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A Rapid Efficient Method for DNA Isolation from Plants with High Levels of Secondary Metabolites

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Abstract: This research was designated to optimize a rapid, inexpensive method to isolate desirable DNA from plants with high concentrations of secondary products. DNA extraction procedures of plants with high levels of secondary metabolites e.g., Tea (*Camellia sinensis*) Pokeweed (*Phytolacca dodecandra*), Broad bean (*Vicia faba* L.) and most of medicinal plants is so complicated due to polyphenolics, tanins, alkaloids and other metabolites that makes it difficult to obtain high quality DNA with good maintenance time. To overcome this problem, we designed a simple but efficient method. In this method, an extraction buffer is primarily used to reduce the secondary metabolite levels followed by a separate lysis buffer comprising of a sarcosyl detergent (N-lauryl sarcosine sodium salt), 2 different reducing agents and higher concentrations of chelating agents. CTAB/NaCl precipitation of polysaccharides and elimination of proteins through chloroform:isoamyl alcohol extraction resulted a more pure DNA. Presented method consists of few steps. Furthermore, protein and polysaccharide contamination was noticeably reduced. This method does not require expensive reagents and modern laboratory equipments and it is possible to isolate several DNA samples per day in a general biology lab. DNA derived using this method was tested electrophoretically and then was examined spectrophotometrically: A260/280 (protein contamination) and A260/230 (Polyphenol and polysaccharide residuals). Polymerase Chain Reaction (PCR) amplification was performed using different RAPD primers and *Eco*RI, *Bam*HI and *Hind*III were used for restriction enzyme reactions at different digestion conditions. Results indicated that DNA produced in this method is desirable for must of genetic assays and because polyphenolic compounds were removed or neutralized, maintenance time of DNA samples increased.

Key words: DNA extraction, medicinal plants, RAPD, restriction enzyme, secondary metabolites

INTRODUCTION

Isolation of pure, intact and high quality DNA is so crucial for any plant genetic studies, especially because of high amounts of compounds present in plant tissues that may interfere with subsequent DNA manipulation. Most of plant species contain high levels of polysaccharides, polyphenols, several pigments and other secondary metabolites (Wen and Deng, 2002), which make DNA unusable for downstream work in molecular biology research (Levi *et al*., 1992; Michiels *et al*., 2003; Qiang *et al*., 2004). Although the new DNA-based methods are highly specific, reproducible and sensitive and characterized by high discriminatory power, rapid processing time and with low costs, they are strongly limited by the presence of inhibitors in plant tissues. These secondary metabolites that are particularly abundant in fruit trees, medicinal plant and some desert shrubs are not completely removed during classical extraction protocols remaining as contaminants in the final DNA preparations. Polysaccharides make DNA viscous, glue-like and non-amplifiable in the PCR reaction by inhibiting *Taq* enzyme activity and also interfere accurate activity of restriction enzymes (Porebski *et al*., 1997). When cells are disrupted, these cytoplasmic compounds can come into contact with nuclei and other organelles (Loomis, 1974). In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and reduce their maintenance time, making it useless for most research applications (Katterman and Shattuck, 1983; Guillemaut and Maréchal-Drouard, 1992). The presence of these compounds renders studies difficult due to long and tedious extraction procedures and often does not result in good standards in terms of yield and quality.

Although a number of methods for DNA isolation from plants containing high levels of secondary metabolites have been developed (Van Driessche *et al*., 1984; Porebski *et al*., 1997; Peterson *et al*., 1997; Cheng *et al*., 2003; Michiels *et al*., 2003; Qiang *et al*., 2004). Zeng and Yang (2002) also developed an efficient

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method for RNA isolation from samples with high levels of polysaccharides and polyphenols. These methods are generally expensive and time consuming. The need for a rapid and efficient procedure for plants having high contents of secondary metabolites is necessary when hundreds of samples need to be analyzed rapidly, such as in genome mapping and Marker Assisted Selection (MAS) programs. High purity DNA is required for PCR and restriction-based techniques like RAPD, SSR, AFLP and RFLP.

We have optimized an efficient, rapid method that yields polysaccharide and polyphenol-free high quality genomic DNA from most of plant species with secondary metabolites. The obtained DNA also had a very low content of proteins. In this study, we describe DNA isolation of plant species such as Tea (*Camellia sinensis*), Pokeweed (*Phytolacca dodecandra*) and Broad bean (*Vicia faba* L.) that are typical plants with high levels of secondary metabolites. Two other standard methods, Dellaporta *et al*. (1983) and Saghai-Maroof *et al*. (1984) were included for comparison.

MATERIALS AND METHODS

Plant materials: This study was conducted summer of 2005 at Sari faculty of agriculture, Mazandaran University. Leaf samples of Tea, Pokeweed and Broad bean were collected and were transferred to laboratory in an icebox and stored at −20°C.

Solutions:

- Extraction Buffer: 120 mM Tris-HCl (pH 8.0), 80 mM EDTA (pH 8.0), 0.5% Triton x-100 (v/v) and 0.5% β-mercaptoethanol (v/v).
- Lysis buffer: 120 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 2% (w/v) N-lauryl sarcosine sodium salt (Sarkosyl), 0.8 M NaCl, 2% PolyVinyl Pyrrolidone (PVP) and 0.2% β-mercaptoethanol (v/v).
- CTAB solution: 0.7 M NaCl, 1% Cetyltrimethy-lammonium bromide (CTAB).
- Chloroform: Isoamyl alcohol (24:1).
- Absolute ethanol.
- Ethanol 70%.
- E: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
- High salt TE : 10 mM Tris- HCl, pH 8.0, 1 mM EDTA, 1.0 M NaCl.
- Sodium acetate 3 M, pH 5.2.
- TBE 5X: 54 g Tris, 27.5 g boric acid, 4.65 g EDTA per liter.
- Isopropanol.

Extraction procedure: Grind 0.4 g of leaf tissue to a fine powder using a mortar, pestle and liquid nitrogen. Transfer the powder to an Eppendorf 1.5 mL tube. Add 700 μL of extraction buffer. Incubate the tube at 40-45°C water bath for 20 min vortex the tube vigorously 5 min interval. Centrifuge the tube at 8000 g for 5 min and gently discard the supernatant by a pipette. Add 600 μL of lysis buffer to the pellet and after resuspension incubate at 65°C water bath for 30 min with occasional inversion (after this step to end do not vortex or DNA will be degraded). Add 500 mL of chloroform:isoamyl alcohol (24:1) and after several inversion, centrifuge it at 12000 g for 10 min. Transfer the upper phase (aqueous) to a clean tube and add 1/10 vol. prewarmed CTAB solution and mix by inversion. Add 1 vol. of Chloroform: isoamyl alcohol, mix by inversion and centrifuge at full speed for 12 min. Gently transfer the supernatant to a new clean tube and add 0.6 vol. of cold isopropanol and 0.1 vol. (considering supernatant and isopropanol) of 3 M sodium acetate (pH 5.2), mix well and incubate for 5 min at room temperature. Centrifuge at 10000 g for 8 min. Discard the supernatant and wash the pellet with 500 μL 70% ethanol and let dry. Resuspend the pellet in 400 μL of high salt TE buffer. Add 40 μL of 3 M sodium acetate (pH 5.2) and 880 μL cold absolute ethanol. Incubate on ice for 20 min. Centrifuge at 12000 g for 10 min. Discard the supernatant and wash the pellet with 500 μL 70% ethanol and let dry. Resuspend the pellet in 50 μL buffer TE by incubating the tube at 65°C water bath. Quantify the DNA spectrophotometrically at 260 nm and electrophoretically in 0.8% agarose gel to verify the DNA purity and intactness.

PCR amplification: For each of isolated DNA samples, PCR was carried out in a 25 μL vol. of reaction mixture. A reaction tube contained 20 ng of template DNA, 1 U of *Taq* DNA polymerase enzyme (Cinnagen Co., Iran), 0.2 mM of each dNTP (Fermentas Co., Lithuania), 1× *Taq* amplification buffer (Cinnagen Co., Iran), 1.5 mM $MgCl_2$ and 10 pmol of random decamer (RAPD) primers (Operon Technologies Inc., USA). Amplifications were carried out by using a DNA thermal cycler (Eppendorf, mastercycler gradient) programmed as: 94°C for 5 min; 38 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and a final extension at 72°C for 8 min. PCR products were separated on 1.5% agarose gel and stained in ethidium bromide, observed under UV light and photographed using gel documentation unit (Vilber-Lourmat, France).

Endonuclease digestion: Isolated genomic DNA of each of Tea, Pokeweed and Broad bean (5 μg) were incubated with 10 units each of *Eco*RI, *Hind* III and *Bam*HI

(Fermentas Co., Lithuania) in the SPK buffer (Fermentas Co., Lithuania) at 37°C for 2 h. Another digestion was performed using Tango buffer (Fermentas Co., Lithuania) and 2 units of each of *Eco*RI, *Hind* III and was incubated overnight to test the probable existence of inhibitor compounds through assessment of reaction accuracy and velocity. DNA digestion was assayed by visual inspection after agarose gel electrophoresis.

RESULTS AND DISCUSSION

DNA suitable for a broad range of molecular biology applications was successfully extracted from 3 different plant species containing high levels of secondary metabolites. The protocol development is a combination of earlier described protocols. The development procedure was optimized by making several changes when compared to two standards protocols. A large part of inhibitor compounds in the first step was eliminated. Triton x-100, β-mercaptoethanol and high concentration of EDTA in the extraction buffer, made some temporary pores in the cytoplasmic membrane, so a noticeable portion of soluble polysaccharides, polyphenols and pigments were removed. The use of high levels of PVP and β-mercaptoethanol in the lysis buffer had a considerable effect on neutralization of polyphenols and prevented oxidization of the secondary metabolites. More polysaccharides were removed by CTAB/NaCl solution and primary DNA elution in high salt TE, could remove more polysaccharides.

This procedure yielded 100-250 μg of DNA per gram of fresh leaf tissue that was lower than 2 other methods (200-400 for Dellaporta method and 350-550 for Saghai-Maroof method). Although we did not use RNase, there were little RNA residues in the DNA solutions (Fig. 1).

There was neither RNA contamination nor any sign of degraded DNA in all samples. It seems that RNA is degraded during the extraction process. Standard DNA isolation methods Dellaporta *et al*. (1983) and Saghai-Maroof *et al*. (1984) yielded DNA of desired quantity but with a higher amounts of contamination by proteins and polyphenols. An A260/A280 ratio of our protocol was 1.69-1.91 indicating very low levels of contaminating proteins (this ratio for Dellaporta method and Saghai-Maroof method was 1.62-1.82 and 1.60-1.88, respectively). The A260/A230 ratio, indicating the contamination by polysaccharides and polyphenols, was 2.07 in our method while it was 0.78 and 1.16 for Dellaporta and Saghai-Maroof methods, respectively. There was not any significant differences between the 3 methods in PCR amplification (Fig. 2 and 3).

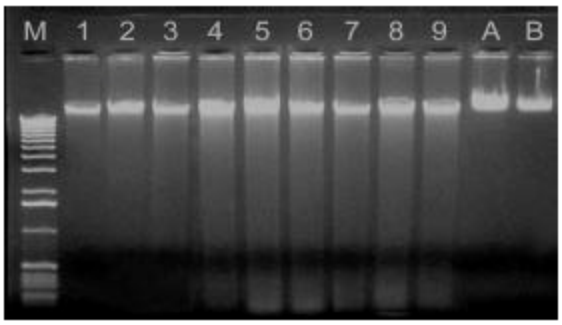

Fig. 1: Genomic DNA isolated from three plant species. The lanes, numbering from left to right, are: M: Weight marker, lanes 1-3: New modified method, lanes 4-6: Saghai-Maroof method, lanes 4-6: Dellaporta method, A and B: uncut lambda DNA, 100 and 50 μg mL^{-1}

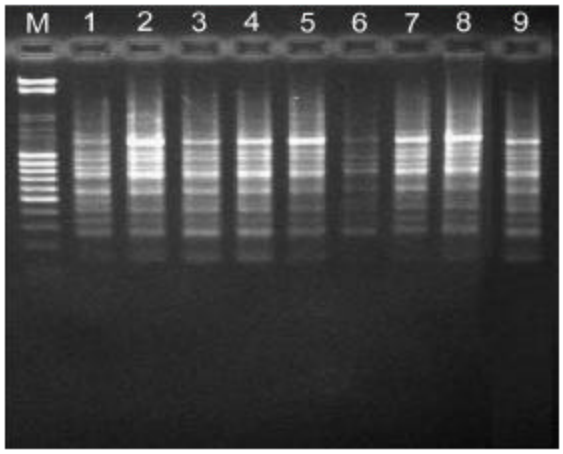

Fig. 2: PCR amplification of genomic DNA from three plant types extracted by three methods with primer OPA-06. M: weight marker, lanes 1-3: New modified method, lanes 4-6: Saghai-Maroof method, lanes 7-9 Dellaporta method

After double treatment with *Eco*R I and *Hind* III with Tango buffer overnight, the DNA of each of methods was well digested (Fig. 4), indicating the absence of large amounts of impurities and inhibitors in all 3 methods, but when digestion with 3 enzymes at limited time was performed (*Eco*RI, *Hind* III and *Bam*HI digestion with SPK buffer for 2 h) some differences was observed. DNA isolated using the modified method was digested more efficiently than 2 other standard methods, possibly due to lower levels of contamination with polysaccharide and polyphenols (Fig. 5).

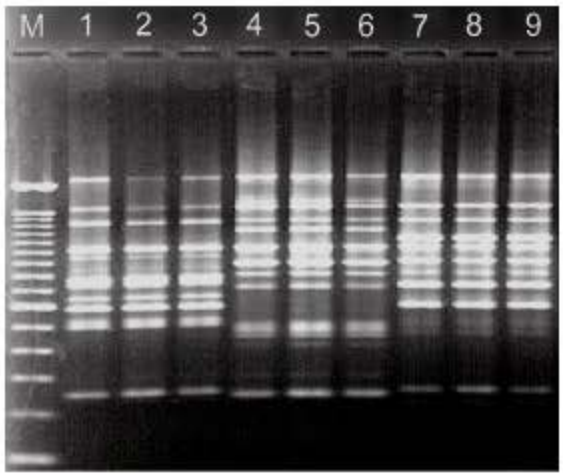

Fig. 3: PCR amplification of genomic DNA from three plant types extracted by three methods with primer OPQ-17. M: weight marker, lanes 1-3: New modified method, lanes 4-6: Saghai-Maroof method, lanes 7-9 Dellaporta method

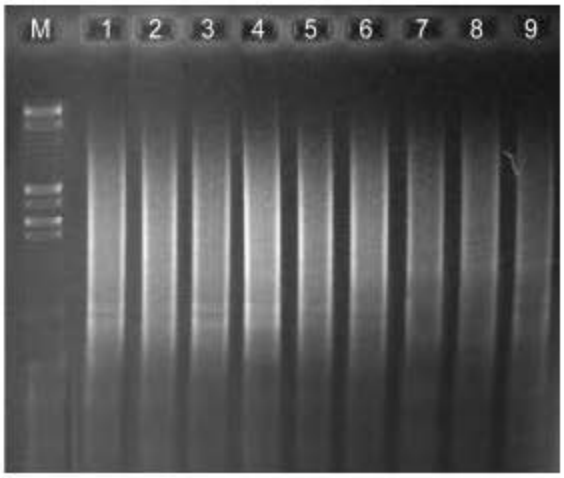

Fig. 4: Restriction endonuclease digestion of DNA isolated form three plant species with *Eco*RI, *Hind* III in Tango buffer, incubated overnight. M: weight marker, lanes 1-3: New modified method, lanes 4-6: Saghai-Maroof method, lanes 7-9 Dellaporta method

In conclusion, this protocol allows for simultaneous DNA extraction from numerous samples and does not need expensive reagents or equipments while producing clean and high quality DNA that is suitable for most genetic analysis purposes. The protocol should be applicable to other recalcitrant plant species with some modifications.

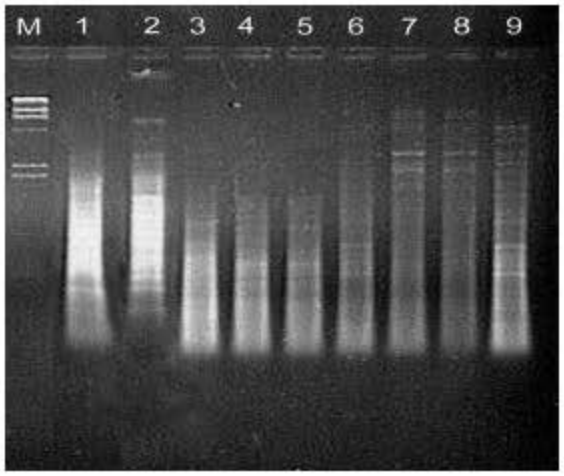

Fig. 5: Restriction endonuclease digestion of DNA isolated form three plant species with of *Eco*RI, *Hind* III and *Bam*HI in the SPK buffer and incubation for 2 h. M: weight marker, lanes 1-3: New modified method, lanes 4-6: Saghai-Maroof method, lanes 7-9 Dellaporta method

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