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RESEARCH ARTICLE

Optimization of DNA extraction and PCR protocols for plants with high Phenolics: Bael, Mango, Pomegranate as examples

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Abstract: The genetic studies of tropical tree species containing high amount of phenols are greatly hampered by the inability to extract sufficient quantities of high quality DNA and the presence of PCR inhibitors in extracted DNA samples. While there are some DNA extraction and PCR protocols for such species, no consistency in results. Hence, optimization of such protocols is a prerequisite for conducting research studies on tropical flora. This study reports the optimization of cheaper DNA extraction and PCR procedures for plants having high amount of phenolic compounds and PCR inhibitors. Three fruit crop species, Bael (*Aegle marmelos* L. Correa), Pomegranate (*Punica granatum* L.) and Mango (*Mangifera indica* L.), with distinctly diverse secondary metabolic profiles, were used as examples. The CTAB DNA extraction method, with some modifications, was compared with two commercially available DNA extraction kits namely; Promega Wizard® Genomic DNA purification kit and QIAGEN DNeasy® Plant Mini kit. DNA from three to five genotypes from each species was extracted from each method and the quality and quantity were assessed. Spermidine was added to the PCR mix at the rate of 0.8 µM to block the PCR inhibitors and the DNA samples were amplified using universal plant barcoding primer pair *rbcL*, and SSR or ISSR primers. The modified CTAB method resulted significantly higher quantity of quality DNA in all samples compared to two commercial kits. Henceforth DNA extracted from CTAB method, and the two commercial kits were used to precede PCR. However, expected bands were not generated in regular PCR. Interestingly, the inclusion of spermidine amplified the relevant band/s in relatively easy PCR reactions such as *rbcL*, as well as trickier reactions such as SSR and ISSR. These results suggest that cheaper alternative procedures used in this research study could be used successfully for the range of applications in plants with array of secondary metabolic profiles.

Keywords: *Aegle marmelos* L. *Correa, Punica granatum L., Mangifera indica* L., DNA extraction, Spermidine, PCR inhibitors, phenolic compounds.

INTRODUCTION

Optimization of DNA extraction and PCR protocols are the basic and most important preliminary steps of any molecular biological application. Secondary metabolites such as phenolic compounds present in plants play important roles in their defence mechanisms against predators and pathogens (War *et al*., 2012). These secondary metabolites often hinder the extraction of high quality DNA and successful PCR amplification (Pirttila *et al*., 2001). While some of these compounds directly bind or intercalate with DNA molecule, others inhibit the activity of DNA manipulating enzymes such as *Taq* DNA polymerase (Manoj *et al*., 2007; Schrader *et al*., 2012).

There are many DNA extraction methods available from quick and dirty to highly purified column-based commercial kits. Most of these methods and commercial extraction kits are optimized for model plant species such as *Arabidopsis thaliana and* Rice (*Oryza sativa*) (Roychowdhury *et al*., 2012). Therefore, DNA extraction protocols have to be optimized for non-model plants. If one protocol works for a range of species, that would save time and cost required for such optimization steps and expedite the genetic improvement of such plant species.

The polyamines such as spermine and spermidine have been found to increase the success of PCR for DNA extracted from plants such as barley (Ahokas and Erkkila, 1993), cotton (Wan and Wilkins, 1993) and pomegranates (Ono *et al*.,2012). Although these studies reported different concentrations of PCR enhancers, it would be beneficial to optimize a protocol applicable for range of plant species with phytochemical diversity. Cetyltrimethyl ammonium bromide (CTAB), based methods originally described by Doyle (1987) are more popular among available DNA extraction protocols. However sometimes CTAB method does not yield good quality DNA especially for tropical plant species and therefore in the present study, the original CTAB method was further optimized and it was compared with the commercially available kits. PCR protocol was optimized by incorporating spermidine (Analytical grade) at the concentration of 0.8 µM.

Three tropical fruit crop species bael (*Aegle marmelos* L), mango (*Mangifera indica*) and pomegranate (*Punica granatum*) with high total phenol contents and distinct secondary metabolic profiles were used for the protocol optimization in the present study. Bael consists of alkaloids, coumarins, terpenoids, fatty acids amino acids, γ-sitosterol, aegelin, lupeol, rutin, marmesinin, β-sitosterol, flavone, glycoside, oisopentenly lhalfordiol, marmeline and phenyl

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ethyl cinnamamides (Yadav and Chanotia, 2009) while mango contains protocatechic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids cycloart-24-en-3β,26diol, 3-ketodammar-24 (E)-en-20S,26-diol, C-24 epimers of cycloart-25 en 3β,24,27-triol and cycloartan-3β,24,27-triol (Shah *et al*., 2010). Punicalagin α and β are considered as major compounds contribute to the high phenolic activity in pomegranate (*Punica granatum*) (Mohammad and Kashani, 2012; Viuda-Martos *et al*., 2010). Morever these three species are important medicinally and economically awaiting further research studies from genetic characterisation to crop improvement in local context. Therefore, objective of the present study was to optimize the DNA extraction and PCR protocols for bael, mango and pomegranate.

MATERIALS AND METHODS

Plant materials collection

Immature leaves from five elite bale accessions (Department of Agriculture, Sri Lanka, 2017), four pomegranate selections (two Sri Lankan selections, a Chinese selection and an exotic variety; Wonderful) and stem bark from three *Karthakollomban* mango accessions were collected. They were ground into fine powder using liquid nitrogen and stored in -80 °C.

DNA Extraction

CTAB method

About 300 mg of finely ground plant materials were mixed with 500 µL pre-warmed 2× CTAB extraction buffer (50 mM CTAB, 950 mM NaCl, 100 mM Tris pH 8.0 and 20 mM EDTA pH 8.0 and 0.2% β mercaptoethanol) and incubated for 1h at 60 °C while mixing at every 15 mins. Then 500 µL of chloroform/ isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 15 min. The supernatant was transferred to a fresh tube and 500 µL of isopropanol was added. Then it was incubated at -20 °C for 30 min. and centrifuged at 12000 rpm for 5 mins. The supernatant was discarded and the DNA pellet was washed with 500 µL of 70% alcohol followed by air drying. The DNA pellet was re-suspended using 30 µL of nuclease free water and stored at -20 °C. Chloroform/isoamyl alcohol (24:1) extraction was repeated one more time as the minor modification to the originally described method.

Commercial extraction kits

The genomic DNA extraction from ground leaf and stem bark materials were also conducted using two commercially available kits, Promega Wizard® Genomic DNA Purification (Cat. No: A1120) and QIAGEN DNeasy® Plant Mini (Cat. No.: 69104), following the manufacturers guidelines.

DNA quantification and assessment of quality

The quantity of isolated total genomic DNA was determined using NanoDrop spectrophotometer (Nano2000, Thermoscientific). The quality of samples was assessed with the 260/280 and 260/230 readings given by the NanoDrop spectrophotometer and further verified by using 1% agarose gel electrophoresis.

PCR

Two different PCRs were carried out for each sample. PCR amplification was done with universal barcoding primer pair *rbc*L, (Forward: 'ATGTCACCACAAACAGAGACTAAAGC3', and Reverse: 'CTTCTGCTACAAATAAGAATCGATCTC3') (Kress and Erickson, 2007) and ISSR primers or SSR primer pairs (Table 01). PCR was carried out in a 25 µL reaction volume containing l× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP (Promega, Cat No: U1515), 0.2 µM of each primer (Integrated DNA technologies), 50 ng of DNA, 0.8 µM spermidine and 1 Unit Go*Taq* FlexiDNA polymerase (Promega, Cat No: M8295). Same reactions were performed in the absence of spermidine. The PCR cycle consisted of 94 °C of initial denaturation for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 sec and 72 °C for 1 min and final extension at 72 °C for 5 min. A total of 10 µL from each PCR product was separated using 2% Agarose gels containing 0.5 µg/mL ethidium bromide.

RESULTS AND DISCUSSION

Applications of PCR in plant research are unlimited and isolation of high quality DNA is a prerequisite for PCR amplification (Pirttila *et al*., 2001). However, unlike in animals or microbes, DNA isolated from plants is usually contaminated with various secondary metabolites.

Table 1: SSR and ISSR markers used for the PCR.

Species	Primer	Sequence
Bael	SSR (Rutaceae family specific)	*NTCP9* Forward 5'CTTCCAAGCTAACGATGC3' *NTCP9* Reverse 5'CTGTCCTATCCATTAGACAATG3'
Pomegranate	ISSR 842	5'GAG AGA GAG AGA GAG AYG3'
Mango	SSR 84 (*Mangifera indica* species specific)	Forward 5'ACTGCCACCGTGGAAAGTAG3' Reverse 5'ACTGCCACCGTGGAAAGTAG3'

Co-isolation of polysaccharides, polyphenols, RNA and other secondary metabolites interfere with PCR amplification (Demeke and Jenkins, 2010, Sahu *et al*., 2012). Polyphenolic compounds interact irreversibly with DNA, resulting inability of different modifying enzymes to manipulate the DNA (Manoj *et al*., 2007). Polysaccharides present in isolates make the DNA solution highly viscous and make it difficult for pipetting (Fang *et al*., 1992).

The removal of PCR inhibitors (Fredricks and Relman, 1998) and use of spermidine (Wang *et al*., 2012) to improve the PCR efficiency are previously reported. For example, in barley (Ahokas and Erkkila, 1993), cotton (Wan and Wilkins, 1993), pomegranate (Ono *et al.,* 2012) and human stool samples (Roperch *et al*., 2015, Kikuchi *et al*., 2010). However, the quantity per reaction varies in different applications reported. Therefore, here we present and discuss an optimized CTAB based DNA extraction protocol and spermidine unified PCR protocol applicable for a wide range of plant species with different secondary metabolic profiles.

DNA extraction

Since allocation of funds for research work is limited in developing countries like Sri Lanka, scientists should track into low-cost methodologies. In that regard, it would be more beneficial to improve CTAB extraction procedure rather than optimizing expensive column based methods to collect good quality DNA. Even in the long run, low

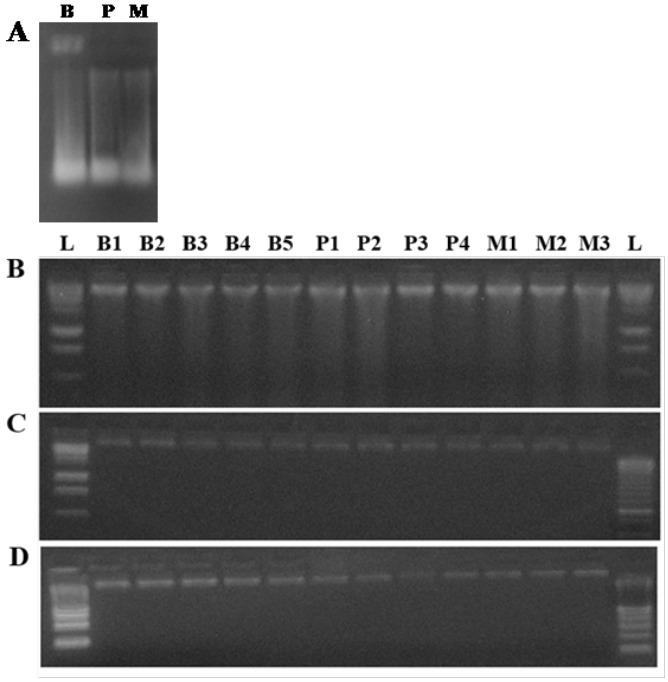

Figure 1: Agarose (1%) gel electrophoresis of genomic DNA extracted from different methods A: CTAB Method (ladders are not shown and DNA samples of three species were separately electrophoresed); B: Modified CTAB method, C: Promega Wizard® Genomic DNA Purification kit, D: QIAGEN DNeasy® Plant Mini kit. L: 1kb DNA molecular weight marker (cat no. promega G571A), B1, B2, B3, B4, B5: five elite accessions of Bael, P1, P2, P3, P4 : four selections of Pomegranate, M1, M2 and M3: three selections of mango.

Table 2: Assessment of quality and quantity of extracted DNA

Species	DNA extraction method	DNA concentration (ng/µL)	260/280	260/230
	CTAB*	1250[a]	1.71	0.92
Bael	Promega Wizard® Genomic DNA Purification kit	85[b]	1.75	1.12
	QIAGEN DNeasy® Plant Mini kit	82[b]	1.79	1.31
	CTAB*	1300[a]	1.65	0.82
Pomegranate	Promega Wizard® Genomic DNA Purification kit	65[b]	1.76	0.92
	QIAGEN Dneasy® Plant Mini kit	87[b]	1.81	0.87
	CTAB*	1150[a]	1.50	0.85
Mango	Promega Wizard® Genomic DNA Purification kit	78[b]	1.72	0.98
	QIAGEN Dneasy® Plant Mini kit	99[b]	1.76	0.87

* The modified CTAB method was used.

Means denoted by the same letter within the column are significantly different at P< 0.05.

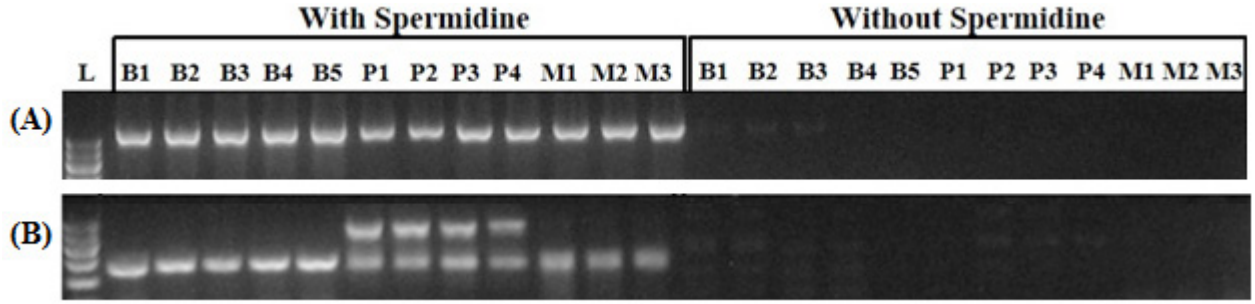

Figure 2: Comparison of the effect of spermidine in PCR amplification (A) plant barcoding region using rbcLprimer(550 bp band was detected). (B) SSR (300 bp band) and ISSR marker regions (300-600 bp band/s) L: 100 bp DNA molecular weight marker (cat no. QIAGEN 239035); B1, B2, B3, B4, B5: five elite selections of Bael; P1, P2, P3, P4: four selections of Pomegranate; M1, M2, M3: three selections of Mango.

cost methods preferred for large scale genotyping and other applications as commercial kits are not affordable in that context.

NanoDrop readings and agarose gel electrophoresis confirmed the presence of DNA in all the samples (Table 2 and Figure 1). For CTAB method, mostly low intense and smear like bands were observed (Figure 1A). However, the modified CTAB method showed significantly higher NanoDrop readings than those of commercial kits with mean DNA concentrations of 1233 ng/µL and 83 ng/µL respectively. Similarly, when same volumes of DNA loaded into an agarose gel, high intense bands were observed for CTAB method compared to kits (Figure 1B, 1C and 1D). NanoDrop readings of 260/280 and 260/230 values were more or less similar among different methods (Table 2). However, DNA extracted with CTAB method showed presence of relatively higher phenolic content and other low molecular weight contaminants (Figure 1B).

The original CTAB protocol (Doyle and Doyle, 1987) has modified a lot over time. Here, other than minor modifications in incubation time and volumes, the key modification was repeating the 24:1 chloroform/ isoamyl alcohol step. This additional step increased the quality of extracted DNA drastically, compared to single step. It is mainly due to removal of phenolic compounds and other secondary metabolites present in the samples. Though accuracy is less in spectrometer based concentration measurements, it is still used widely. According to the results, modified CTAB protocol has given significantly higher concentration of DNA compared to the commercially available kits with same amount of starting materials. Though there is no observable RNA contamination, there is little polyphenol or other substances remaining in the sample, appear as a smear. Higher concentrations achieved with modified CTAB protocol are obviously beneficial for applications such as genotyping needed to use the same sample with different primer combinations.

PCR

The universally accepted plant barcoding region; *rbcL* has been amplified successfully from many plant species (Group, CBOL Plant Working e*t al* 2009). However, neither modified CTAB extracted DNA samples nor the commercial kits successfully amplified the *rbcL* region in the absence of spermidine (Figure 2A). However, inclusion of spermidine into the reaction had positive effect on PCR amplification and resulted the expected band of ~550bp in all samples.

Both quality and quantity of DNA are critical for the applications such as SSR, ISSR and RAPD. Therefore, in addition to amplification of *rbcL* region, same samples were amplified with either SSR or ISSR primers. While for bael, Rutaceae family specific SSR markers 12 (R) 13 (F) which supposed to result a ~300 bp fragment were used, mango samples were amplified with species specific SSR marker 84 (R & F) with expected band size of ~300 bp. Pomegranate DNA samples were amplified with ISSR primer 842 and it resulted two products in the range of 300 -600 bp. As in the *rbcL* example, no PCR amplification was observed for SSR and ISSR for all samples without adding spermidine. Incorporation of spermidine resulted clear, repeatable amplification in all the samples (Figure 2B). The results confirm that the effect of remaining contaminants in DNA extracted with modified CTAB method could suppress with the inclusion of spermidine.

A concentration series of spermidine was tested with similar PCR conditions and 0.8 µM was en-counted as the optimum concentration. Optimization of PCR amplification conditions by the addition of spermidine significantly enhanced the amplification of universally accepted bar coding region *rbcL*, SSR and ISSR markers in all three fruit species. Other than the selected species, PCR amplification of *Gotukola* (*Centella asiatica*), *Kithul (Cryota urenus), Strobilanthes* and *Sandalwood* (*Santalum album*) with high phenolic content was successful in the presence of spermidine (data not shown). Thus, the broad spectrum applications indicate that the possibility of routine supplement of spermidine for PCR reactions to overcome inhibition generated from secondary metabolites. Further, inclusion of spermidine had no negative effect on PCR reactions with high quality DNA extracted from species such as *Arabidopsis* or *Oryza*. Previous findings suggest that spermidine attenuate the DNA intercalater, actinomycin D, which is a natural inhibitor of transcription and replication (Wang *et al*., 2012). Further, the polyamines such as spermine and spermidine are known to induce autophagy in cell lines (Madeo *et al*., 2010), improve responses to chemotherapy (Allen *et al*., 2007) and found to present in

breast milk as an important ingredient (Atiya-Ali *et al*., 2014).

Overall, optimized CTAB DNA extraction together with incorporation of spermidine successfully amplified relatively easy PCR reactions as well as difficult PCR reactions from all three plant species considered. Therefore, the results of this research provide evidence for the wide range of applications of optimized protocols, for different plant species and different types of PCR amplifications.

CONCLUSIONS

DNA extraction from the plant species that consist of high amount of phenolic compounds could be accomplished with all three extraction methods tested here. However, with the same amount of starting materials, the modified CTAB method yielded higher amount of total DNA than commercial kits, without sacrificing the quality. Inclusion of spermidine at the rate of 0.8 µM improved the efficiency of PCR for easily amplifiable and difficult primer pairs in all the species reported. It could be concluded that, CTAB method with necessary modifications yields high quality DNA and the inclusion of spermidine facilitates successful amplification in PCR for all the three species implying the importance of alleviating PCR inhibitors.

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