GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

DNA isolation protocol for the medicinal plant lemon balm (*Melissa officinalis*, Lamiaceae)

S. Ghaffariyan, S.A. Mohammadi and S. Aharizad

Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Corresponding author: S.A. Mohammadi
E-mail: mohammadi@tabrizu.ac.ir

Genet. Mol. Res. 11 (2): 1049-1057 (2012)
Received February 22, 2011
Accepted June 21, 2011
Published April 27, 2012
DOI http://dx.doi.org/10.4238/2012.April.27.3

ABSTRACT. Lemon balm (*Melissa officinalis*) is a medicinal plant that is widely used as a sedative or calmant, spasmolytic and antibacterial agent and sleep aid. This has led to a high demand for lemon balm products, resulting in the extinction of this species in some of its natural habitats. Molecular techniques have increasingly been used in plant diversity conservation and isolation of PCR amplifiable genomic DNA is an important pre-requisite. Lemon balm contains high levels of polyphenols and polysaccharides, which pose a major challenge for the isolation of high-quality DNA. We compared different genomic DNA extraction protocols, including traditional phenol-chloroform DNA extraction protocols and two commercial kits for DNA purification for their ability to produce good-quality DNA from fresh leaves of five lemon balm genotypes. Quality and quantity of the DNA samples were determined using 0.8% agarose gel electrophoresis and a spectrophotometer. The DNA purity was further confirmed by PCR amplification using barley retrotransposon LTR base primers. The spectral quality of DNA as measured by the A_{260}/A_{280} ratio ranged from 1.46 to 2.37. The Fermentase genomic DNA purification kit and the CTAB extraction protocol using PVP and ammonium acetate to overcome the high levels of polyphenols and polysaccharides yielded high-quality DNA with a mean A_{260}/A_{280} ratio

of 1.87. The quantity of DNA and its PCR purity were similar with all the protocols, but considering the time and cost required for extraction of DNA from a large number of samples, the CTAB protocol using PVP and ammonium acetate is suitable for lemon balm.

Key words: DNA extraction; Lemon balm; Medicinal plants; Retrotransposon markers

INTRODUCTION

Lemon balm (*Melissa officinalis* L.), an important medicinal plant species mainly grown in natural flora, is native to southern Europe and northern Africa, and east as far as the Caucasus and northern Iran. Its wild types grow in all Mediterranean countries and the southern part of the Alps (Davis, 1982; Ilisulu, 1992). It is used as an additive in food and herb tea, and as an ingredient in cosmetics, ornaments and medicines (Adinee et al., 2008). As a medicinal plant, lemon balm has traditionally been used due to its memory enhancing properties, but it is currently more widely used as a sedative or depressant, spasmolytic and antibacterial agent, and sleep aid (Coleta et al., 2001; Sadraei et al., 2003; Kennedy et al., 2003, 2004; de Sousa et al., 2004). In Iran, lemon balm and many medicinal plant species and populations are threatened by over-harvesting, land conversion and habitat destruction. This situation necessitates the development of proactive approaches for medicinal plant collection and the incorporation of integrated principles to ensure sustainable use of the plant. The application of DNA markers in the conservation of rare and endangered medicinal plants was explicated, including identification of germplasm resource, population structure and diversity, sampling strategies of *ex situ* conservation, evaluation of the conservation effects of rare and endangered medicinal plants, as well as elucidation of their endangered mechanism, etc. The information could help in drawing up conservation strategies and conservation measures for references (Joshi et al., 2004).

Successful application of a DNA marker system requires pure, intact and high-quality DNA. 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. Most of medicinal plant species contain high levels of polysaccharides, polyphenols, several pigments, and other secondary metabolites such as tannins, alkaloids, phenolics, and terpenes (Wen and Deng, 2002), which make DNA unusable in amplification and restriction reactions (Michiels et al., 2003; Qiang et al., 2004). The secondary metabolites are not completely removed using common extraction protocols and remain as contaminants in the final DNA preparations. Polysaccharides make DNA viscous, glue-like and non-amplifiable in the polymerase chain reaction (PCR) by inhibiting *Taq* DNA polymerase activity and also interfere in the accuracy and activity of restriction enzymes (Porebski et al., 1997).

To isolate pure and intact DNA from plant tissues, numerous protocols have been established (Saghai Maroof et al., 1984; Doyle and Doyle, 1990; Scott and Playford, 1996; Sharma et al., 2000; Pirttilä et al., 2001; Drábková et al., 2002; Shepherd et al., 2002; Mogg and Bond, 2003; Haymes et al., 2004). However, these DNA extraction protocols are not suitable for all medicinal plants, since each medicinal plant species contains specific secondary

metabolites (Ribeiro and Lovato, 2007). Lemon balm also contains high amounts of phenolics, polysaccharides and organic constituents that interfere with DNA isolation and purification (Adinee et al., 2008). The aim of this study was to compare various DNA isolation protocols and identify the optimal DNA extraction protocol for lemon balm.

MATERIAL AND METHODS

Plant materials

The plant materials included two lemon balm varieties from Japan and Germany and three Iranian wild genotypes. For each genotype, leaf samples were collected from 15 field grown plants. The leaves were frozen in liquid nitrogen and transported to laboratory and stored at -80°C until use.

DNA extraction

Total genomic DNA was extracted from leaf tissue by means of the three modifications of the CTAB protocol and two kit-based methods.

CTAB extraction method 1

Total genomic DNA from leaf tissue was extracted in CTAB isolation buffer as described by Saghai Maroof et al. (1984) with minor modifications on concentration and amount of the components.

Solutions

- CTAB extraction buffer (20 mM sodium EDTA, 100 mM Tris-HCl, 1.4 M NaCl, 2.0% (w/v) CTAB)
- 0.2% β-mercaptoethanol
- TE
- Wash solution 1 (2.5 M NaOAc, 76% EtOH)
- Wash solution 2 (1 M NH_4OAc, 76% EtOH)
- Chloroform:isoamyl alcohol (24:1)
- Isopropanol
- 5 M NaCl
- RNase

Protocol

- Preheat CTAB solution to 60ºC and add 0.2% β-mercaptoethanol just before use.
- Grind 0.5 g leaf tissue in liquid nitrogen using mortar and pestle, transfer to 2.0-mL Eppendorf tube containing 800 µL extraction buffer and swirl.
- Incubate at 65°C for 60 min and mix every 5 min during incubation.
- After 5-min incubation at room temperature, add 800 µL chloroform-isoamyl alcohol (24:1, v/v) and mix.

- Centrifuge for 15 min at 3000 rpm.
- Transfer 700 µL supernatant to new Eppendorf tube, add equal volume of cold isopropanol and leave at -20°C until DNA mass appears (1-24 h).
- Centrifuge for 5 min at 10,000 rpm.
- Discard supernatant and wash pellet three times with wash solution 1 for 10 min.
- Wash pellet three times with wash solution 2 for 10 min.
- Air dry pellet and dissolve it in 300 µL TE.
- Add 1 µL RNase per tube and incubate at 37°C for 1 h.

CTAB extraction method 2

This protocol was proposed by Lodhi et al. (1994) and utilizes polyvinylpyrrolidone (PVP) for removing phenolic pollutions.

Solutions

- CTAB buffer (2.0% CTAB, 20 mM sodium EDTA, 100 mM Tris-HCl, 1.4 M NaCl 2.0% (w/v))
- 0.2% β-mercaptoethanol
- TE buffer
- 2.0% PVP
- Chloroform-isoamyl alcohol (24:1)
- Isopropanol
- 5 M NaCl

Protocol

- Grind the leaf tissue in liquid nitrogen using mortar and pestle, transfer to 2.0-mL Eppendorf tube.
- Add 800 µL CTAB buffer and 120 µL PVP and mix.
- Incubate at 60°C for 30-60 min and mix every 5 min during incubation.
- Incubate at room temperature for 5 min and add 950 µL chloroform-isoamyl alcohol (24:1). Mix slowly to form an emulsion.
- Centrifuge for 15 min at 8000 rpm at room temperature.
- Transfer supernatant to new tube. Repeat centrifugation.
- Add 250 µL NaCl to the aqueous solution and invert several times to mix well.
- Add 1 mL cold isopropanol and incubate at -20°C until DNA mass appears.
- Centrifuge for 5 min at 5000 rpm at room temperature.
- Discard supernatant and wash DNA pellet with 70% cold ethanol for 5-10 min.
- Dry DNA pellet and dissolve it in 300 µL TE.
- Add 1 µL RNase and incubate at 37°C for 1 h.

Modified Lodhi et al. (1994) method

This method is similar to method 2, except that it uses ammonium acetate.

Protocol

- Add 100 µL 7.5 M ammonium acetate and incubate in ice for 20 min to sediment proteins.
- Incubate at -20°C for 24 h.
- Centrifuge for 20 min at 13,000 rpm at 4°C, discard supernatant and air dry pellet.
- Dissolve DNA pellet in 200 µL TE.

Fermentase PCR cloning kit

In this method, the DNA extracted using methods A, B or C was purified by means of a PCR cloning kit.

Solutions

In addition to solutions required for DNA extraction, Fermentase PCR cloning kit, 95% ethanol and ddH_2O were also used.

Protocol

To purify DNA, 500 µL binding solution was added to 100 µL DNA dissolved in TE. Other steps were according to the Fermentase protocol.

Fermentase genomic DNA purification kit

Solutions

- Lysis solution (part A + part B)
- Precipitation solution
- Binding solution
- Wash solution
- 100% EtOH

Protocol

The method was according to the Fermentase genomic DNA purification kit protocol for extraction of total genomic DNA. Approximately 0.1 g leaf tissue was ground in liquid nitrogen with mortar and pestle, powder was transferred to a 1.5-mL Eppendorf tube and 200 µL TE was added. Other steps were according to the kit protocol. Finally, extracted DNA was treated with RNase for 1 h at 37°C.

Determination of DNA quality and quantity

The quantity of DNA extracts was estimated via spectrophotometry (BioPhotometer, Eppendorf AG, Germany) at 260 nm assuming that an absorbance of 1.0 U corresponds to a

DNA concentration of 50 µg/mL (Surzycki, 2000). In addition, DNA purities were evaluated via the absorbance ratios A_{260}/A_{280}. Agarose gel (0.8%) electrophoresis was also performed to determine DNA quality.

Amplification quality of DNA

To assess the amplification quality of the extracted DNA, PCRs were carried out with three different protocols in a 10-µL volume using primers designed based on barley retrotransposon Sukkula and Nikkita LTRs. The amplifications were carried out using a Mastercycler gradient thermocycler (Eppendorf).

In protocol I, reaction mix consisted of 40 ng genomic DNA, 1 U *Taq* DNA polymerase (Cinagen, Iran), 2 mM dNTP mix, 1.5 mM $MgCl_2$ and 12 pmol of each primer. The PCR program comprised one cycle of initial denaturation at 94°C for 4 min followed by 35 cycles including denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and one cycle of final extension at 72°C for 7 min. The second protocol was similar to protocol I, except that 0.01% BSA was added to the reaction mix. In the third protocol, the reaction mix was the same as that of the second protocol, but the PCR program consisted of one cycle of initial denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min and extension at 74°C for 2 min and one cycle of final extension at 74°C for 9 min. The amplified products were separated using 4% polyacrylamide gel electrophoresis on GelScan 3000 (Corbett Robotics, Australia).

RESULTS AND DISCUSSION

High-quality and -quantity DNA are necessary for successful DNA amplifications, Southern blot analysis and library construction. Failure to isolate large molecular weight DNA of sufficient purity from medicinal plants could be attributed to the presence of polysaccharides, polyphenolic compounds, tannins, alkaloids, phenolics, terpenes, etc. (Michiels et al., 2003). Although various protocols for simple isolation of high-quality DNA from plant tissues are available (Saghai Maroof et al., 1984; Doyle and Doyle, 1990; Scott and Playford, 1996; Sharma et al., 2000; Pirttilä et al., 2001; Drábková et al., 2002; Shepherd et al., 2002; Mogg and Bond, 2003; Haymes et al., 2004), none of them is fully applicable for a large range of plants. In the present study, we focused on the analysis of PCR and PCR temperature program, and their requirements for efficient PCR quality DNA extraction.

The quality of DNA was assessed by its physical appearance, spectrophotometry, gel electrophoresis, and PCR amplification. When the physical appearance of the extracted samples was compared, the methods C, D and E performed best and resulted in absolutely transparent DNA solutions, while the DNA obtained from methods A and B showed yellowish and dark contaminations mainly due to the fast oxidation of the extracts. High viscosity of DNA extracted using methods A and B may be due to high endogenous levels of polysaccharides, phenolics and other organic constituents that interfere with DNA isolation and purification (Sarwat et al., 2006). Ribeiro and Lovato (2007) also reported colored DNA solutions while comparing different DNA extraction protocols using fresh and herbarium specimens of the genus *Dalbergia*. It seems that addition of ammonium acetate in method C, compared to methods A and B could solve the problem of color contamination in DNA.

The yields (A_{260}) and purities (A_{260}/A_{280}) of the DNA solutions were assessed using a

spectrophotometer and the average of five samples for each method is summarized in Table 1. The A_{260}/A_{280} ratio is an indication of protein contamination. For high-quality DNA, A_{260}/A_{280} ratios should range between 1.8 and 2.2 and A_{260}/A_{230} ratios between 1.5 and 1.8 (Moyo et al., 2008). In our study, the A_{260}/A_{280} ratio ranged between 1.46 (Method A: Saghai Maroof et al., 1984) and 2.37 (Method C: modified Lodhi et al., 1994) indicating large amounts of protein impurities in the DNA samples isolated by these methods. Method B (Lodhi et al., 1994) and E (Fermentas genomic DNA purification kit) yielded high-quality DNA with an average A_{260}/A_{280} ratio of 1.87 and range of 1.81-1.98 and 1.81-1.99, respectively (Table 1). Method B (Lodhi et al., 1994) used PVP to remove contamination. PVP forms complex hydrogen bonds with phenolic compounds and coprecipitates with cell debris upon lysis. When the extract is centrifuged in the presence of chloroform, the PVP complexes accumulate at the interface between the organic and the aqueous phases (Michiels et al., 2003). Quantification of DNA by spectrophotometer may be affected by residual contamination; therefore, it can only be considered a very rough estimate. DNA yields ranged from 46.0 ng/µL (Fermentas PCR cloning kit) to 1050.6 ng/µL (Method A: Saghai Maroof et al., 1984). The quantity of DNA extracted via Methods A and E was 698.4 and 570.2 ng/µL, respectively. As evidenced by agarose gel electrophoresis, all methods yielded relatively high molecular weight DNA without sharing (Figure 1). RNA pollutions were removed by RNase treatment.

Table 1. DNA yields (A_{260}) and purities (A_{260}/A_{280}) of samples isolated via A (Saghai Maroof et al., 1984), B (Lodhi et al., 1994), C (modified Lodhi et al., 1994), D (Fermentas PCR cloning kit), and E (Fermentas genomic DNA) protocols.

Samples	A		B		C		D		E	
	260	260/280	260	260/280	260	260/280	260	260/280	260	260/280
1	937	1.88	804	1.82	112	2.15	40	-	497	1.81
2	817	1.80	386	1.81	116	3.50	35	1.03	817	1.87
3	1454	1.86	544	1.89	77	1.85	72	1.75	595	1.85
4	1091	1.78	745	1.98	115	1.85	61	3.14	520	1.81
5	954	1.74	1013	1.85	53	2.50	22	3.60	422	1.99
Mean	1050.6	1.46	698.4	1.87	94.6	2.37	46.0	1.90	570.2	1.87

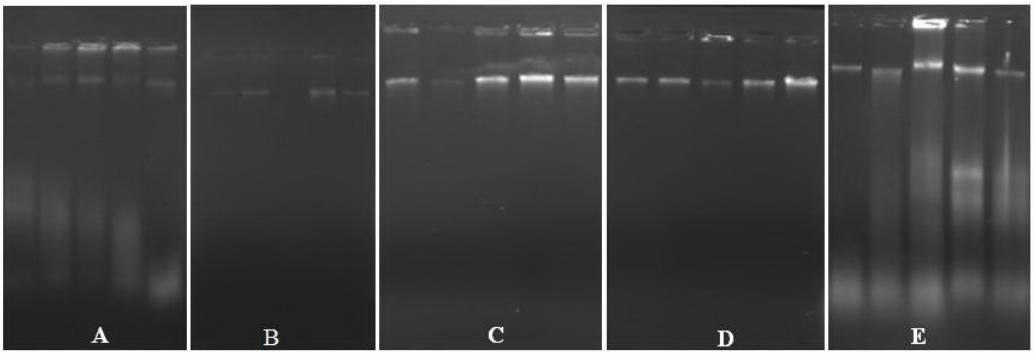

Figure 1. Agarose gel electrophoresis of DNA samples isolated via A (Saghai Maroof et al., 1984), B (Lodhi et al., 1994), C (modified Lodhi et al., 1994), D (Fermentas PCR cloning kit), and E (Fermentas genomic DNA) protocols.

The suitability of extracted DNA for downstream molecular processes was further verified by PCR amplification. Three PCR conditions were used to amplify genomic fragments from lemon balm genotypes using primers designed based on barley Sukkula and Nikkita LTR retrotransposons. DNA samples extracted using all the assessed protocols were amplified, but the quality and quantity of amplification were unsatisfactory when PCR was preformed based on PCR protocol I. Addition of BSA resulted in improved amplification. The DNA extracted using protocols C and D showed poor amplification even in the presence of BSA, while the DNA isolated by protocols A, B and E amplified efficiently especially using PCR protocols using BSA (Figure 2). Successful extraction of genomic DNA that can be amplified by PCR can lead to the establishment of DNA fingerprinting for the individual genotypes for various molecular approaches. BSA is often used as a carrier protein and stabilizing agent in enzymatic reactions. In PCRs, BSA has been shown to enhance enzyme activity and therefore amplification efficiency.

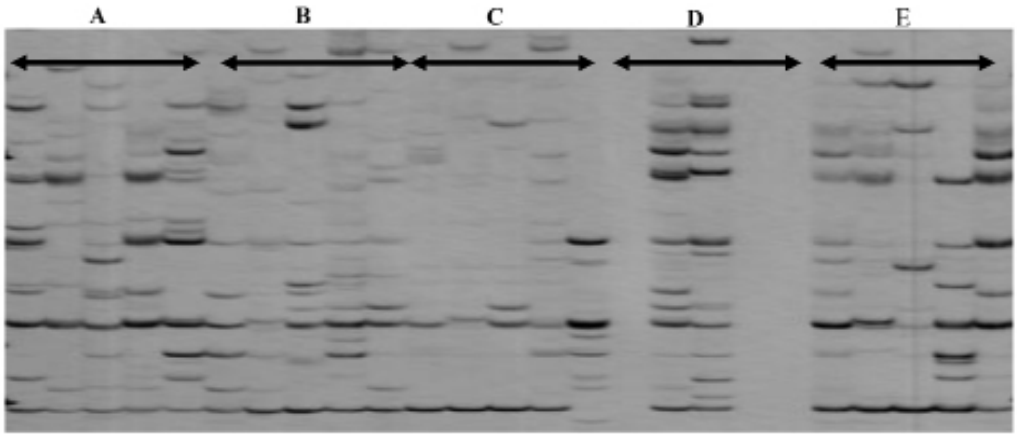

Figure 2. Gel electrophoresis (4% polyacrylamide) showing PCR profiles of amplified DNA from A (Saghai Maroof et al., 1984), B (Lodhi et al., 1994), C (modified Lodhi et al., 1994), D (Fermentas PCR cloning kit), and E (Fermentas genomic DNA) protocols using barley LTR retrotransposon primers.

Five different protocols were assessed to optimize an efficient method for DNA extraction from lemon balm with high secondary metabolite components. High-quality DNA was successfully isolated using a genomic DNA purification kit. The quantity of DNA extracted using Saghai Maroof et al. (1984) and Lodhi et al. (1994) protocols was high and the quality of DNA obtained via Lodhi et al. (1994) method was comparable to that of the genomic DNA purification kit as revealed by spectrophometer analysis. Considering the time and cost required for extraction of DNA from a large number of samples, the protocol described by Lodhi et al. (1994), with minor modification, is suitable for lemon balm.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the laboratory facilities provided by Genomics & Molecular Breeding Lab, Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Iran.

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