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Full Length Research Paper

Two mini-preparation protocols to DNA extraction from plants with high polysaccharide and secondary metabolites

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Were standardized two previously reported standard plant DNA extraction methods, but improved them on mini preparations to use the samples for population genetic analysis. The combination of CTAB lysis procedure-solvent extraction and DNA column purification (DNeasy plant mini kit modification) enables a faster and reliable DNA extraction from all samples tested: *Piper, Quercus, Zea* and cacti species (considered "hard" extraction species), and this protocol uses smaller tissue samples than other mini or midi-prep protocols. We obtained high quality and DNA yields in all samples tested. This alternative protocol (CTAB lysis-solvent extraction based) is an excellent option if there are many samples to process and it is also a non expensive protocol. This method also produces good DNA quality but fewer yields. Both two protocols produce reproducible PCR pattern-bands amplification with all the genetic markers tested (RAPD's and microsatellites). The DNA obtained was used in other molecular biology standard analysis methods, like enzymatic restriction patterns, ligation, sequencing and cloning with good results too.

Key words: DNA extraction, mini preparations, CTAB methods, anion-exchange membranes, plants.

INTRODUCTION

Several methodological problems have been documented previously for DNA extraction from some difficult plant samples, like tropical species, coriaceous leafs and tissues with high polysaccharide and secondary metabolites contents (Doyle and Doyle, 1990; Guillemaut and Maréchal-Drouard, 1992; Stewart and Via, 1993; De la Cruz et al., 1997; Csaikl et al., 1998; Chen and Ronald, 1999) and many of these species are characterized like "hard" or difficult tissues to extract DNA from. Also, there exist very complex protocols with high salt and detergents concentrations to clean polysaccharide and secondary metabolites and these causes background problems because many of those chemicals inhibit PCR reactions (Pandey et al., 1996) and affect the accuracy from some analyses like PCR, DNA cloning and sequencing.

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However, some studies involve screening large samples, such as population genetic analyses or evolutionary studies require high quality DNA. For such large scale work, it is necessary to use mini-preparations of DNA (Stewart and Via, 1993; Chen and Ronald, 1999; Doulis et al., 2000). Most of the normal DNA isolation protocols are inefficient with some tissues like *Quercus* and cacti species that we have worked with.

We improved two methods for DNA extraction in mini-preparations. One is a CTAB-based protocol, modified from De la Cruz et al. (1997) and the second is a combined CTAB and anion exchange chromatography-based protocol, modified from DNeasy™ Plant Mini Kit (Quiagen, 1999). Both protocols were tested in many difficult species with large quantities of samples: five species of tropical trees (genus *Piper*), two cacti species, 36 species from *Quercus* and three species from *Zea*. Both methods described here are faster than those reported previously and we obtained DNA yields from 500 ng to 1.5 µg, using 100 - 500 mg from initial leaf or

parenchyma tissue. The DNA samples obtained were used for RAPDs, microsatellite, restriction and sequencing analyses, and both protocols were evaluated for reproducibility, speed of preparation, reliability and final DNA quality obtained.

MATERIALS AND METHODS

Plant material

Several fresh, and some silica dried and frozen samples from each species (*Piper*, cacti, *Quercus* and *Zea*) were subjected to DNA extraction with our two protocols. All samples (except *Zea*) are considered "hard species" due to different secondary metabolites and high polysaccharide content, and are difficult for DNA extraction. We used leaf tissue (*Piper*, *Quercus* and *Zea*) and fresh parenchyma (cacti species) for the DNA extraction. Samples were frozen in liquid nitrogen and pulverized in a mortar. 500 mg per sample was used for CTAB-based protocol, and 200 mg for DNeasy Plant Mini Kit modification.

CTAB-based mini-preparation method

This is a modification from protocol to DNA extraction reported by De la Cruz et al. (1997), adapted for mini-preparations. This was optimized to a final volume of 2 ml.

Chemicals and solutions:

CTAB extraction buffer: 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 2% CTAB, 1.5 M NaCl, 4% PVP-40, 5 mM ascorbic acid, 5 mM DIECA and 10 mM 2-mercaptoethanol (De la Cruz et al., 1997).
STE buffer: 100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM NaCl, 10 mM 2-mercaptoethanol (De la Cruz et al., 1997).
20% SDS.
3 M potassium acetate.
TE buffer, pH 8.0.
Sterile and deionized water.
3 M sodium acetate, pH 5.2.
Cold isopropanol.
Chloroform:octhanol (24:1).

Protocol:

To 500 mg frozen and grounded plant tissue material, add 200 µl of CTAB extraction buffer and homogenize it 3-4 min.
Add 800 µl of STE buffer to homogenate and transfer to 2 ml polypropylene tube.
Add 50 µl of 20% SDS solution, with vigorous shaking for 7 min.
Incubate all samples in a water bath to 65°C for 15-20 min, with occasional gentle shaking.
Add 415 µl of cold potassium acetate 3 M and incubate in ice bath for 40 min.
Spin tubes at 12,000 rpm for 20 min to remove cellular debris and recover the supernatant carefully.
Add 7/10 volume of cold isopropanol, mix gently and incubate at –20°C for 40 min to precipitate genomic DNA.
Spin the samples at 15,000 rpm for 15 min and discard the supernatant. Air-dry the pellet and resuspend in 500 µl of TE pH 8.0.
Extract all samples with 1 volume of chloroform:octhanol (24:1), mix to with homogenate and spin at 10,000 rpm for 10 min. Extract again until the supernatant becomes clear.
Transfer the supernatant to a new tube and add 65 µl of 3 M sodium acetate and 600 µl of cold isopropanol and mix gently.
Incubate at –20°C overnight.
Spin at 15,000 rpm for 15 min and wash the DNA pellet with 1.2 ml of 96% ethanol.
Spin again at 15,000 rpm for 5 min and wash the pellet with 1.2 ml of 75% ethanol.
Air-dry the DNA pellet (be careful to not over dry it) and resuspend in 150 µl of sterile/deionized water.

CTAB and anion exchange chromatography-based method (DNeasy Plant Mini Kit modification)

This protocol is faster, and more reliable and efficient than the original QIAGEN protocol.

Chemicals and Solutions:

CTAB-based Carlson lysis buffer: 2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 1% polyethylenglycol 8000, 20 mM EDTA pH 9.5.
2-mercaptoethanol.
RNAase A (10 mg/ml).
DNeasy™ Plant Mini Kit.
Chloroform:octhanol (24:1).
Litmus paper.
20% Chlorhidric acid.

Protocol:

To 200 mg frozen and ground tissue plant material, add 900 µl of Carlson buffer (preheated to 65°C), 50 µl of 2-mercaptoethanol and 20 µl of RNAase A. Homogenize the sample 2-3 min.
Incubate all samples for 20 min at 65°C with gentle shake.
Cool 8 min to room temperature.
Extract the samples with one volume of chloroform:octhanol (24:1) and spin at 9,000 rpm for 10 min. Repeat until the supernatant become clear.
Recover the aqueous phase (upper) into a new sterile tube (2 ml) and add one volume of sterile water. Adjust the pH to 7.0 with 20% HCl (verify it with litmus paper).
Transfer the sample to the DNeasy column (white column), pippeting each time 650 µl and spinning at 9,000 rpm for 1 min. Repeat until the entire sample will be filtered. Keep the pellet.
Change the DNeasy column to a new collection tube and add 500 µl of AW buffer to the column and spin 2 min at 9,000 rpm. Keep the pellet.
Repeat a new wash with 500 µl of AW buffer and spin again 2 minutes at 15,000 rpm. Verify that the column wil be alcohol free.
Transfer the DNeasy column to a new sterile tube (1.5 ml) and add 50 µl of preheated (65°C) AE buffer, directly on the DNeasy membrane. Incubate for 5 min at room temperature and then spin for 1 minute at 8,000 rpm to elute the DNA.
Repeat the elution with 100 µl more of preheated AE buffer (like the previous step).

DNA amplification

For DNA quantification we used fluorometer and densitometer readings for band intensity of DNA samples separated on agarose gel (Figure 1). DNA quality was estimated by PCR amplification with three genetic markers: RAPDs (*Zea*, cacti and *Piper*) and nuclear microsatellites (*Quercus* spp.). We used 20 ng per 25 µl PCR reaction. The amplification protocols used are standard protocols previously reported; RAPDs for cacti (De la Cruz et al., 1997) and nuclear microsatellites for *Quercus* (Steinkellner et al.,

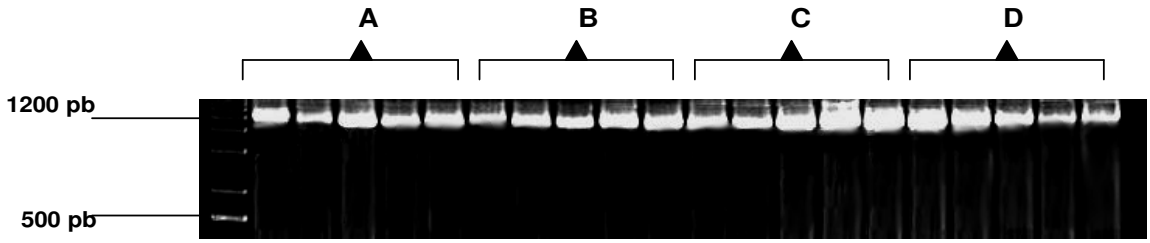

Figure 1. DNA samples extracted with both two protocols, first two samples in each case correspond to protocol 2, and last two samples correspond to protocol 1. The protocols were tested in *Piper* (A), *Quercus* (B), *Zea* (C) and cacti (D) species.

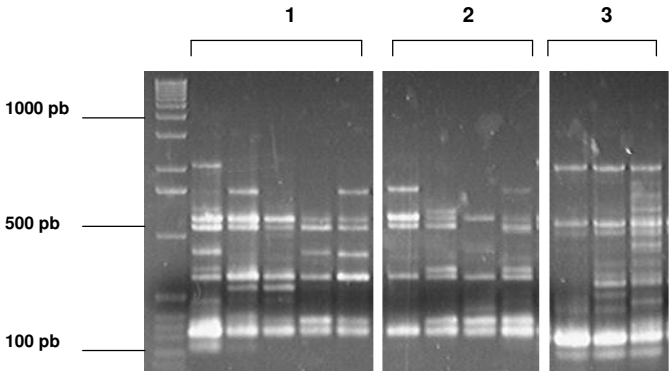

Figure 2. DNA samples from *Piper* (1), *Zea* (2) and cacti (3) species were amplified with RAPD's primers, and all samples shown clear and well differentiated band patterns.

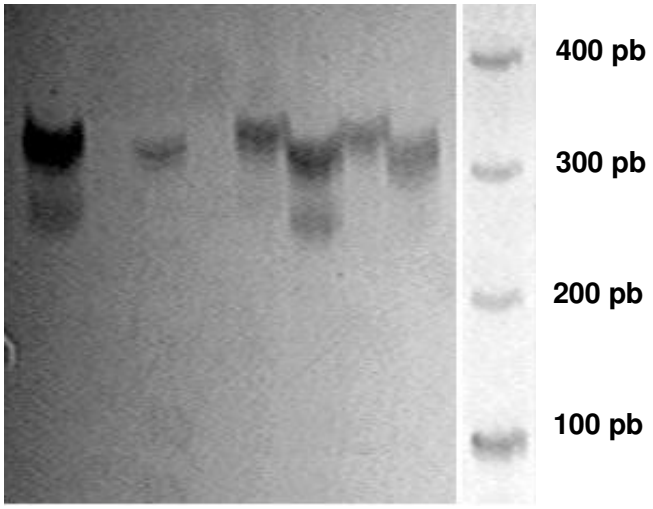

Figure 3. DNA samples from *Quercus* species were used to test some nuclear microsatellites, previously reported for the genus.

1997), and all samples were digested with three digestion enzymes (HindIII, Sma I and EcoRI). All *Quercus* samples were used too for DNA sequencing for five nuclear microsatellites (ssr 110, ssr 119, ssr 46, ssr 58 and ssr 1/5; Steinkellner et al., 1997). RAPD's were stained with ethidium bromide (Figure 2) and microsatellite bands were stained with silver nitrate (Figure 3), using standard procedures.

RESULTS AND DISCUSSION

Ground of plant tissue

The homogenization, pulverization and uniformity grinding of plant tissue were essential when we extracted DNA from these species. The use of liquid nitrogen has a double function: the maintenance of frozen tissue and the prevention of nucleic acid degradation and secondary metabolites oxidation that may form some complexes that could form a coprecipitate with DNA; and a better mechanical disruption from tissue in the mortar. Although this step is the most time consuming part of every DNA plant extraction method, the small requirement of tissue (200-500 mg) make this step faster.

DNA quality

DNA yield was determined by quantification in fluorometry and densitometry readings direct from agarose gel. We obtained DNA concentrations from 500 ng to 1.5 µg per microliter with both two protocols. These yields represent a better recovery compared with other mini-preparation methods suggested previously (Stewart and Via, 1993; Lodhi et al., 1994; Khanuja et al., 1999; Chen and Ronald, 1999; Doulis et al., 2000). Complete digestion with restriction endonucleases and amplification in PCR indicate the absence of polysaccharides.

DNA amplification

DNA quality is an essential feature for most molecular applications (Pandey et al., 1996). The DNA obtained was evaluated by performing two PCR-based techniques, enzyme digest and sequencing. All tests were done with at least in ten samples extracted with both two protocols. In all amplifications we used 20 ng of genomic DNA, into a final reaction volume of 25 µl.

The RAPDs amplification was tested using four decamer primers (Operon™ A18, A19, A20 and G16). All samples showed a consistent banding pattern, and PCR products with good quality. All amplifications were reproducible and consistent. RAPDs amplifications were done with all species tested.

Other markers like nuclear and chloroplast microsatellites were also used to test all *Quercus* species. High quality and reproducibility patterns in all samples were obtained too. We used some *Zea* samples to evaluate AFLPs amplification, and good results were also obtainned (data not shown).

Comparison analysis with other DNA extraction protocols

Other standard procedures previously reported (Doyle and Doyle, 1990; Guillemaut and Maréchal-Drouard, 1992; Porebski et al., 1997; De la Cruz et al., 1997; Tel-Zur et al., 1999) requires large quantities of tissue for DNA extraction. There are other protocols standardized for mini-preparations (Stange et al., 1998; Chen and Ronald, 1999; Doulis et al., 2000) but these are inefficient as they produce low DNA yields and bad quality samples for PCR amplification when we tested them. One of the best options was the DNeasy plant mini kit; however, when it was used to extract some samples like cacti parenchyma and mature oak leafs, we obtained DNA contaminated with polysaccharide, and with low PCR amplifications ranges.

As mentioned before, we needed a reliable protocol for low quantities of tissue (mini-preparations) and, mostly times, with good yields and DNA quality. The DNeasy kit modifications were the best protocol to obtain very good yields and DNA quality. The combination of CTAB extraction buffer (Carlson buffer), light solvents (chloroform : octhanol) and the anion-exchange chromatography (DNeasy membrane) for DNA extraction proved to be the best combination for an excellent, fast and efficient protocol. Previously, other authors have reported methods that combine CTAB Carlson buffer and the anion exchange Quiagen membranes (Csaikl et al., 1998) but like a midi-preparation requiring more time and more cost-expensive than this new (our) modification to the DNeasy kit. Moreover, the combination with light solvents gives our protocol the capability to extract DNA with high quality from very small tissue samples. We also found that it provides a faster DNA extraction with these modifications in comparison to the original kit's protocol. This is inspite of fact that we did not use the QIAshredder column, making the procedure less expensive and faster. For this protocol, it is necessary to buy all the kit, which is expensive, because the provider (Quiagen) does not sell the DNA columns separately.

The first protocol (CTAB-based method) is economical, but more time-consuming (2 days) and produces good DNA quality like Quiagen method modification. Reagents like CTAB and PVP remove polyphenols and polysaccharides, while the ascorbic acid, DIECA and 2-mercaptoethanol reduce oxidation (De la Cruz et al., 1997). This ensures an efficient and easily reproducible method. This protocol is much more economical compared with the modification to the DNeasy plant mini kit. Although we did not obtain high DNA yields like the Quiagen kit modifications protocol, the DNA concentration (500 ng) and quality obtained with this method is enough to obtain reproducible PCR amplifications with RAPDs and microsatellites in all species tested (Figure 3) as well as enzyme digest and sequencing.

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