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Short communication

Optimising DNA isolation for medicinal plants

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Abstract

In African traditional health care systems medicinal plants have long been known to contain pharmacologically active compounds. This has led to an excessively high demand of these plant products resulting in the extinction of some plant species. With the application of molecular techniques in plant diversity conservation becoming increasingly popular, the isolation of PCR amplifiable genomic DNA becomes an important pre-requisite. However, medicinal plants are known to contain high levels of polyphenols and polysaccharides posing a major challenge in the isolation of high quality DNA. The objective of our research was to optimize a cetyl-trimethyl ammonium bromide (CTAB)-based protocol for the extraction of genomic DNA from a range of medicinal plant species, namely *Sclerocarya birrea* (tree), *Barleria greenii* (shrub), *Aloe polyphylla* and *Huernia hystrix* (both succulent plants). The quantity of DNA (µg/g) isolated using the modified CTAB protocol was higher for the lower plant tissue amounts (0.1 and 0.2 g) per 500 µl of extraction buffer. The spectral quality of DNA as measured by the A_{260}/A_{280} ratio ranged from 1.76 to 2.14 for *S. birrea*, *B. greenii* and *A. polyphylla* and 1.39 to 1.74 for *H. hystrix*. The DNA purity was further confirmed by restriction endonuclease digestion and PCR gel electrophoresis using operon arbitrary decamer primers (OPB-05, OPB-06 and OPG-07). The results show that the optimization of the amount of plant tissue per extraction buffer volume is a critical factor in genomic DNA isolation. In all cases the isolated DNA yielded high quantities from small amounts of plant tissue, and had good spectral qualities amenable to restriction endonuclease digestion and PCR amplification.
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Keywords: DNA isolation; Gel electrophoresis; Medicinal plants; PCR amplification; Restriction endonuclease digestion; Secondary metabolites

1. Introduction

Medicinal plants are in high demand in African health care systems due to their accessibility and efficacy, leading to many of these plants becoming threatened with extinction. These medicinal and aromatic plants have long been known to contain high levels and different types of secondary metabolites, which are often responsible for their therapeutic and pharmacological activities. Application of molecular techniques would increase and facilitate production of these substances, help in plant conservation (Pirttilä et al., 2001) as well as prevent biological piracy. Furthermore, techniques such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) are useful in studying plant diversity, genetic transformation and clonal fidelity determination of micropropagated plants. The isolation of high molecular weight genomic DNA is a pre-requisite for these molecular techniques. However, high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins in tree species usually interfere with DNA isolation (Mishra et al., 2008). Similarly, Diadema et al. (2003) observed that genomic DNA extraction from succulent plants is difficult. These authors reported that the difficulty could be attributed to small cell density in succulent tissues and high levels of contaminants (polyphenols or polysaccharides) that co-precipitate with DNA. Polyphenols released from the vacuoles during the cell lysis process are oxidized by cellular oxidases and undergo irreversible interactions with nucleic acids causing browning of the DNA (Varma et al., 2007). The presence of gelling polysaccharides prevents complete dissolution of nucleic acids and imparts a viscous constituency to the DNA making it stick to the wells during gel electrophoresis (Barnell et al., 1998; Diadema

Abbreviations: CTAB, Cetyl-trimethyl ammonium bromide; EDTA, Ethylene diamine tetraacetic acid; PCR, Polymerase chain reaction; PVPP, Polyvinyl polypyrrolidone; RAPD, Random amplified polymorphic DNA.

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et al., 2003; Varma et al., 2007). According to these authors, residual polyphenols, polysaccharides and secondary metabolites interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases.

Though several successful genomic DNA isolation protocols for high polyphenol and polysaccharide containing plant species have been developed, none of these are universally applicable to all plants (Varma et al., 2007). Researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma et al., 2007). A good isolation protocol should be simple, rapid and efficient, yielding appreciable levels of high quality DNA suitable for molecular analysis. Križman et al. (2006) were of the opinion that, among other factors, the amount of plant sample extracted could be critical in keeping an extraction procedure robust. Our objective was to optimize a cetyl-trimethyl ammonium bromide (CTAB)-based extraction procedure amenable for the isolation of high quality DNA in a diverse range of plant species. Four plant species with high polyphenol content and/or gelling polysaccharides were used in this study and they included a tree species [*Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae)], a shrub [*Barleria greenii* M. Balkwill & K. Balkwill (Acanthaceae)], and two succulent plants [*Huernia hystrix* (Hook.f.) N.E.Br. (Asclepiadaceae) and *Aloe polyphylla* Schönland ex Pillans (Asphodelaceae)].

2. Materials and methods

2.1. Plant material

Leaves were obtained from *S. birrea* seedlings grown under controlled temperature of 30 °C in a Conviron (Controlled Environments, Canada) and *in vitro* raised *A. polyphylla* plants. Leaf and stem materials from *B. greenii* and *H. hystrix* respectively were collected from plants growing in the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. Leaf material was collected from actively growing parts of the plants. The plant materials were collected fresh just before extraction was done.

2.2. Reagents and chemicals

The chemicals and reagents used in the isolation of DNA were: CTAB extraction buffer [2% (w/v) CTAB; 20 mM EDTA, pH 8.0; 100 mM Tris–HCl, pH 8.0; 1.4 M NaCl]; CTAB/NaCl solution [10% (w/v) CTAB; 0.7 M NaCl mixed at 65 °C with stirring]; CTAB precipitation solution [1% (w/v) CTAB; 50 mM Tris–HCl, pH 8.0; 10 mM EDTA]; high salt TE buffer [10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1.0 M NaCl]; TE buffer [10 mM Tris–HCl, pH 8.0; 1.0 mM EDTA, pH 8.0]; chloroform: isoamyl alcohol (24:1, v/v); *iso*-propanol; absolute ethanol; 80% ethanol; polyvinylpolypyrrolidone (PVPP); 2-mercaptoethanol (2ME) and liquid nitrogen.

2.3. DNA extraction procedure

1. Five hundred microlitres of CTAB extraction buffer heated to 65 °C was added to 3% PVPP in a 1.5 ml sterile Eppendorf tube. 2-Mercaptoethanol (10 µl) was added to this mixture just before the start of the extraction process.
2. Plant materials were frozen in liquid nitrogen (−196 °C) in a sterile mortar and ground to a fine powder. The plant tissue amounts used were 0.1, 0.2, 0.3 and 0.4 g except for *H. hystrix* where 0.5 g was included.
3. The ground frozen tissue was then added to a warm (65 °C) PVPP/2-ME/CTAB extraction solution (prepared in step 1) followed by incubation at 65 °C for 30 min in a warm water bath. The mixture was regularly mixed by gentle inversion.
4. Chloroform:isoamyl alcohol (24:1, v/v) was added in equal volume to the homogenate and mixed gently by inversion, followed by centrifugation at 10,000 rpm (7500 ×*g*) for 5 min. The upper phase was pipetted into a sterile Eppendorf tube. This step was repeated twice.
5. CTAB/NaCl solution (0.1 volumes at 65 °C) was added to the recovered upper phase and mixed gently by inversion. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added to the mixture. After mixing and centrifuging for 5 min at 10,000 rpm (7500 ×g) the upper phase was recovered. This step was repeated twice.
6. One volume of CTAB precipitation solution at 65 °C was added to the recovered supernatant. A precipitate formed at this stage and the mixture was centrifuged at 2700 rpm (500 ×g) for 5 min. DNA pellet was recovered by decanting the supernatant.
7. The DNA pellet was dissolved in 500 µl of high-salt TE buffer. The DNA was then precipitated by adding 0.6 volumes (300 µl) of ice-cold *iso*-propanol followed by gentle mixing and centrifugation for 15 min at 10,000 rpm (7500 ×g).
8. The DNA pellet was recovered by decanting the supernatant followed by washing in 80% ethanol and 100% ethanol. After drying, the pellet was re-suspended in TE buffer and stored at −20 °C until further use.

2.4. DNA quantification

The quantification of genomic DNA was achieved using a spectrophotometer (UV–Visible spectrophotometer, Varian, Australia). The yield was determined by measuring the absorbance at A_{260}, A_{280} and A_{320} nm. The level of DNA purity was determined by the A_{260}/A_{280} absorbance ratio. DNA purity was further tested by running the extracted genomic DNA samples on 0.8% agarose gel stained with 0.25 µg/ml ethidium bromide in 1× TAE (Tris base, glacial acetic acid, 0.5 M EDTA) gel buffer. A DNA molecular weight marker (GeneRuler™ DNA Ladder Mix) was included on each gel. The gels were visualized and photographed under UV light (BTS-20 M model, UVItec Ltd, Cambridge, UK).

2.5. Restriction endonuclease digestion

The suitability of the extracted genomic DNA for downstream molecular analysis was further determined by restriction digestion using *Eco*RI (Fermentas). The digestion was performed at 37 °C for 4 h in a 40 µl reaction volume containing 5 µg genomic DNA, 4 µl of 10× restriction buffer and 40 U of restriction

Table 1
Effect of sample weight on genomic DNA quality and quantity

Plant species	Sample weight (g)	A_{260}/A_{280} ratio	DNA yield (μg/g fresh weight)
Sclerocarya birrea	0.1	1.76[a*]	408.7[b]
	0.2	1.95[b]	198.8[a]
	0.3	1.99[b]	217.4[a]
	0.4	2.00[b]	179.1[a]
Barleria greenii	0.1	1.99[a]	486.2[c]
	0.2	2.01[a]	390.9[bc]
	0.3	2.03[a]	257.2[ab]
	0.4	1.95[a]	173.7[a]
Huernia hystrix	0.1	1.39[a]	99.6[ab]
	0.2	1.73[a]	205.3[b]
	0.3	1.74[a]	103.3[ab]
	0.4	1.50[a]	72.0[a]
	0.5	1.46[a]	11.2[a]
Aloe polyphylla	0.1	2.15[b]	145.0[a]
	0.2	1.86[a]	125.9[a]
	0.3	1.78[a]	64.9[a]
	0.4	1.76[a]	68.6[a]

*Values followed by different letters in a column per plant species are significantly different at α=0.05 according to Duncan's Multiple Range Test (DMRT) (n=5 for *H. hystrix*, n=4 for other species).

endonuclease enzyme. The digested DNA was confirmed on 0.8% agarose gel.

2.6. PCR amplification

The PCR for *S. birrea* contained approximately 50 ng of genomic DNA (Dawson et al., 1995), 1 U of Taq DNA polymerase (Bioline™ DNA polymerase), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Bioline), 0.2 μM each of OPB-05 and OPB-06 random decamer primers (Operon Technologies, Alameda, USA), 1.5 mM $MgCl_2$ and 10× NH_4 reaction buffer. The reaction volume was adjusted to 50 μl using sterile HPLC grade water and overlaid with 50 μl paraffin oil. Amplification was carried out using a Hybaid Thermal Reactor (Hybaid 1991 Model, Hybaid Ltd., UK) programmed for 45 cycles of 92 °C for 1 min, 36 °C for 2 min, 72 °C for 2 min, followed by a final extension step of 72 °C for 5 min (Dawson et al., 1995). DNA amplification reaction was performed for *H. hystrix* in 25 μl reaction volumes containing 10 ng genomic DNA, 0.1 mM each of dATP, dCTP, dGTP and dTTP (Bioline), 0.2 μM of OPG-07 random decamer primer, 1.5 U of Taq DNA polymerase and 2.5 μl of PCR reaction buffer. The programme for the reaction was 45 cycles of 94 °C for 1 min, 38 °C for 1 min, 54 °C for 30 s, 72 °C for 2 min, followed by an extension step of 72 °C for 15 min (De la Cruz et al., 1997). The amplification products for both plant species were resolved on 1.5% agarose gel.

3. Results and discussion

The effect of different plant tissue amount on the quality and quantity of isolated DNA is shown in Table 1. The quality of DNA was assessed by spectrophotometry, gel electrophoresis, restriction endonuclease digestion and PCR amplification. A ratio of absorbance (A_{260}/A_{280}) in the range 1.8–2.0 indicates a high level of purity (Pašakinskienė and Pašakinskienė, 1999; Weising et al., 2005). The $A_{260}/A_{280\ nm}$ absorbance ratio ranged from 1.76–2.0 for *S. birrea* and *B. greenii* for plant tissue mass in the range 0.1–0.4 g, indicating high purity of the isolated DNA. However, the yield decreased with increasing plant tissue amount per extraction buffer volume. The highest yields in *S. birrea* and *B. greenii* (408.7 and 486.2 μg/g respectively) were obtained from a sample weight of 0.1 g per 500 μl of extraction buffer (Table 1). In the case of *H. hystrix* and *A. polyphylla*, the optimum tissue amount for good quality and quantity genomic DNA was 0.2 g per 500 μl of extraction buffer (Table 1). Plant tissue amounts below and above the 0.2 g-threshold resulted in a reduction in spectral qualities.

Gel electrophoresis of the isolated DNA further showed intact genomic DNA bands of high molecular weight (greater than 10,000 base pairs) with little or no RNA contamination

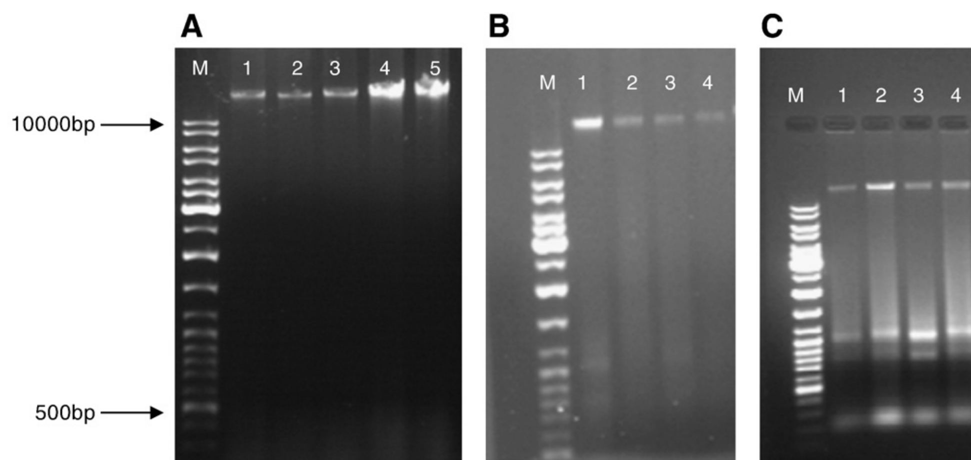

Fig. 1. Genomic DNA resolved on 0.8% agarose gel showing (A) *H. hystrix* (B) *B. greenii* and (C) *S. birrea* subsp. *caffra*. 'M' represents the Molecular Weight Marker — GeneRuler™ DNA Ladder Mix. Lanes numbering from left to right represent isolated DNA at increasing sample weights — 0.1, 0.2, 0.3, 0.4 and 0.5 g.

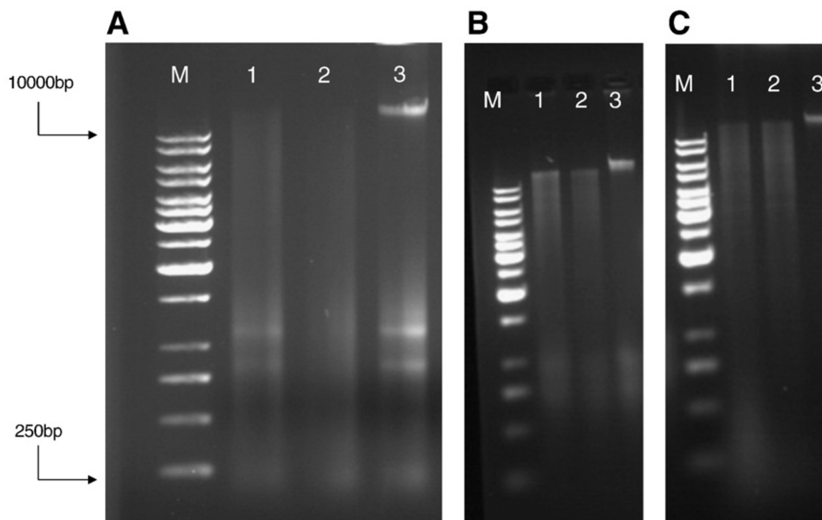

Fig. 2. Restriction endonuclease digestion of DNA resolved on 0.8% agarose gel (A) *B. greenii* (B) *A. polyphylla* and (C) *H. hystrix*. 'M' represents the Molecular Weight Marker — GeneRuler™ 1 kb DNA ladder. Lanes 1 and 2 represent DNA restricted by *Eco*R I. Lane 3 is the unrestricted DNA.

(Fig. 1). Križman et al. (2006) postulated that the plant tissue amount per volume of extraction buffer has an effect on DNA quality and yield. Since the extraction buffer is responsible for the lysis of membranes and liberation of DNA from cellular organelles (Weising et al., 2005), the smaller the quantity of plant tissue per unit volume, the more optimal the lysis process. Striking the correct balance between plant tissue amount and extraction buffer volume would reduce the probability of co-precipitation of contaminants with the DNA pellet as the saturation concentration during precipitation is less likely reached or exceeded (Križman et al., 2006).

The suitability of extracted DNA for downstream molecular processes was further verified by restriction endonuclease digestion and RAPD-PCR amplification. As shown in Fig. 2, the isolated DNA was amenable to restriction digestion using *Eco*RI. The genomic DNA of *S. birrea* and *H. hystrix* was highly amplifiable by PCR as indicated by the amplification products resolved on 1.5% agarose gel (Fig. 3). This further confirmed the purity of the DNA, free of polysaccharide and polyphenol contamination, which would otherwise inhibit Taq DNA polymerase and restriction endonucleases (Ahmad et al., 2004). Plant molecular applications such as RAPD and AFLP necessitate the successful isolation of high quality DNA (Michiels et al., 2003; Ahmad et al., 2004), devoid of contaminants. Without high quality DNA such downstream molecular manipulations are not feasible (Varma et al., 2007).

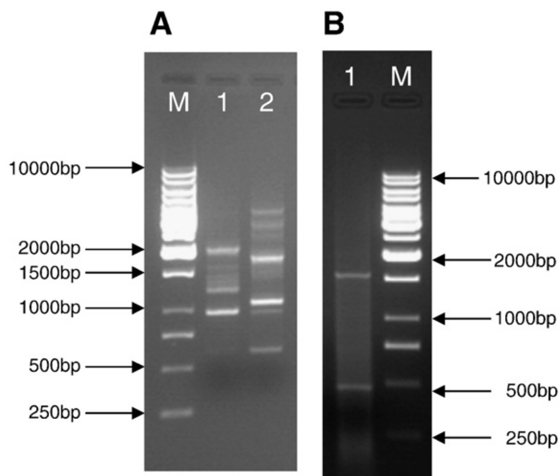

Fig. 3. Gel electrophoresis (1.5% agarose) showing PCR profiles of amplified DNA from (A) *S. birrea* subsp. *caffra* using arbitrary primers OPB-05: 5′-TGCGCCCTTC-3′ (Lane 1), OPB-06: 5′-TGCTCTGCCC-3′ (Lane 2); (B) *H. hystrix* using arbitrary primer OPG-07: 5′-GAACCTGCGG-3′ (Lane 1). 'M' represents the Molecular weight marker — GeneRuler™ 1 kb DNA ladder.

Our results show that the optimization of the amount of plant tissue per unit volume of extraction buffer is one of the most critical factors in plant DNA isolation procedures. The isolated genomic DNA for *S. birrea*, *H. hystrix*, *B. greenii* and *A. polyphylla* yielded high quantities from small amounts of plant tissue, and had good spectral qualities amenable to restriction endonuclease digestion and PCR amplification. The protocol uses small amounts of plant tissue, can be performed within 2–3 h and allows for the simultaneous extraction of a large number of samples. Moreover, the procedure eliminates the need for RNAse treatment and can be applied to different plant species.

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