sample. Vortexed tubes for 30s and placed in a pre-heated water bath at 60°C for 30 minutes. Centrifugation of tubes was done at 9,000 rpm for 5 min. 400 µL supernatant was moved to a new falcon tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added and tubes were shaken slightly and were centrifuged for 7 minutes at 9,000 rpm. Supernatant (400µL) was collected after centrifugation and tubes were kept in freezer (-40°C). 3M Sodium acetate (20 µL) + cold isopropanol (1mL) was added followed by centrifugation for 10 minutes at 11,000 rpm. Pellets were settled in the bottom and supernatant was discarded. 500 µL of 70% cold ethanol was added and tubes were again centrifuged for 6-7 minutes at 7,000 rpm. After discarding the 70% ethanol, tubes were kept for air dried overnight at room temperature. 200 µL of deionized water was added. All the experiments were repeated thrice with three biological repeats.

Using DNAzol: Ground 2g plant leaf sample in liquid nitrogen and 5ml plant DNAzol reagent was added, vortexed thoroughly and then placed on rotator for 30 minutes. Added 5ml chloroform and placed back on rotator for 20 minutes. Centrifugation was performed for 7 minutes at 4,000 rpm and supernatant was shifted to a new tube. 2/3 volume of absolute ethanol was added in supernatant and tubes were placed on rotator for 20 minutes which resulted in precipitation of DNA. The precipitated DNA was spun for 7 minutes at 4,000 rpm and supernatant was discarded. DNA pellets were air dried overnight at room temperature and finally dissolved in 200ml d_3H_2o.

DNA concentration and purity checking: Concentration and purity of extracted DNA were checked using nanodrop (ND 2000, Thermo Scientific USA)) following method described by Jamil *et al*., (2020 a,b).

Results and Discussion

Results for DNA yield and purity using different lysis buffers are presented in Fig.1 and Fig. 2, respectively. It was observed that different buffers used for DNA isolation gave quite different results. DNA yield obtained was observed to be higher for buffers A and C compared with buffers B and D (Fig. 1). The concentration of DNA ranged from 88.62 ng/µl to 119.34 ng/µl. It was highest for buffer A and lowest for buffer D. DNA purity regarding protein contamination was estimated with ODs proportion at 260/280 nm, as proteins particularly aromatic amino acids generally absorbed light at 280 nm wavelength. This ration indicated the nucleic acid impurity in protein preparations precisely (Sambrook & Russell, 2001). Ratio for pure DNA ~1.8 is generally acceptable. If the ratio is lower than 1.8, it indicates presence of impurities i.e., phenolic compounds and protein that absorb light sturdily at 280 nm wavelength. Values for ODs at 260/280 nm ranged from 1.4 to 1.84 for all the buffers and buffer A showed the maximum mean value 1.84.

PVP has high affinity for binding with polyphenolic compounds, resulting in their precipitation; LiCl has the tendency to remove RNA (Jobes *et al*., 1995). Formation of complex hydrogen bonds of PVP with these polyphenolic compounds causes their detachment from DNA by centrifugation (Maliyakal *et al*., 1992). Previously, it was reported that polyphenolic compounds could be removed by using PVP in the process of genomic DNA isolation (Lodhi *et al*., 1994). However addition of PVP (Buffer B) in lysis solution did not affect DNA purity as well as its yield as compared to addition of NaCl alone (buffer A) (Fig. 1). Precipitation of RNA was found to be more reliable than treatment with RNAase, therefore LiCl was used for elimination of precipitated RNA and ensuring RNA-free DNA. Addition of 1 M NaCl enhanced polysaccharides solubility in ethanol and helped in their removal so that polysaccharides may not precipitate out along with genomic DNA (Fang *et al*., 1992). Lodhi *et al*., (1994) further reported that addition of NaCl up to 2.5 M might lead to increase in DNA quality and yield. Present study witnessed that addition of NaCl (buffer A) alone in lysis buffer provided DNA of higher quality in comparison to addition of PVP and LiCl. This shows that addition of lysis buffer an alone is sufficient for extraction of good quality DNA from date palm.

Table 1. Constituents of lysis buffer (A-D).

Lysis buffer	Main components,	Additives,
A	"CTAB (2.0g) + Na_2EDTA (0.4g)'+ Trizma'(1.2g),	NaCl (8.1g)
B	CTAB'(2.0g) + Na_2EDTA (0.4g),+ Trizma'(1.2g)	PVP'(2.0g)+ NaCl (8.1g)
C	"CTAB (2.0g) + Na_2EDTA (0.4g)'+ Trizma'(1.2g),	LiCl'(0.2g) + NaCl'(8.1g)
D	CTAB'(2.0g) + Na_2EDTA (0.4g),+ Trizma'(1.2g)	LiCl'(0.2g) + PVP'(2.0g) + NaCl'(8.1g)

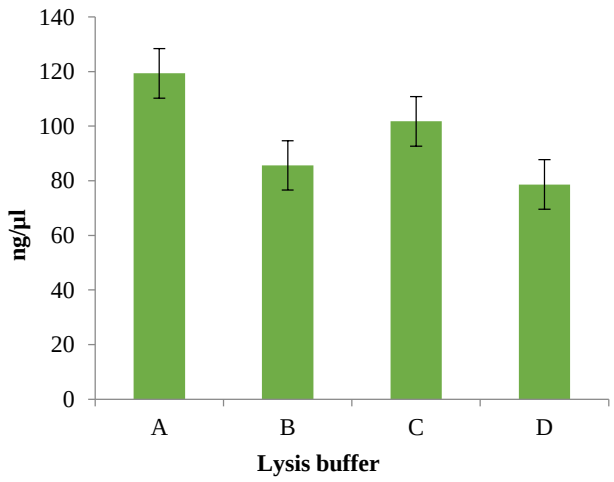

Fig. 1. Effect of various lysis buffers on DNA quantity/yield.

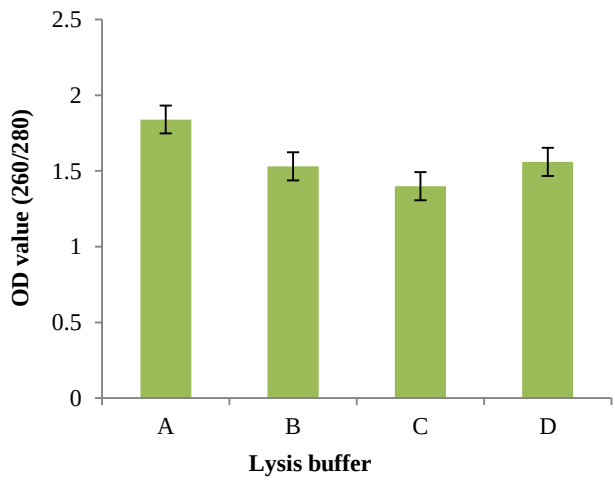

Fig. 2. Quality of extracted DNA as affected by various lysis buffers.

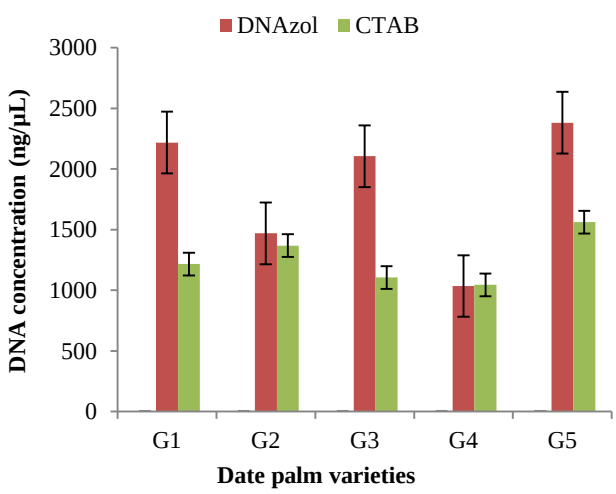

Fig. 3. DNA concentration of date palm varieties using different protocols.

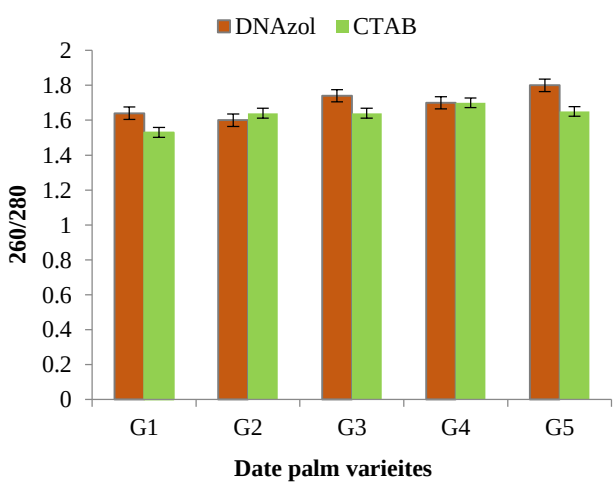

Fig. 4. DNA purity and date palm varieties using different protocols.

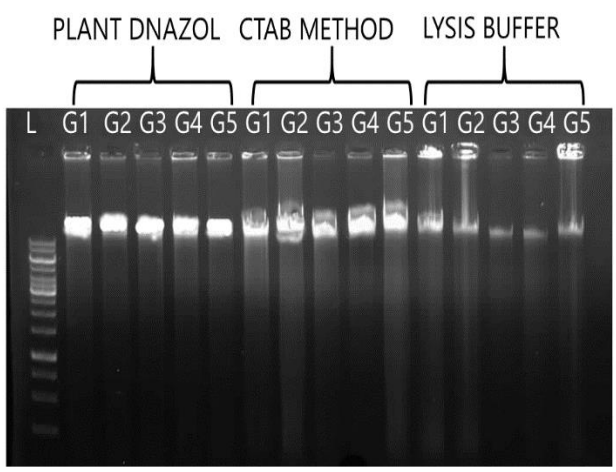

Fig. 5. DNA purity and concentration of five date palm varieties using different DNA extraction protocols.

The results related to DNA concentration and purity obtained by using modified DNAzol protocol is presented in Fig. 3 and 4. DNA yield ranged from 1035.8 ng/µL to 3105.4 ng/µL while purity from 1.6 to 1.8. The 260/A280 absorbance ratio was greater than 1.6 in all date palm samples. In comparison with routine CTAB extraction method, DNAzol method was quick procedure of DNA isolation (less than 2 hours from DNA extraction to DNA solubilization) and provided higher concentration of genomic DNA and an appropriate 260/280 ratio. Clear DNA bands of higher molecular weight were observed in agarose gel for DNA samples obtained from extraction using the DNAzol compared with other two methods (Fig. 5). DNA isolation using DNAzol reagent has been successfully used in many other monocots like *Oryza sativa* (Garg *et al*., 2002) and *Poa pratensis* (Lickfeldt *et al*., 2002). Thus, this DNAzol method has the ability to

isolate genomic DNA from various plant species for molecular research and novel biotechnological applications. Figure 5 represents the DNA purity and concentration of five date palm varieties obtained from modified DNAzol extraction method (1-5), Liquid Nitrogen Grinding+CTAB method (6-10), Sand Grinding+CTAB method (11-15).

Conclusion

Modified DNAzol protocol provides the best results in isolation of good quality genomic DNA as well as high yield. However use of sterile sand for leaf grinding and addition of NaCl in lysis buffer provides sufficient DNA yield along with good quality.

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