Isolation of high-quality DNA in 16 aromatic and medicinal Colombian species using silica-based extraction columns

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ABSTRACT

Aromatic and medicinal plant species are a valuable resource for research and development of pharmaceutical, cosmetic, crop protection and nutritional agents, due to the high amount of bioactive phytochemicals that they contain. However, these compounds are a major obstacle in the isolation of high-quality DNA suitable for genetic analyses. In this paper, we report a protocol that optimizes the use of the cationic detergent CTAB and the reductant β-mercaptoethanol in cell lysis. The elimination of plant secondary metabolites such as polysaccharides and polyphenols, that typically co-isolate with DNA, was achieved using the chemical denaturing properties of the guanidinium cation, which together with the adsorbent chemical specificity of the silica, resulted in the purification of high-quality DNA suitable for digestion with restriction enzymes and optimal for PCR amplification of AFLP-type molecular markers. This protocol was evaluated on 16 Colombian aromatic and medicinal plant species promising for their essential oils. The results allow suggesting that this procedure might be appropriate for other species, tissues and sample types recalcitrant to DNA extraction.

Key words: chaotropic agent, guanidinium cation, silica-based extraction columns, nucleic acid extraction kit, guanidinium salts, molecular markers.

Introduction

Worldwide, aromatic and medicinal plant species are used as sources of new molecules for the synthesis of chemopreventive and anti-cancer drugs (Balunas and Kinghorn, 2005), medicinal, cosmetic, antibacterial, antiparasitic (Bakkali et al., 2008), antimicrobial, antioxidant and anti-inflammatory agents (Cowan, 1999; Svoboda and Hampson, 1999). Their importance lies in that they contain many bioactive phytochemicals, plant secondary metabolites and essential oils (Balunas and Kinghorn, 2005; Cowan, 1999), significant for the pharmaceutical, crop protection, cosmetic and food industries (Bakkali et al., 2008), and certain research areas such as pharmacognosy (Balunas and Kinghorn, 2005), biotechnology, plant breeding, genetics, molecular and evolutionary biology (Canter et al., 2005; Gómez-Galera et al., 2007; Kumar and Gupta, 2008).

In Colombia, there is an increasing interest in studying the composition and biochemical properties of essential oils from different aromatic and medicinal native plants, and their potential uses (Meneses et al., 2009; Nerio et al., 2009). The research in this kind of promising species must be accompanied by: \textit{i}) the development of cultivable lines in conventional plant breeding programs and/or assisted

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by molecular markers from wild materials to ensure the proper agronomic exploitation and the conservation of genetic resources (Gómez-Galera et al., 2007; Kumar and Gupta, 2008), ii) the application of molecular and genomic tools appropriate for handling the modulation in the production of these oils and plant secondary metabolites (Gómez-Galera et al., 2007), and iii) the development of quality control testing to prevent piracy or adulteration of commercial products (Breton et al., 2004; Marieschi et al., 2009).

The use of molecular markers such as AFLPs (Vos et al., 1995), to assist the selection process in plant breeding (Bernardo, 2008; Collard et al., 2005) and quality control procedures (Breton et al., 2004; Marieschi et al., 2009), requires the development of protocols for isolation of high-quality DNA that allows enzymatic digestion with restriction endonucleases and amplification of regions or fragments with high reproducibility (Mace et al., 2003; Matasyoh et al., 2008; Sarwat et al., 2006). However, the isolation of DNA in aromatic and medicinal plant species is a difficult, tedious and time-consuming procedure (Khanuja et al., 1999; Michiels et al., 2003; Padmalatha and Prasad, 2006; Pirttilä et al., 2001). Basically, the problem is the formation of complexes between DNA and plant secondary metabolites such as polyphenols and polysaccharides. Among these metabolites are tannins, flavonoids, quinones, terpenes and alkaloids, that make not only attractive this group of plants but also the procedure of isolation difficult, because they degrade the extracted DNA and directly or indirectly inhibit the action of enzymes used in downstream molecular applications such as PCR amplification (Ivanova et al., 2008; Khanuja et al., 1999; Michiels et al., 2003; Padmalatha and Prasad, 2006; Pirttilä et al., 2001; Porebski et al., 1997).

The commonly reported methods for the extraction of DNA in medicinal and aromatic plants are modifications of classical procedures such as the CTAB of Doyle & Doyle and SDS of Dellaporta (Dellaporta et al., 1983; Doyle and Doyle, 1990), which must be adjusted for each sample type, tissue and species. Variable results are obtained when these procedures are applied in other species, even those closely related (Marva et al., 2007; Pirttilä et al., 2001; Sangwan et al., 2000; Sarwat et al., 2006). Because of this, the aim of this work was to develop a high quality DNA extraction protocol for recalcitrant or difficult samples, that it is chemically simple, fast, reproducible and optimal for aromatic and medicinal plant species, based on the use of chaotropic agents and membrane-silica-based extraction columns.

The use of chaotropic agents for protein denaturation dates back to the work of Svedberg in 1937, and Greenstein in 1938 (Greenstein, 1938; Greenstein, 1939a; Greenstein, 1939b; Greenstein and Edsall, 1940; Svedberg, 1937). Among the chaotropes more commonly used in chemistry and molecular biology for the structural study of proteins and for nucleic acid isolation are the guanidinium cation (Gdm⁺)-salts (Bowtell, 1987; Castellino and Barker, 1968; Cox, 1968; Greenstein and Edsall, 1940; Mason et al., 2003). These salts, made up of Gdm⁺ or Gdm⁻-substituted and an anion such as Cl⁻, I⁻, Br⁻ or thiocyanate (SCN⁻), constitute the strongest denaturing agents known in nature, and its denaturation strength depends on the accompanying anion, being Cl⁻ the weakest and the SCN⁻ the most powerful reported (Castellino and Barker, 1968; Greenstein, 1939b; Greenstein and Edsall, 1940; Mason et al., 2003). Thus, the denaturation or dissociation and solvent properties of these salts on proteins are in function of the type of cation, the accompanying anion and the concentration in solution (Castellino and Barker, 1968; Greenstein, 1939a; Greenstein, 1939b). On the other hand, silica is known for its ability to adsorb specifically nucleic acids (Alexander et al., 2007; Boom et al., 1990; Rogstad, 2003; Sarwat et al., 2006).

Boom et al. (1990) reported for the first time the use of Gdm⁺ and silica particles jointly in samples of human serum and urine for the isolation of DNA and RNA. This work demonstrated the potentiality of the method, simple chemistry and a basic foundation: in presence of any chaotropic agent and solvent as Gdm⁺-salts, nucleic acids are specifically adsorbed by silica particles (Boom et al., 1990; Boom et al., 1999).

The procedure reported in this paper is reproducible, simple, fast, with minimal requirements of specialized equipment and chemistry of easy handling, which proved to be suitable for obtaining high-quality DNA in aromatic and medicinal plant species. This protocol allows recovering on average 4.35 µg of DNA per 150 mg of tissue, with spectral quality of 1.85 for the ratio A₂₆₀/A₂₈₀ and 2.08 for the ratio A₂₆₀/A₂₃₀. The extracted DNA was appropriate for digestion with restriction enzymes, PCR amplification with primers of importance in population genetic studies and amplification of AFLP-type molecular markers.

Materials and methods

Plant material
Young leaves were harvested from 16 medicinal and aromatic Colombian species of importance for its content of essential oils (represented by 20 accessions of interest, see
Buffers and reagents

The protocol of DNA extraction for aromatic species called A-2X is described in detail in Tab. 1. The buffers and reagents used in the procedure are as follows: i) Buffer A-2X: 2% CTAB w/v, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 1% β-mercaptoethanol (add just before use), ii) Chloroform : isoamyl alcohol (24:1, v/v), iii) Isopropanol at -20°C, iv) Gdm⁺-salt solution: a) Guanidinium Hydrochloride (GdmHCl) Solution: 2 M or 4 M GdmHCl dissolved in sterile distilled water or b) Guanidinium Thiocyanate (GdmSCN) Solution: 2 M or 4 M GdmSCN, v) Ethanol at 90 and 70%, vi) Low salt TE buffer pH ≥ 8.0, and vii) Stock RNase A in TE buffer at a concentration of 1 μg μL⁻¹.

Chemistry of the protocol A-2X

The A-2X extraction buffer contains the reagents CTAB, EDTA, NaCl and β-mercaptoethanol. The CTAB is a cationic surfactant used as a detergent for both selective precipitation of nucleic acids and reduction of contamination by polysaccharides and denatured proteins (Cheng et al., 1997; Križman et al., 2006; Michiels et al., 2003; Pirrtilä et al., 2001; Sarwat et al., 2006). The EDTA is used as a chelate to inhibit DNases (Matasyoh et al., 2008). The NaCl in high concentrations prevents co-precipitation of DNA together with polysaccharides (Jobes et al., 1995; Križman et al., 2006; Porebski et al., 1997). The β-mercaptoethanol is a reducing agent used to break di-sulfide bridges, inhibit different types of enzymes by destabilization and prevent oxidation of polyphenols (Cheng et al., 1997). The buffer typically has pH between 7.0 and 8.0 for reducing contaminant RNA (Chomczynski and Sacchi, 1987).

The DNA purification is achieved using Gdm⁺-salts and silica-based extraction columns. The salts denature and break any inter- and intra-molecular interaction between DNA and proteins. We suggest the use of GdmHCl and GdmSCN, two of the most commonly used salts and with different denaturation powers, the first being the weakest and the other about 2.5 times more powerful (Castellino and Barker, 1968). The silica in the extraction columns efficiently adsorbs high molecular weight DNA after treatment with Gdm⁺-salts, which has been used successfully (Alexander et al., 2007; Ding et al., 2008; Ivanova et al., 2008; Sassa, 2007).

Quantification of extracted DNA

Two microliters of each extracted sample were used in order to determine its concentration, quantity and purity in a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Additionally, five microliters of each sample were loaded in a 1% agarose gel to determine the integrity of DNA. The staining of the products was carried out with EZVision™ Three (Amresco®) and each gel was run at 90 V for 30 min. The gels were documented using a gel documentation system Gel Doc XR (Bio-Rad Laboratories Inc.).

Enzymatic digestion with MseI

The restriction analysis was performed in nine of the 20 accessions under study. For this, 200 ng of extracted DNA were incubated for each of the samples with 1 U of MseI enzyme (Invitrogen™) for 2.5 h, following the manufacturer’s instructions. The samples were run at 90 V for 50 min and visualized in a 1% agarose gel.
PCR using primers of importance in genetic population studies

Four pairs of universal primers were used to amplify different non-coding chloroplast regions, \textit{trnL} (UAA) – \textit{trnF} (GAA), \textit{trnL} (UAA) intron (Taberlet et al., 1991), petA – \textit{psbE} (Fofana et al., 1997), and \textit{trnS} (GCU) – \textit{trnG} (UCU) (Hamilton, 1999), on six of the 20 accessions under study. Each PCR reaction contained 1 U of recombinant Taq DNA polymerase (Fermentas Inc.), 1X PCR Buffer with KCl, 4 mM MgCl₂, 200 μM of each dNTP, 0.2 μM of each primer, for a final reaction volume of 25 μL. The reaction was performed on a C1000 Thermal Cycler (Bio-Rad Laboratories Inc.), using the following amplification protocol: 1 cycle at 94°C for 4 min, 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 60 s, and a final extension step at 72°C for 5 min. The products were run at 90 V for 45 min and analyzed by 1% agarose gel electrophoresis.

Amplification of AFLP-type molecular markers

The kit AFLP® Analysis System I (Invitrogen™) was used to amplify three selective primer combinations (E-ACC – M-CAA, E-ACC – M-CAC, and E-ACC – M-CAG), on the same six accessions used previously to amplify non-coding regions of the chloroplast DNA. Two genotypes per accession were taken in order to reveal the presence of polymorphisms. The AFLP amplification was carried out following the manufacturer's instructions. The visualization of the products was performed as recommended by the manufacturer, although the staining of 6% PAGE was performed in silver nitrate. The gel was documented with a digital camera Olympus® SP-350.

Results

The concentration, quantity, quality and usefulness of the DNA extracted with the protocol A-2X were evaluated. The whole extraction process, from the harvesting of tissue until DNA quantification in NanoDrop™ 2000 and visualization in agarose gel on the 16 species (represented by 20 accessions of interest), was repeated three times independently for statistical support (Tab. 2). Similar results in quality and quantity were obtained in all the repetitions performed.

An average of 4.35 μg of DNA with an average concentration of 21.73 ng μL⁻¹ was extracted for all species evaluated using 150 mg of fresh tissue, being \textit{L. alba} the species with

<table>
<thead>
<tr>
<th>CA</th>
<th>Species</th>
<th>EO (%)</th>
<th>DNA (ng μL⁻¹)</th>
<th>SD DNA (ng μL⁻¹)</th>
<th>DNA (ng/150 mg)</th>
<th>DNA (μg/150 mg)</th>
<th>A₂₆₀/A₂₈₀</th>
<th>A₂₆₀/A₂₃₀</th>
<th># PCR</th>
<th># AFLPs</th>
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<td>Tagetes caracasana</td>
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<tr>
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<td>24.07</td>
<td>7.01</td>
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<td>4.81</td>
<td>1.85</td>
<td>2.23</td>
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<tr>
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<td>29.54</td>
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<td>2.70</td>
<td>640</td>
<td>64</td>
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TABLE 2. Production and quality data of the DNA extracted using the protocol A-2X in 16 aromatic and medicinal Colombian plant species. The table displays the accession number (CA), species, family, percent concentration of essential oils extracted by hydrodistillation (EO%), average concentration of extracted DNA from 150 mg of initial tissue, standard deviation (SD), and absorbance measurements A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀. The columns #PCR and #AFLPs indicate the number of PCR and AFLP reactions that could be carried out using 10 and 100 ng per reaction, respectively. Data shown correspond to the average of three independent repetitions made in the extraction process of the same individual in each accession.
On the other hand, the full restriction of DNA with the frequent-cutter enzyme *MseI* resulted in a high production of fragments lower than 2 Kb (Fig. 2). Different non-coding regions of the chloroplast DNA were amplified (Fig. 3), as well as three combinations of selective primers using the kit AFLP® Analysis System I (Invitrogen™) (Fig. 4).

**Discussion**

The isolation of high quality DNA for genetic analyses in aromatic and medicinal plant species is a difficult and time-consuming procedure (Ivanova et al., 2008; Khanuja et al., 1999; Matasyoh et al., 2008; Michiels et al., 2003; Pirttilä et al., 2001), which is strengthened by the use of conventional protocols that are not easily adaptable to other systems (Marva et al., 2007; Pirttilä et al., 2001; Sangwan et al., 2000; Sarwat et al., 2006). For this reason, we used the potentiality of the chemistry of nucleic acid extraction with Gdm⁺-salts and silica (Boom et al., 1990).

The quality and the molecular weight of the extracted DNA can be seen in Fig. 1. The DNA is clean in all evaluated species and does not present degradation or residual RNA. Furthermore, if we consider that the lambda DNA sequence is 48.5Kb, the molecular weight of the DNA extracted by the protocol A-2X would be close to the molecular weight of lambda DNA.

The highest amount of DNA (9.29 µg / 150 mg of tissue at a concentration of 46.46 ng µL⁻¹, SD = 20.01 ng µL⁻¹), and *H. atrorubens* the species with the lowest amount (2.41 µg / 150 mg of tissue at a concentration of 12.04 ng µL⁻¹, SD = 2.8 ng µL⁻¹). The average absorbance measurements to qualify the purity of the extracted samples were: $A_{260}/A_{280} = 1.85$ and $A_{260}/A_{230} = 2.08$. For each accession the number of PCR and AFLP reactions that could be performed using 10 ng and 100 ng, respectively, were calculated from the total amount of extracted DNA per accession. On average, 434 PCR reactions and 43 AFLP reactions could be carried out from 150 mg of tissue sample using the extraction protocol A-2X.

The quality and the molecular weight of the extracted DNA can be seen in Fig. 1. The DNA is clean in all evaluated species and does not present degradation or residual RNA. Furthermore, if we consider that the lambda DNA sequence is 48.5Kb, the molecular weight of the DNA extracted by the protocol A-2X would be close to the molecular weight of lambda DNA.

**FIGURE 1.** Visualization in 1% agarose gel of extracted DNA in 16 medicinal and aromatic Colombian plant species using the protocol A-2X. Right: pattern of lambda DNA (Invitrogen™); the concentrations (ng µL⁻¹) are indicated for comparison. In the samples, CA is the number of accession per species (Tab. 2). Note that the DNA extracted using the protocol A-2X is neither degraded nor contaminated with RNA. The staining was performed with EZVision™.
FIGURE 3. PCR amplification of different chloroplast regions in five aromatic and medicinal Colombian species. See materials and methods for references for the primers and regions used. L: molecular weight marker 100 bp Ladder (Axygen Biosciences); for the number of accession (CA), see Tab. 2. The accession CA-46 was included in the amplification because it is a *L. alba* chemotype of interest. Notice that the amplified bands are sharp, and with the exception of the products of CA-10, CA-14 and CA-44 in the region *trnS* – *trnG*, all the amplicons are suitable for sequencing. The samples were loaded on a 1% agarose gel and visualized with EZVision™.

FIGURE 2. Restriction digestion of nine medicinal and aromatic Colombian plant species with the enzyme *MseI*. DNA λ: lambda DNA (Invitrogen™); for the number of accession (CA), see Tab. 2. In each sample, the lane on the left and labeled with (+) corresponds to the restriction with *MseI*, while the other lane corresponds to the negative control. The samples were loaded on a 1% agarose gel and visualized with EZVision™.
Using the procedure described in Tab. 1, we were able to extract high quality and high molecular weight DNA in 16 aromatic and medicinal Colombian plant species with variable yields of essential oils (Tab. 2). The DNA extracted by the protocol A-2X is of high molecular weight and high quality which is probably explained by the chemistry used both in the extraction buffer and during the DNA purification (Fig. 1). Furthermore, the use of solutions with Gdm⁺ and the nature of the silica-based extraction columns allowed efficient elimination of contaminants and denatured proteins and recovering of large amounts of high molecular weight DNA.

The absorbance measurements of DNA at 280 nm, 260 nm and 230 nm, allowed to calculate two types of ratios widely adopted and qualify the DNA as “pure” or of high-quality (in average, $A_{260}/A_{280} = 1.8$ and $A_{260}/A_{230} = 2.0$). Nevertheless, some accessions exhibited ratios slightly away from these.
values. Digestion with the restriction enzyme Msel carried out in nine accessions with variable yields of essential oils and different ratios of purity (Tab. 2), showed that the potential contaminants detected by these ratios did not affect the use of the extracted DNA in restriction digestion and suggest that these ratios per se are not reliable or accurate indicators of the usefulness of the extracted DNA in this study. Amplification of four non-coding chloroplast regions in the six evaluated accessions resulted to be successful (Fig. 3) and suitable for sequencing and for subsequent population genetic studies. Likewise, PCR amplification with three selective AFLP primer combinations resulted to be optimal and showed several polymorphisms for each of the accessions evaluated (Fig. 4), which is desirable for the study of genetic variation and the development of molecular markers.

In summary, the DNA extracted using the protocol A-2X met the requirements of quality and quantity to apply different molecular techniques such as PCR, SSRs, RAPDs, ISSRs, AFLPs, sequencing, construction of genomic libraries, among other applications (Mace et al., 2003; Marva et al., 2007; Sarwat et al., 2006). As a more efficient and less expensive alternative to the use of commercial kits for nucleic acid extraction, the protocols that use Gdm* salts in conjunction with membranes or particles of silica and report the chemistry of the extraction may be easily implemented in the isolation of DNA from recalcitrant samples (Ivanova et al., 2008).

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Literature cited


