
An efficient protocol for genomic DNA extraction from *Alcea rosea*

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High quality DNA is essential for many molecular biology techniques. However, the reagents used for that purpose usually are expensive and/or cause a high environmental impact. A protocol proposed that the use of inexpensive reagents and are not hazardous to the environment. The spectral quality of genomic DNA isolated using this method as measured by the A260/A280 absorbance ratio ranged from 1.88 to 2 for all species of *Alcea Rosea*. The DNA obtained was free of any contaminating proteins, polysaccharides and coloured pigments. The isolated genomic DNA was found suitable for restriction digestion and PCR amplification. This method does not require RNAase, ammonium acetat and this method does not require for fixation or grinding in liquid nitrogen, making it advantageous over common protocol.

Key words: DNA extraction, *Alcea rosea*, polysaccharides, RAPD, phenol

Introduction

Herbal and aromatic plants are attracting more attention among contemporary plant researchers because some human diseases resulting from bacterial antibiotic resistances have gained worldwide concern. Hollyhock is one of the most important medicinal plants (Makambila *et al.*, 2010). These plants contain exceptionally high amounts of polysaccharides, polyphenols and other secondary metabolites that have medicinal properties (Oznur Akcin and Ozbucak, 2006 ; Umamaheswari and Govindan, 2007). Application of molecular technology would increase and facilitate production of these substances (Stöckigt *et al.*, 1995) and help save natural resources. However, species of *Alcea rosea* contain polysaccharides and polyphenols posing a major problem in the isolation of high quality DNA (Odukoya *et al.*, 2007). Isolating high quality DNA is essential for molecular research. Polysaccharide

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contamination is a common problem in higher plant DNA extraction. DNA samples are often contaminated with polysaccharides, polyphenols, which are almost insoluble in water or TE buffer. This can affect manipulation, inhibit enzyme reactions (Porebski *et al.*, 1997; Schlink and Reski, 2002) and hinder the downstream work in molecular biology research. DNA samples are unstable for long-time storage (Lodhi *et al.*, 1994; Sharma *et al.*, 2002). Several plant DNA extraction protocols for removing polysaccharides have been reported (Möller *et al.*, 1992; Cruz *et al.*, 1997; Porebski *et al.*, 1997; Schlink and Reski, 2002; Sharma *et al.*, 2002). However, in Herbal and aromatic plants that contain high polysaccharide levels and phenol, such as *Alcea Rosea* the protocols could only be used on vigorous tissue (Porebski *et al.*, 1997; Olotuah, 2006), and the DNA isolated was not of high enough quality to be used in PCR and RAPD analyses. Although several successful DNA extraction protocols for plant species containing polyphenolics and polysaccharides compound have been developed, none of these are universally applicable to all plants (Varma *et al.*, 2007) and the published protocols are also limited because of degradation of DNA by DNases and other nucleases (Sharma and Sharma, 1980). Therefore, 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. The common procedure is to grind plant tissue in liquid nitrogen and transfer to a preheated extraction buffer (Dellaporta *et al.*, 1983; Mohapatra *et al.*, 1992). Liquid nitrogen can be difficult to procure in remote locations. Thus, a method not requiring use of liquid nitrogen would be helpful to researchers in remote area. In this paper we describe a DNA isolation method suited for isolation of genomic DNA in *Alcea Rosea* leaves young that can be stored for a longer duration, lasting for several PCR reactions and also does not require RNAase, ammonium acetate and also does not require for fixation or grinding in liquid nitrogen, making it advantageous over common protocol. The method has used no expensive and toxic chemical. The aim to develop this method was to make this technique readily available in low-facility laboratories. This method is suitable for genomic DNA isolation from Herbal and aromatic plants containing high polysaccharide levels.

Materials and methods

Plant material

Accessions of Hollyhock native (38 accessions) to Iran have been gathered from different parts of Iran (Table 1) . Seed all these accessions were

grown in the same conditions in a greenhouse. Seed accessions in a greenhouse were germinated and total genomic DNA was isolated from the very young of plants.

Table 1. *Alcea rosea* populations, their collection sites, codes, elevation, latitude, longitude and collection dates during the year 2009-2010

Population Name/Collection Site	Population Code	Elevation (m)	latitude East	Longitude North
Kermanshah A,B,C	7-9	1389	34.314	47.065
Kurdistan A,B,C	10-12	1464	35.31	46.999
Bushehr A,B,C	13-15	9	28.974	50.834
Hamedan A,B,C	16-18	1824	34.795	48.514
AzarbajjanWest A,B,C	19-21	1267	35.58	44.03
Golestan A,B,C	22-24	183	36.828	54.439
Gilan A,B,C	25-27	1	37.278	49.595
Mazandaran A,B,C	28-30	52	36.568	53.059
AzarbajjanEast A,B,C	31-33	1395	38.08	46.292
Qazvin A,B,C	34-36	1290	36.262	50.017
Lorestan A,B	37-38	1147.8	33.26	45.17

Reagents and chemicals

An extraction buffer consisting of 2% CTAB (w/v), Tris HCl pH 8.0 (2 M); Sodium EDTA pH 8.0 (0.5 M); NaCl (5.0 M), PVP (2 gr) was prepared.

- Phenol:Chloroform:Isoamylalcohol (25:24:1 v/v/v)
- Ethanol (70%).
- TE buffer

DNA isolation protocol

- Submerge 1 g of tissue very young in 5 mL of alcohol and Grind the tissue with a mortar and pestle.
- Transfer the homogenized tissue to prewarmed DNA extraction buffer.
- Incubate for 15 min in a 65°C water bath, occasionally mixing by gentle swirling.
- Remove from water bath. Add 1 mM of Phenol: Chloroform: Isoamylalcohol (25:24:1 v/v/v).

Mix by inversion for 5 min.

- Spin at 13,000 rpm [10,000 g] for 10 min.
- Transfer the aqueous phase to another tube.
- Add 500 μ L of isopropanol to precipitate the DNA.
- centrifuge briefly to pellet the DNA (5 min).
- Wash with 70% alcohol. Invert the tubes and drain on a paper towel for approximately 10 min.
- Dissolve the dried DNA in TE buffer.

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV Spectrophotometer (Perkin-Elmer- EZ-201) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The quality was also examined by running the extracted DNA samples on 0.8 % agarose gel stained with 10 mg/ml ethidium bromide in 1 \times TBE (Tris base, Boric acid, EDTA) buffer. The gel was visualized and photographed under UV light (Biorad).

PCR amplification

For the optimization of RAPD reaction using DNA extracted from various *Alcea Rosea* species, oligonucleotide primers from TibMolBiol series were used for amplification to standardize the PCR conditions. Polymerase chain reactions (PCRs) for amplification of DNA preparations were carried out in a 25 μ l volume. The reaction tube contained 25 ng of genomic DNA template, 1.25U Taq polymerase, 1.25 mM of each dNTP , 10 pmol random 10-mer primer, 1.5 Mm MgCl₂, 1X PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl₂). Amplifications were performed in a DNA Thermocycler (Bio-Rad model i-Cycler) programmed as follows: an initial denaturizing at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min, followed by one final extension at 72°C for 4 min. After amplification PCR product was resolved by electrophoresis in 1/2% agarose gel for 2.16 h at 70 V with IX TBE buffer. Bands were visualized by staining with ethidium bromide (0.5 μ g mL⁻¹) under UV light(Figure1).The Band size was determined with the DNA size marker (SM1553 Fermentas).

Results

We successfully isolated DNA from *Alcea rosea* using the protocol described above. The A260/ A280 values were in the range of 1.88-2. White DNA pellets formed (Figure 2) and were quickly soluble in TE buffer. Results of the agarose gel test and PCR or RAPD analysis indicated that polysaccharides had been efficiently removed and DNA quality had been enhanced (Figures 1-3). Result showed that our method was efficient in removing polysaccharides, polyphenols and RNA. The DNA obtained was free of any contaminating proteins, polysaccharides and coloured pigments. Several modified DNA protocols that remove polysaccharides have recently been reported (Fang *et al.*, 1992; Porebski *et al.*, 1997). All were almost unsuccessful in removing polysaccharides from Herbal and aromatic plants. In our method, the extraction buffer contains high amounts of PVP and b-mercaptoethanol, prevent oxidation of the secondary metabolites in the disrupted plant material and also CTAB is used as a detergent in the extraction buffer to separate polysaccharides from DNA. In this protocol, we found the combined use of CTAB and PVP during the precipitation of DNA efficient in removing most of the secondary metabolites and polysaccharides from the DNA. In our study a little amount of RNA was usually obtained, but it did not show any effect on molecular biology applications. The present protocol does not include the need for liquid nitrogen for crushing the plant material. A similar procedure for isolation of DNA from date palms has been followed by Ouenzar *et al.* (1998). Because we found the methods described in this paper functional for plants that were otherwise recalcitrant to DNA isolation, we believe that these methods will be of help for molecular biological studies of many other aromatic and herbal plants.

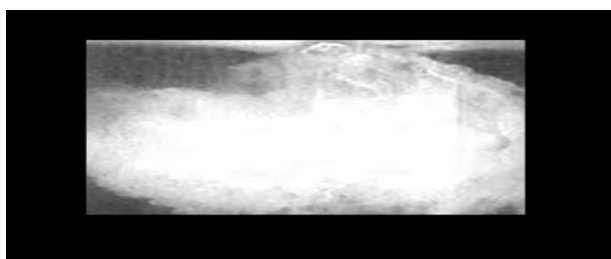


Fig. 1. Example of a White DNA pellet of young *Alcea Rosea* leaves formed in isopropanol



Fig. 2. Quality test of 10 DNA samples of *Alcea Rosea*. on 0.8% agarose gel.

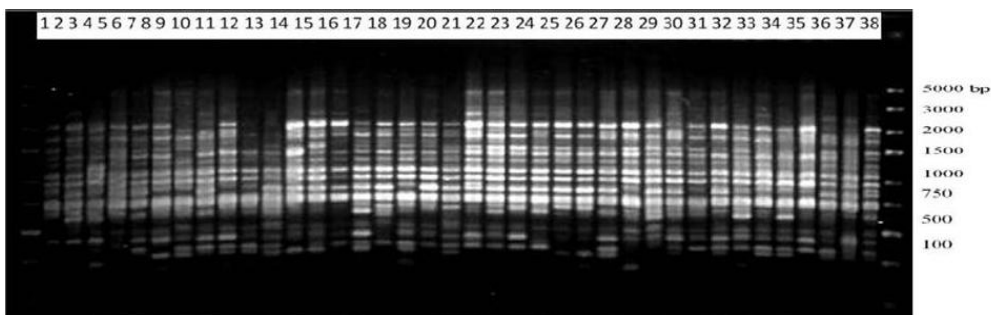


Fig. 3. Example of an agarose gel showing the amplified DNA patterns obtained with a RAPD-PCR reaction with primer BB01 (ACACTGGCTG).

Discussion

The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications (Katterman and Shattuck, 1983; Peterson *et al.*, 1997; Porebski *et al.*, 1997; Hemphill *et al.*, 2006). Tannins, terpenes and resins considered as secondary metabolites are also difficult to separate from DNA (Ziegenhagen and Scholz, 1993). Certain polysaccharides are known to inhibit RAPD reactions. They distort the results in many analytical applications and therefore lead to wrong interpretations (Kotchoni *et al.*, 2003; Beatriz *et al.*, 2010). Addition of PVP along with CTAB may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent. Published methods of DNA isolation including those of Doyle and Doyle (1990), Rogers and Benedich (1985) and Dellaporta *et al.* (1983) proved unsuccessful and unreliable for Herbal and aromatic plants as the DNA obtained was dirty yellow in appearance and with high viscosity. This may be due to high endogenous levels of polysaccharides, phenolics and other organic constituents that interfere with DNA isolation and purification. The isolated DNA could not be subjected to even agarose gel electrophoresis (Padmalatha *et al.*, 2006). Polyphenol contamination of DNA makes it resistant to restriction enzymes as also shown in other taxa where polyphenol copurify with DNA (Katterman and Shattuck, 1983) and interact irreversibly with proteins and nucleic acids (Cheng *et al.*, 2003). The protocol described here is efficient, inexpensive, and yields clean genomic DNA, amplifiable by PCR, as indicated by the results of the RAPD technique. We do not depend on liquid nitrogen to grind leaf material for DNA isolation. This method is very simple and reliable for plant species like *Alcea rosea*

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