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A Protocol for the Isolation of DNA from *Trochetia boutoniana*

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ABSTRACT

Various problems are encountered during DNA extraction from plant species harbouring high levels of secondary metabolites and polysaccharides. Trochetia species are known to synthesize a wide spectrum of polyphenols including flavonoids. These compounds represent a significant barrier to the extraction of pure genomic DNA. This study describes, for the first time, a reliable protocol for extracting good quality DNA in reasonable amount from *Trochetia boutoniana*, the National flower of Mauritius. DNA was isolated using the Porebski *et al.* (1977), method but with various modifications made. This modified protocol used 1.6 M NaCl, 2% CTAB and 2.5% PVP of molecular weight 40,000, 0.5% β- mercaptoethanol and an incubation period of 35 min at 60°C. The purity of the extracted DNA was assessed by spectrophotometry. The quantity of DNA obtained was equivalent to 238.5µg per gram of fresh leaf material and it was amenable to restriction digestion.

Key Words: DNA isolation; DNA purification; Leaf material; Polyphenols; Polysaccharides; *Trochetia boutoniana*
Abbreviations: CTAB, cetyltrimethylammonium bromide; IUCN, International Union for the Conservation of Nature and Natural Resources; Mr; molecular weight; PVP, polyvinylpyrrolidone; RT, room temperature; SDS, sodium dodecyl sulphate.

INTRODUCTION

The genus Trochetia, member of the Sterculacea family, is an important endemic group to the Mascarene Islands and consists of six recognised species, namely, *Trochetia granulata, Trochetia uniflora, Trochetia triflora, Trochetia boutoniana, Trochetia blackburniana* and *Trochetia parviflora* (Strahm, 1993). Out of these, the last five are from Mauritius. *T. boutoniana* is a shrub 2-3 m high with a very short trunk which branches profusely at the base. It bears spectacular earring-shaped carmine red colour flowers locally known as "Boucle d`oreille" (Fig. 1).

Its magnificent flowers have greatly captured the interest of botanists and it was declared the National flower of Mauritius in 1992. The most important population is located on the cliffs of Le Morne Brabant in the South-west of Mauritius but other discrete individuals scattered over the island have also been reported. Recently, a renewed interest has spurred after a tantalizing finding regarding the capability of the flowers of *T. boutoniana* to produce coloured nectar. Unfortunately, the plant is on the red list of critically endangered species (IUCN, 1994). The invasion of "goyave de chine" species has stifled the Trochetia species by competing with them for space and sunlight. Also, removal of flowers by visitors and attack by monkeys are the key reasons for the dwindling of the Trochetia`s population. Recently, the Ministry of Agriculture Food Technology and Natural Resources have been working in the regeneration of *Trochetia boutoniana* by conventional seed propagation. However, proper identification of the species seems to be a problem. Prior to envisaging the use of molecular tools for the proper identification of this species and to elucidate phenomena such as genetic variation, gene flow and hybridisation, the establishment of a proper DNA extraction protocol remains the foremost step. The quantity and quality of genomic DNA will undeniably determine the outcome of any molecular studies following. Basically a DNA extraction protocol involves lysis of the cell wall and membranes by use of detergents such as SDS or CTAB to release the genetic material in an extraction buffer. However, the extraction of DNA is sometimes compounded by the presence of complex polysaccharides and phenolic compounds which affect the yield and quality of DNA. Polysaccharidelike contaminants are particularly problematic (Scott & Playford, 1996) and more difficult to remove. Polysaccharides can cause anomalous re-association kinetics (Merlo & Kemp, 1976). They can also coprecipitate with DNA after alcohol addition during DNA isolation to form highly viscous solutions (Do & Adams, 1991). The DNA is unsuitable for restriction and Southern hybridization and often remains in the wells during electrophoresis (Sharma *et al*., 2002). Polysaccharides can inhibit the activity of certain DNA-modifying enzymes and may interfere in the quantification of nucleic acids with spectrophotometric methods (Wilkie *et al.,* 1993).

Antioxidants are commonly used to address problems related to phenolics. Examples include the use of β-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA), sodium azide, and polyvinylpyrrolidone (PVP) (Dawson & Magee, 1995; Clark, 1997). Phenol extractions coupled with SDS are also helpful. However, SDS-phenol tends to produce low DNA yields of plants rich in polyphenolics (Rezaian & Krake, 1987).

Although a plethora of plant DNA isolation protocols exist, there is a paucity of information regarding extraction

procedures for Trochetia species. Preliminary HPLC studies have shown the presence of high level of polyphenolic compounds in *T. boutoniana,* mainly flavonoids that might interfere with the extraction of large quantity of pure DNA. This study describes an optimised protocol suited specifically for DNA extraction in *Trochetia boutoniana*.

MATERIALS AND METHODS

As a preliminary step, a modified Dellaporta *et al.* (1985) - (Aljanabi *et al.,* 1999) protocol was used for the extraction of DNA. However, the protocol yielded DNA which was brown in colour. Consequently, the Porebski *et al.* (1997) method with some modifications was used.

Plant materials for DNA isolation. Young, tender and unbruised leaves of *Trochetia boutoniana* were used as plant materials. For all DNA extraction procedures fresh materials were used to avoid any DNA degradation which might occur during freezing. The leaves were chosen for DNA extraction due to their continued availability whole year round.

Solutions. An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris-HCl (pH 8.8), 20 mM EDTA (pH 8.8), 1.6 M NaCl, 2.5% PVP (Mr 10,000), and 0.5% β-mercaptoethanol was prepared. Chloroform: isoamylalcohol (24:1), 70 and 95% ethanol, 3 M NaCl and a TE buffer consisting of 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8.4) were also needed.

DNA Isolation and purification. 0.5 g of fresh leaves was ground in liquid nitrogen using a mortar and a pestle. 15 mL of preheated (60°C) extraction buffer were then added and the tubes mixed by inversion. After mixing for some minutes the tubes were incubated in a water-bath at 60°C for 35 min. The tubes were then allowed to cool to room temperature before adding an equal volume of chloroform:octanol (24:1). After mixing of the tubes by inversion to form an emulsion, the tubes were centrifuged at 8000 g for 15 min. at room temperature (RT). After centrifugation the top aqueous solution was transferred to new tubes using a micropipette. The chloroform: octanol extraction step was repeated to remove cloudiness in the aqueous phase. The tubes were gently inverted about 12 times. To accentuate precipitation of DNA, the mixtures were placed in freezer (-20°C) for 10 min. The DNA was pelletted by centrifugation at 8000 g for 10 min at 4°C. Then the supernatant were poured off the resulting pellets were washed twice with 70% ethanol and allowed to air dry for 1 h. The pellets were then dissolved in 300 µL TE buffer. To remove any RNA from the preparation, 7 µL Rnase A was added, and the tubes were incubated at 37°C for 1 h. An equal volume of chloroform-isoamylalcohol was added and the mixture centrifuged at 8000 g for 5 min. and the upper aqueous layer was gently removed with the help of a micropipette. To this supernatant 1/10 vol. of 3 M NaCl was added followed by 2 vol. of cold absolute ethanol. The mixture was placed in freezer at -20°C for 1 h. followed by centrifugation at 12000 g for 10 min. The resulting pellets were washed with 70% ethanol, air dried and resuspended 100 µL of 0.1x TE.

Measurement of amount and purity of DNA. The DNA yield per gram of leaf tissue was measured by using a UV-VIS Spectronic Genesys 5 (Milton Roy) spectrophotometer at 260 nm. DNA purity was determined by calculating the absorbance ratio $A_{260/280}$. Pure DNA has a ratio of 1.8 ± 0.1 (Clark, 1997). Polysaccharide contamination was assessed by calculating the absorbance ratio $A_{260/230}$ (Wilson & Walker, 1997). DNA samples from the leaf tissues were electrophoresed on a 1% agarose gel.

RESULTS AND DISCUSSION

The modified Dellaporta method of extraction (Aljanabi *et al.,* 1999) gave maximum DNA yield of 332.6 µg per gram of leaf material. However, it was heavily contaminated with polysaccharides and polyphenols as indicated by $A_{260/280}$ and $A_{260/280}$ ratios of 0.488 and 1.530, respectively. The samples were very viscous and brown in colour. Upon electrophoresis on a 1% agarose gel, the DNA extracted by this method showed uneven migration producing thick fire-like trailing smears with traces of contaminants (Fig. 2).

When the Porebski *et al.* (1997) extraction protocol was used the DNA yield was reduced to 306.2 µg per gram of leaf material. The purity on the other hand improved ($A_{260/280}$ and $A_{260/280}$ ratios of 1.257 and 1.640, respectively) and the bands following electrophoresis were sharper. Several modifications were made to the Porebski *et al.* (1997) protocol. Use of 0.5% β- mercaptoethanol, 1.6 M NaCl and 2.5% PVP (Mr 10000) were found to be most appropriate. Although the yield of the DNA was further reduced to 283.5 µg per gram of leaf material, the $A_{260/280}$

Fig. 1. *Trochetia boutoniana* flower

Fig. 2. Electrophoresis of genomic DNA of *Trochetia boutoniana* obtained by the modified Dellaporta method on 1 % agarose gel. From the three lanes thick fire-like trailing smears were obtained.

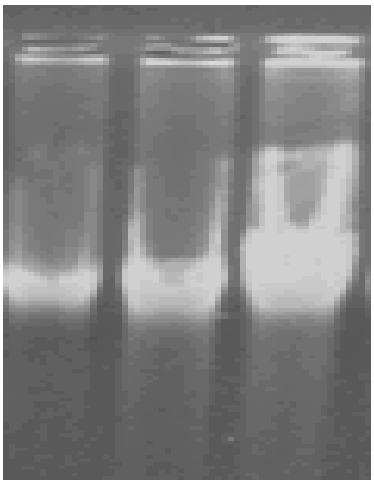
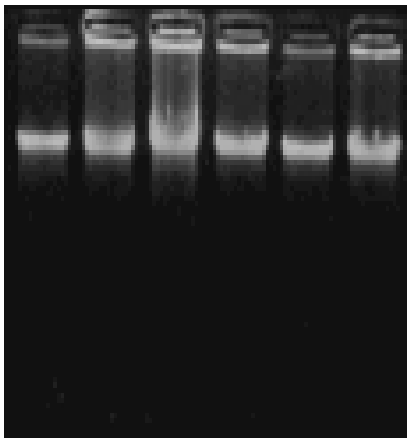


Fig. 3. Electrophoresis of genomic DNA of *Trochetia boutoniana* obtained by the modified Porebski method on 1 % agarose gel. From the six lanes thick distinct genomic bands were obtained showing no sign of degradation.



and $A_{260/280}$ ratios improved (1.842 & 1.740, respectively). Upon electrophoresis on 1% agarose gel, thick and distinct bands were obtained (Fig. 3).

The absence of smears indicates a high purity in the nucleic acids extracted. While the modified Dellaporta protocol was not efficient in getting rid of the polysaccharides and phenolic compounds, the modified Porebski protocol gave high quality DNA free from polyphenols and polysaccharides. Basically the Dellaporta and the modified Porebski protocols make use of more or less the same reagents but the differences lie in the concentration of certain reagents, namely NaCl and β-mercaptoethanol. A higher concentration of NaCl (1.6 M) together with CTAB in the Porebski method helped to remove more polysaccharides thus resulting in good quality DNA. The addition of NaCl at concentrations higher than 0.5 M, together with CTAB, is known to remove polysaccharides (Murray & Thompson, 1980; Paterson *et al*., 1993). The concentration ranges mentioned in the literature varies between 0.7 M (Clark, 1997) and 6 M (Aljanabi *et al*., 1999) and is dependent on the plant species under investigation. Some protocols replace NaCl with KCl (Thompson & Henry, 1995). 1.6 M of NaCl seems to work best for DNA extraction in *T. boutoniana*. The high quality DNA obtained by the modified Porebski method can also be attributed to the use of a higher concentration of PVP (2.5%) of lower molecular weight (10,000) rather than 40,000 as used in the Dellaporta protocol. A number of workers (Couch & Fritz, 1990; Chaudhry *et al*., 1999) recommend the use of PVP (Mr 10000) at 2% (w/v) to address the problem of phenolics. Others (Stewart & Via, 1993; Porebski *et al*., 1997; Zhang & Stewart, 2000) recommend the use of a higher-molecular-weight PVP (i.e., 40000 at 2% [w/v]) instead. In our experiments, adding 2.5% of PVP Mr 10000 improved the colour of the nucleic acid obtained. Lower molecular weight PVP has less tendency of precipitating with the nucleic acids as compared to the high molecular weight ones thus yielding sufficient amount of polyphenol-free DNA. Modifying the concentrations of NaCl and β-mercaptoethanol along with the use of low molecular weight PVP and a second extraction using 3 M NaCl were therefore crucial for the isolation of pure DNA from *T. boutoniana*. It is hoped that the method described here will help in characterisation using molecular tools and genetic diversity studies regarding *Trochetia boutoniana*.

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