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Full Length Research paper

Genomic DNA extraction protocols from a Moroccan medicinal and aromatic plant *Artemisia herba-alba* Asso for RAPD-PCR studies

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This is the first report on development of protocol for high purity genomic DNA isolation from the Moroccan *Artemisia herba-alba* schrub leaves and optimization of conditions for RAPD-PCR analysis. Two DNA extraction protocols were specifically developed: QIAgen DNA Kit and protocol developed by Ouenzar et al. (1998). DNA yield and purity were monitored by gel electrophoresis and by determining absorbance at UV (A_{260}/A_{280}). Both ratios were between 1.7 and 2.0, indicating that the presence of contaminating metabolites was minimal. The Ouenzar and collaborators protocol gave higher yield but was more time consuming compared to QIAgen Kit. However, both techniques gave DNA of good quality that is amenable to RAPD-PCR reactions. Additionally, restriction digestion and PCR analyses of the obtained DNA showed its compatibility with downstream applications. Randomly Amplified Polymorphic DNA profiling from the isolated DNA was optimized to produce scorable and clear amplicons. The presented protocols allow easy and high quality DNA isolation for genetic diversity studies within *A. herba-alba*.

Key words: *Artemisia herba- alba*, genomic DNA extraction, PCR-RAPD.

INTRODUCTION

Artemisia herba-alba is a medicinal and aromatic dwarf shrub that grows wild in arid areas of the Mediterranean basin, extending into northern Himalayas (Vernin et al., 1995). This species has a vegetative growth in autumn (large leaves) and then at the end of winter to spring (small leaves). It is traditionally known for its essential oils. It has a very pronounced purgative effect and playing a major role in the control of intestinal worms (Idris et al., 1982). Extracts from *A. herba-alba* have antidiabetic effect (Al-Waili, 1986; Al-Khazraji et al., 1993; Al-Shamaony et al., 1994; Jouad et al., 2001) and strong antibacterial activities (Hatimi et al., 2001; Neerman, 2003). It also shows an allelopathic role against some other plants (Escudero et al., 2000). Flavonoids from this plant have a neurological action (Medhat Salah and Jäger, 2005).

The *A. herba alba* is also a big economic interest in Morocco. Its content in essential oil varies from 1 to 1.5% of dry material. Its essence is intended for the industry of the beauty care and the perfume shop. Two countries, Morocco and Tunisia, were divided in the international market to cater for this oil, but the big part returns to Morocco which holds 90% of the world market. More so, the *A. herba-alba* is recommended for the protection of the pastoral potential and the restoration of the degraded ecosystems.

In view of its medical value, mainly as natural antioxidant plant, *in situ* and *ex situ c*onservation strategies is needed through forestry policies and legislations to protect this species.

DNA molecular techniques were commonly used for studying the genetic relationship among accessions and analysis of genetic diversity between and within the populations. Random amplified polymorphic DNA (RAPD)

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markers have been widely used in the reconstruction of phylogenetic relationships for many organisms (Williams et al., 1990; Devos and Gale, 1992; Echt et al., 1992; Bakkali et al., 2010). However a commonly experienced problem with RAPD analysis, is its poor reproducibility (Devos and Gale, 1992). It is therefore essential to optimize the PCR to obtain reproducible and interpretable results.

Furthermore, isolation of high quality of DNA for use in many molecular markers is one of the most important and time-consuming steps (Zidani et al., 2005). Various protocols for DNA extraction have been successfully applied to many plant species (Doyle and Doyle, 1987; Guillemaut and Marechal-Douard, 1992; Ziegenhagen et al., 1993).

In the present work, we report two total genomic DNA isolation protocols: the DNeasy Plant Mini Kit and the protocol of Ouenzar et al. (1998). We also optimized RAPD marker technique that would be used to understand the species genetic diversity and population structure.

MATERIALS AND METHODS

Plant material

The *A. herba-alba* leaves were collected in Oujda region, Morocco, quickly frozen in liquid nitrogen, and then placed in -80°C until genomic DNA extraction.

DNA extraction protocols

Two different genomic DNA extraction methods were tested to recover genomic DNA from *A. herba-alba*. The first procedure assayed to extract genomic DNA from *A. herba-alba* was the DNeasy Plant Mini Kit (QIAGEN). Leaves of *A.* herba-*alba* were ground to a fine powder in CryoMill MM 400. Fifty milligrams of the powder was transferred to a pre-chilled microcentrifuge tube containg 400 µl of lysis buffer AP1 and 4 µl of RNase A (stock solution 100 mg/ml). The suspension was vortexed vigorously until a complete emulsion is formed and then incubated for 10 min at 65°C. Clumped tissues were dispersed using a disposable micropestle to allow higher yields of DNA. 130 µl of buffer AP2 was added to the lysate then the mixture was incubated for 5 min on ice. After centrifugation for 5 min at maximum speed and room temperature, the supernatant was transferred into mini columns filtration "QIAshredder spin column" placed in 2 ml collection tubes using a wide bore pipette and centrifuged for 2 min at maximum speed. The flow-through fraction was transferred into clean eppendorf tube. Occasionally, a light colored cell-debris pellet may appear and must not be disturbed. 0.5 volume of buffer AP3 and 1 volume of ethanol (96 to 100%) were added to the cleared lysate and mixed by pipetting. 650 µl of the mixture was transferred to DNeasy mini spin column and centrifuged for 1 min at 8000 rpm. This step was with the remaining sample and the flow-through and collection tubes were discarded. The DNeasy column was placed in a new collection tube and 500 µl of AW buffer (washing buffer) was added onto the column. After centrifugation for 1 min at 8000 rpm, the supernatant was discarded. An additional 500 µl of AW buffer was added to column and then centrifuged for 2 min at maximum speed. An additional washing step with 500 µl ethanol (96 to 100%) was applied to avoid coloration of the final eluted DNA. The column was placed in a new collection tube and span for 5 min under a vacuum in order to completely dry the column membrane. This additional step was introduced because it is crucial that no residual ethanol is carried over during elution of DNA from the column. Finally, the DNeasy column was transferred to a 1.5 ml microcentrifuge tube and 100 µl of preheated (65°C) buffer AE (elution buffer) was added directly onto the DNeasy column membrane. After incubation for 5 min at room temperature, the DNA was eluted in buffer AE by centrifugation for 1 min at 8000 rpm.

The second procedure used to extract genomic DNA was adapted from Ouenzar et al. (1998) with some modifications. 100 mg of leaves powder were ground in 5 ml of lysis buffer containing 50 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 0.05% BSA, 1% PEG_{6000} and 0.5% beta-mercaptoethanol. The suspension was transferred in tubes containing 60 µl of SDS (20%) and 40 µl of Sodium Acetate (3 M, pH 8), and incubated at 65°C for 30 min. The DNA was extracted once with chloroform-isoamyl alcohol (24/1) and once with phenol/chloroform/isoamyl alcohol (25/24/1), followed by an additional extraction with chloroform-isoamyl alcohol (24/1). Every extraction was preceded by a centrifugation at 10000 × g for 10 min. The final aqueous supernatant was recovered in fresh tubes and mixed with an equal volume (v/v) of cold Isopropanol then was incubated at -20°C for one hour. Finally, the mixture was centrifuged at 10000 × g for 20 min. The resulting pellet was washed twice with cold ethanol (70%), air-dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) then treated with proteinase-K (20 µg/ml) at 37°C for 30 min. Proteinase-K was removed by one extraction with phenol/chloroform/isoamyl alcohol. After centrifugation, nucleic acids were precipitated by adding two volume of absolute cold ethanol and 1/10 of NaCl (5 M), followed by incubation over night at -20°C. The DNA was pelleted by centrifugation at 10 000 × g for 20 min, washed with 70% cold ethanol, air-dried, and then dissolved in 200 µl of TE buffer. The extracted DNA was further treated with 2 µl of RNAase (10 µg/ml) at 37°C for 30 min.

DNA quality was examined following electrophoresis on agarose gel. Spectrophotometric analysis was performed at 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Absorbance at 260 nm and the A_{260}/A_{280} ratio provided an estimate of quantity and purity of extracted DNA, respectively.

Restriction analysis and electrophoresis

One microgram of the extracted DNA was analysed by digestion with *Alu*I, *Cfo*I, *Eco*RI and *Msp*I restriction enzymes according to manufacturer's specifications (Promega, Madison, WI, USA). Effective digestion of DNA by these enzymes was regarded as indicator of absence of polysaccharides (Do and Adams, 1991). The digested genomic DNA was separated on a 3% TBE agarose gel at 80, stained with ethidium bromide, and then viewed under UV illumination and photographed using.

Optimization of RAPD-PCR reaction

For the optimization of RAPD reaction using the DNeasy Plant Mini Kit (QIAGEN) extracted DNA, five decamer primers, OPA-01 (CAGGCCCTTC), OPA-02 (TGCCGAGCTC), OPA-03 (AGTCAGCCAC), OPA-04 (AATCGGGCTG) and OPA-05 (AGGGGTCTTG) from Operon Technologies Inc. (Promega, Madison, WI, USA), were used. The PCR conditions were optimized by varying the quantity of the DNA template, the concentration of $MgCl_2$, the units of *Taq* polymerase, dNTPs and primer concentrations and the annealing temperature (Tm). The initial protocol tested for RAPD reaction was the one recommended

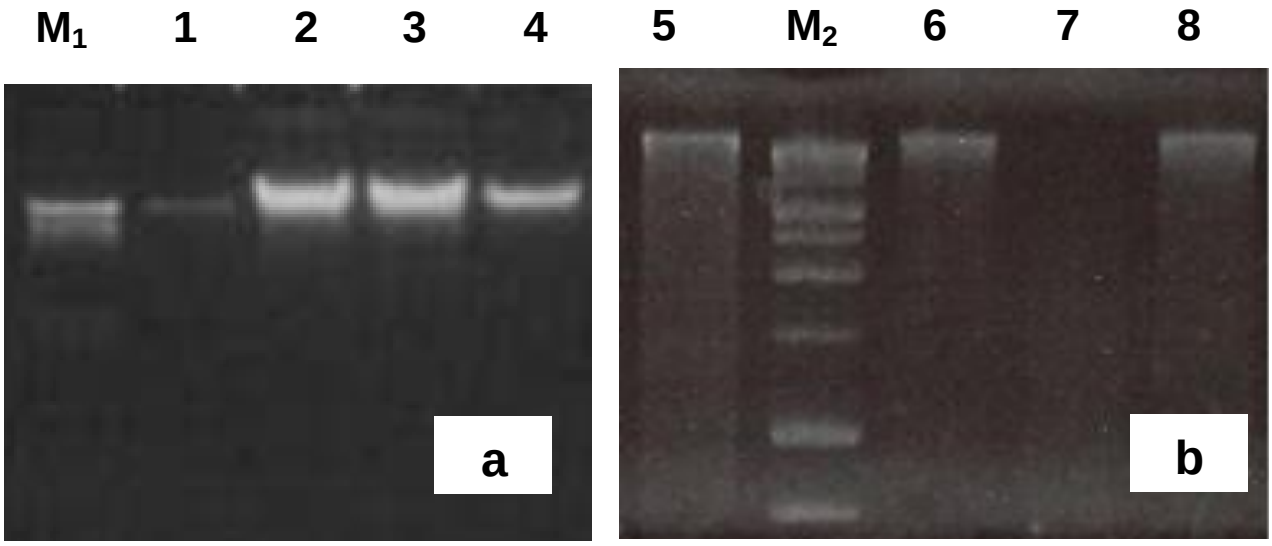

Figure 1. (a) Agarose gel analyses of DNA isolated from *A. herba-alba* leaves obtained by the two different procedures; lanes 1 and 4 represent DNA obtained by the DNeasy Plant Mini Kit; lane 2 and 3 indicate DNA samples isolated by the method described in Ouenzar et al. (1998). (b) Agarose gel analyses of *Artemisia* DNA digested with restriction enzymes. Lane 5, 6, 7, and 8 represent restriction digestion with *Alu*I, *Cfo*I, *EcoR*I and *Msp*I, respectively. M_1: λDNA/Hind III marker. M_2: 10 kb marker.

by Vural and Dageri (2009). Each 20 µl reaction volume contained about 50 ng of template DNA, 4 µl 1x buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM Operon primer and 0.2 units of *Taq* polymerase. The thermal cycles used were: 1 cycle of 3 min at 94°C, followed by 30 cycles of 45 s. at 94°C, 1 min at 37°C, extension was carried out at 73°C for 1 min and final extension at 72°C for 7min and a hold temperature of 10°C at the end. The parameters tested in RAPD-PCR reactions were as follows: 7 different concentrations of DNA template (5, 10, 20, 40, 70, 100, 200 ng), 8 different concentrations of $MgCl_2$ (1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 mM), 9 different concentrations of *Taq* polymerase (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2 unit), 4 different concentrations of primers and 3 different annealing temperatures (33, 37 and 40°C). Amplification products were separated by electrophoresis in 2% agarose gels. It was performed in 1xTBE (trisborate-EDTA) running buffer at 100 volts and then the revelation of RAPD profiles was done by ethidium bromide staining under ultra-violet light. Molecular weights of the amplified products were estimated using 100 bp and 10 kb DNA ladder.

RESULTS

Isolation of good quality DNA from *A. herba-alba* proved difficult because leaves are very rich in polyphenolic compounds. In the present study, two different genomic DNA extractions were performed in order to obtain polysaccharide free genomic DNA from *Artemisia* shrub leaves, namely, the method described in Ouenzar et al. (1998) and the QIAgen DNeasy Plant Mini Kit (Valencia, CA, USA). Both procedures were used with some modifications. The steps and reagents of the first procedure are routinely used for plant genomic DNA extraction. For the second procedure, we improved some modifications according to Bakkali et al. (2010). The concentrations and purity of DNA obtained from those procedures were determined with spectroscopy and gel-electrophoresis. The modified Ouenzar et al. (1998) extraction procedure produced a relatively high yield product (2 µg/50 mg sample tissue). While the average DNA yield by the DNeasy Plant Mini Kit procedure is around 0.8 to 1 µg per 50 mg sample tissue. Our procedures were capable of extracting, high-quality genomic DNA as indicated by the absorbance ratio $(A_{260})/(A_{280})$ of approximately 2.0.

In addition, the suitability of the genomic DNA obtained was also checked in restriction digestion and PCR-RAPD reactions. Digestion reactions with four different restriction enzymes, *Alu*I, *Cfo*I, *EcoR*I and *Msp*I, suggest that the extracted DNA was of low polysaccharide contaminants (Figure 1a and 1b).

In this study, we optimized RAPD-PCR reaction conditions for *Artemisia* shrub. A summary of the parameters tested and the corresponding results is presented in Table 1. The effects of the template DNA quantity (Figure 2), $MgCl_2$ concentration (Figure 3), primer and dNTP concentrations, *Taq* polymerase units (Figure 4) and the annealing temperature (Figure 5) on RAPD-PCR reaction were investigated. Optimum conditions were chosen that give more discriminatory band profiles (Figure 6).

DISCUSSION

Quantity and purity of extracted genomic DNA plays crucial role for analysis of molecular diversity and

Table 1. Summary of the parameters of RAPD-PCR tested and the optimum conditions selected.

PCR parameter	Range tested	Optimum conditions
DNA concentrations (ng)	5-10-20-30-40-50-70-100-200	5 ng
Magnesium chloride (mM)	1.5-2.5-3-3.5-4-4.5-5	1.5 mM
Primer concentration (µM)	0.1-0.3-0.5-0.7	0.3 µM
Taq polymerase (units)	0.2-0.4-0.6-0.8-1- 1.2-1.4-1.6-1.8-2	0.4 unit
dNTPs concentration (mM)	0.05-0.1-0.15-0.2-0.25	0.1mM
Temperatures of annealing (°C)	33, 37 and 40	37°C

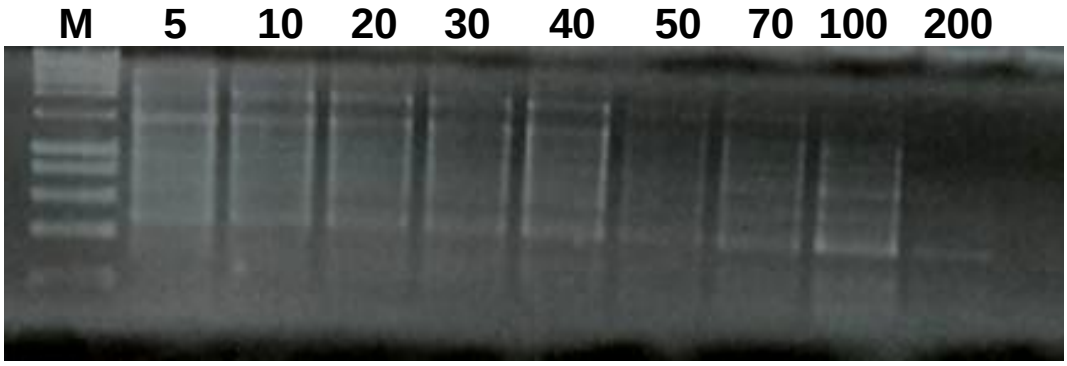

Figure 2. RAPD-PCR profile of nine DNA concentrations ranging from 5 to 200 ng obtained by the Operon primer OPA-05 in 1.8% agarose gel. M: 10 kb DNA ladder.

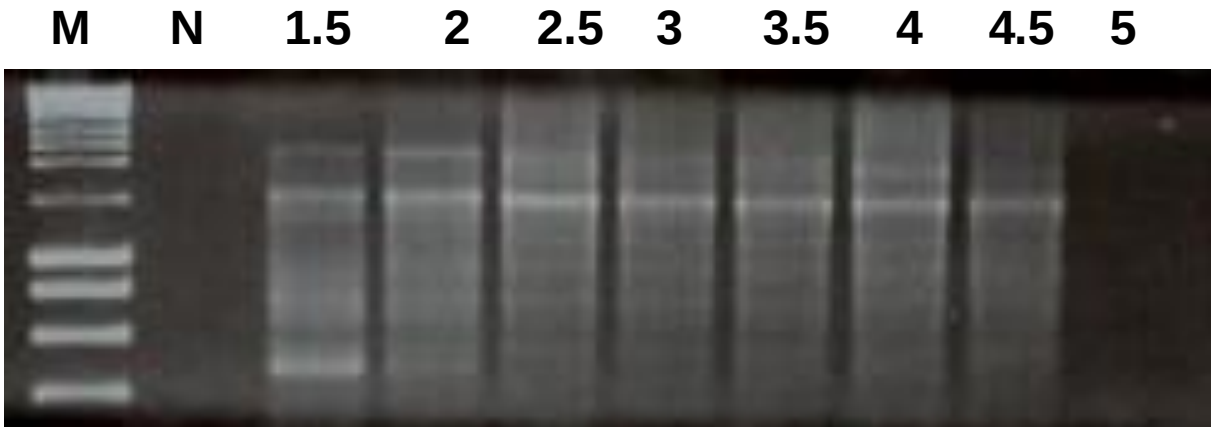

Figure 3. Optimization of RAPD-PCR parameters with eight $MgCl_2$ concentrations using the Operon primer OPA-05 and 5 ng of *A. herba-alba* genomic DNA. M: 10 kb DNA ladder. N: PCR negative control (no template DNA).

optimization of different parameters for PCR (Weeden et al., 1992; Staub et al., 1996). There are difficult to get plant DNA free from contaminating proteins and polysaccharides. These compounds can link covalently to DNA which becomes useless in enzymatic based reactions. It is for that reason that our first efforts concerned the development of an extraction protocol capable of giving a DNA which can be used as support for the enzymatic reactions. To do it, two basic techniques were used. The technique of DNA extraction by the QIAGEN Kit, it is effective, simple and fast. The best adapted to the plant material of *A. herba-alba*. Our modified Kit methods are those proposed by Bakkali et al. (2010). It could be suitable for increasing and standardizing the quality and quantity of genomic DNA extracted. The modified Ouenzar et al. (1998) extraction

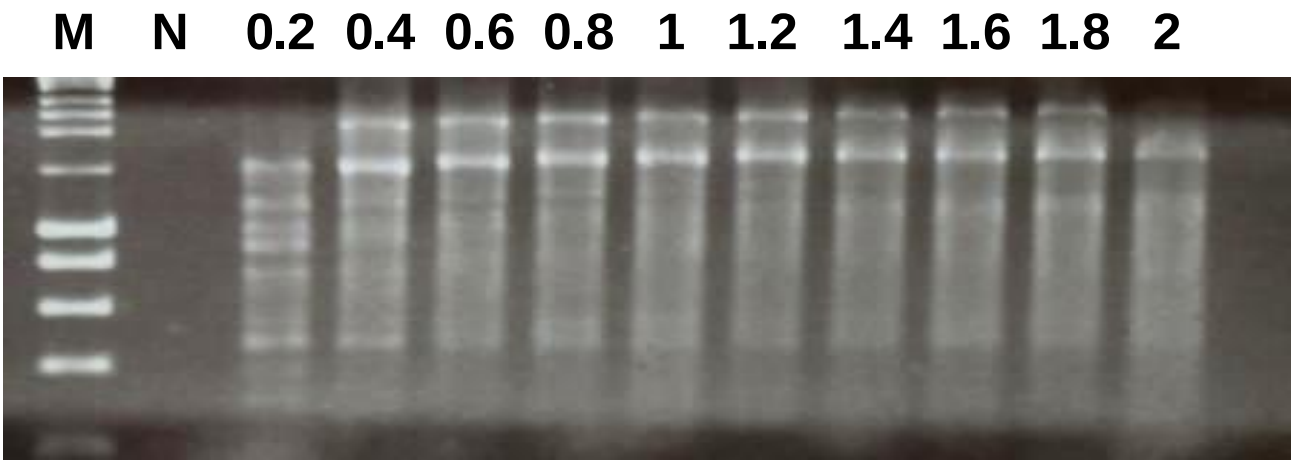

Figure 4. RAPD-PCR profile of *A. herba-alba* DNA (5 ng) using ten different amounts of *Taq* DNA polymerase using the Operon primer OPA-05. M: 10 kb DNA ladder. N: PCR negative control (no template DNA).

protocol allows the obtaining of translucent, good quality and intense genomic DNA. This procedure is a maxi-scale preparation which is very time-consuming. Its adaptation to the *A. herba-alba* leaf would probably be due, in part, to its lysis buffer containing the Polyethylene Glycol (PEG_{6000}) and on the other hand, to a long processes of deproteinisation. The PEG is considered by some authors as a chemical agent having a positive and effective impact on the dissociation of the tannin-protein complex responsible for the anchoring of several elements or cellular constituents of certain plants (Jone and Mangan, 1977; Priolio et al., 2000).

RAPD is one of the molecular marker that had been used in genetic study. RAPD technique had been used in genetic study for Moroccan forest tree (Konaté, 2007; Bakkali et al., 2010) and also had used for diversity analysis for medicinal and aromatic plant (Padmalatha and Prasad, 2006). To obtain reproducible and interpretable results with RAPD analysis, it is essential to optimize some PCR parameters.

In this study, the PCR-RAPD protocol was optimized by introducing several modifications to the original (Vural and Dageri, 2009) protocol in some PCR-components such as template DNA, primer, magnesium chloride, *Taq* polymerase, dNTPs, as well as in amplification cycles especially annealing temperature.

Amount of template DNA strongly influences the outcome of the reaction. More than 30 ng / 25 µl give the premium amplification (Henegariu et al., 1997). In the present study, identical RAPD profiles were found when DNA amounts varied from 5 to 200 ng. DNA concentration of 5 ng / 20 µl was found optimum. Optimization of $MgCl_2$ is an important factor for precise amplification; 1.5 mM of $MgCl_2$ was found optimum in 20 µl final volume. Moreover, concentration of dNTPs in reaction mixture is also strongly correlated to the Mg ions concentration due to the interaction between mononucleotides and the Mg^{2+}. 0.1 mM found optimum in this study.

In PCR, 2 to 2.5 units of Taq Polymerase are normally used in 100 µl final volume. Higher *Taq* Polymerase concentration (above 4 units/100 µl) can generate non-specific products and may reduce the yield of the desired product (Saiki, 1988). However, in the present study, 0.4 unit/20 µl reaction was used to amplify the DNA of *A. herba-alba*. Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 35 to 55°C. The annealing temperature 37°C was found optimum to amplify with primer pairs OPA-01 to OPA-05. The oligonucleotide primers OPA-01 to OPA-05 generate relatively more diverse and reproducible genomic fingerprints for a number of plant species and were used for the optimization of RAPD parameters (Doulis et al., 2000; Padmalatha and Prasad 2006; Bakkali et al., 2010). We used the same primers to optimize the primer concentration for RAPD-PCR analysis of *A. herba-alba*. Results showed that 0.3 µM of the primer generated distinct and reproducible DNA profile.

In this study, the two different extraction methods gave a DNA of quality suitable for RAPD-PCR analysis. The protocol described in Ouenzar et al. (1998), used with some modifications, gave higher DNA yield compared to QIAgen. These modifications were made in both the lysis buffer (5mM EDTA, 0.05% BSA and 0.5% beta-mercaptoethanol) as the various steps in the implementation of the Protocol. The standard reaction developed included: 5 ng of DNA template, 1.5 mM $MgCl_2$, 0.3 mM primer, 1 mM dNTPs (for each) and with 0.4 U of *Taq* polymerase per 20 µl PCR reaction. The protocols described here and the optimized RAPD parameters constitute a strong beginning for future

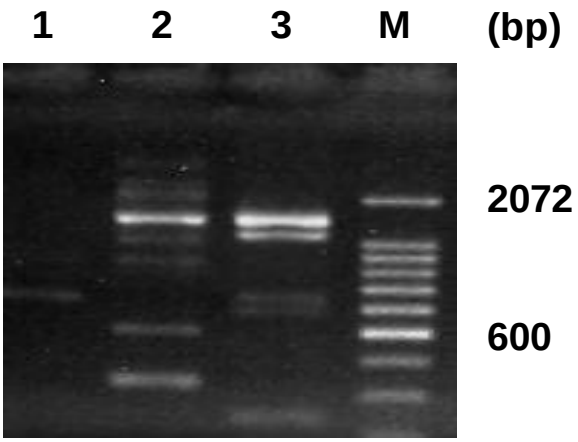

Figure 5. RAPD-PCR profile of *A. herba-alba* DNA (5 ng) in 1.8% agarose gel with three different annealing temperatures: (1) 33°C, (2) 37°C and (3) 40°C. M: 100 bp DNA ladder. N: PCR negative control (no template DNA).

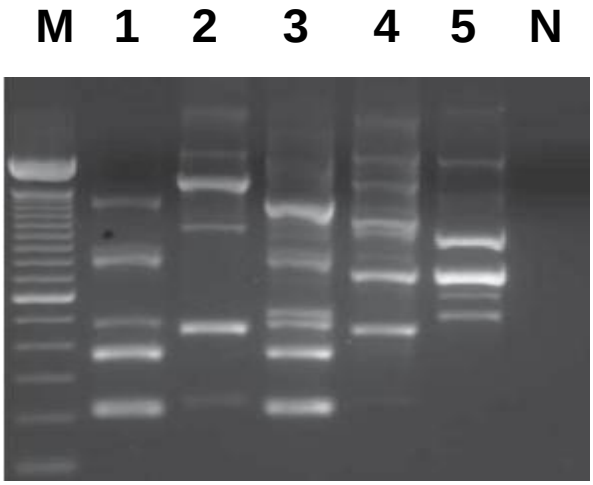

Figure 6. A representative agarose gel fractionation of RAPD amplification products from A. herba-alba genomic DNA (5 ng) derived from a single Artemisia shrub using the Operon primers: OPA-01 (1), OPA-02 (2), OPA-03 (3), OPA-04 (4), OPA-05 (5). Amplification products were fractionated in a 1.8% agarose gel. M: 100 bp DNA ladder. N: PCR negative control (no template DNA).

molecular characterization and genetic improvements studies in *A. herba-alba*.

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