A modified CTAB method for isolation of DNA from mint plants (*Mentha* spp.)

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Abstract

A large number of medicinal and aromatic plants produce secondary metabolites such as alkaloids, flavonoids, phenols, gummy polysaccharides, terpens and quinines. For genetic improvement of these medicinal plants, it is desirable to use molecular markers for screening of accessions, choosing of parents and selection of progeny. The presence of certain metabolites has been observed to interfere with DNA isolation procedures and downstream reactions such as DNA amplification. It has been thought that the RAPD fingerprinting is sensitive to the quality of the DNA template. Therefore, in the present study, we modified the some essential steps of DNA isolation procedures for mints which yielded high quantity and quality of high molecular weight DNA. This procedure is a modified CTAB method described by Murray and Thompson (1980).

Leaves of different *Mentha* species were used for DNA isolation. Extraction buffer containing polyvinylpyrrolidone (PVP) was used for better removal of polysaccharides and secondary metabolites. Polysaccharides and secondary metabolites which are abundant in mint leaves are known to be bound by PVP. For purification, DNA was extracted with phenol:chloroform:isoamylalcohol. The DNA samples isolated by the method were used for RAPD fingerprinting of Iranian mints. The results showed successful reproducibility through PCR amplification. This procedure could be applied to some plant species of medicinal and aromatic plants which produced essential oils and secondary metabolites.

Keywords: DNA isolation, RAPD fingerprinting, Medicinal plants, Mints, RAPD fingerprinting, Secondary metabolites

Introduction

A large number of plant species produce secondary metabolites such as alkaloids, phenols, polysaccharides and terpens that are used in food, confectionary, cosmetics and liquor industries. Molecular marker approaches not only used for breeding of genotypes of medicinal and aromatic plants, but also used for assessing and exploiting the genetic variability of these plants. Developing DNA markers/fingerprints of all the genetic resources of the medicinally and industrially important plants, is a necessity for generating a molecular database as well as to utilize the information in a systematic way.

The presence of certain metabolites can hamper the DNA isolation procedures and reactions such as DNA restriction, amplification and cloning. Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include: (1) degradation of DNA due to endonucleases, consolation of highly viscous polysaccharides, and (2) inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weising *et al*., 1995). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA (Loomis, 1974, Porebski *et al*., 1997).

Plant species belonging to the same or related genera can exhibit enormous variability in the complixity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. The chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weising *et al*., 1995).

Here we described a rapid DNA isolation protocol that can be used for some medicinal and aromatic plants and in particular those taxonomically related with *Mentha* species. The protocol permitted isolation of DNA from tissues of diverse plant species in fairly good yield, and the isolated DNA proved amenable to PCR amplification.

Materials and Methods

Plant materials

Four species of mints including *Mentha spicata, M. piperita, M. longifolia* and *M. suaveolens* were used. Total DNA was isolated from freshly germinated young leaves by following a modification of the CTAB method of Murray and Thampson (1980).

DNA isolation protocol

1. Grind 3 g of fresh tissue to a fine powder using liquid nitrogen and a mortar and pestle.
2. Transfer the powder as fast as possible into 10ml of pre-warmed 2.5× CTAB extraction buffer [2.5% Cetyl- Trimethyl Ammonium Bromide (CTAB), 1.5 M NaCl, 25 mM Na_2EDTA, 100 mM Tris-HCl (pH 8.0), 2% β-mercaptoethanol, 1% polyvinylpyrrolidone) to 50 ml polypropylene tube.
3. Incubate for 60 min at 60˚C in a water bath with slow shaking every 10 min.
4. Add 10ml of chloroform: isoamylalcohol (24:1), and shake slowly for 10 min on a rocker.
5. Spin at 4000 rpm for 15 min at room temperature using a centrifuge rotor.
6. Carefully transfer the upper clear aqueous layer to another 50 ml fresh tube.
7. Repeat step 4 to 6 for more purity of DNA.
8. Transfer the supernatant to a new 50 ml tube and precipitate DNA by adding 2/3 volume of pre-chilled (-20°C) isopropanol.
9. Collect the precipitated DNA by low speed centrifugation (10 min, 1500 rpm), air dried briefly and re-suspended DNA in 1 ml TE buffer (10 mM Tris- HCl, 1 mM Na_2EDTA) in a 15 ml tube.
10. Add RNase A to a final concentration of 100 µg/µl and transfer the tube to 37˚C for 1 h.
11. Add equal volume of processed phenol to the tube containing DNA and shake for 10 min to form an emulsion and then centrifuged at 3000 rpm for 10 min at room temperature to separate the organic and aqueous phases.
12. Transfer the aqueous phase to a fresh 15 ml tube and add 1/2 volume phenol + 3/4 volume chloroform:isoamylalcohol (24: 1) and shake for 10 min and centrifuge at 3000 rpm for 10 min.
13. Transfer the aqueous layer to a fresh 15ml tube and add 1ml chloroform:isoamylalcohol (24:1), mix by rocker for about 10 min and centrifuge at 4000 rpm for10 min.
14. Transfer the aqueous layer to a fresh 15ml tube, add 1/10 volume sodium acetate and 2.5 volume ethanol -20˚C to precipitate DNA.

15. Spool out DNA with glass rod, air dried for 20min and finally suspended in appropriate volume of TE buffer in a 1.5 ml microfuge tube.

For checking the quality of total genomic DNA, 2µl of DNA in TE was fractionated by 0.8% agarose gel electrophoresis. DNA concentration in TE was determined spectrophotometrically by measurement of optical density. The ratio between the readings at 260 nm and 280 nm (OD260/OD280) provided an estimate of the purity of nucleic acid. Pure preparation of DNA has OD260/OD280 values of 1.8.

PCR amplification

Thirty one arbitrary 10-mer primers were used for PCR amplification of the total genomic DNAs. Polymerase chain reaction was performed based on the protocol of Williams *et al*. (1990), with minor modification. Amplification were carried out in 25 µl of reaction mixture containing 2.5 µl of PCR buffer, 200 µM each of dNTPs, 1.5 mM $MgCl_2$, 15 ng of the primer, 0.7 unit of Smar *Taq* DNA polymerase and 25 ng of DNA template. The amplifications were performed using a Eppendorf Mastercycler Gradient termalcycler. The amplified products were loaded in a 1.2% agarose gel containing ethidium bromide. After electrophoreses, the gel was photographed under UV light.

Results and Discussions

The plants that are sources of natural products or bio-active substances also produce large amounts of secondary metabolites and substances of medicinal or industrial importance. Thus while working with a variety of plants it is common to encounter problems arising from the presence of essential oils, polysaccharides, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations. In our experiments we encountered difficulties from the stage of cell lyses to DNA separation in the supernatant and subsequent reactions when following the procedure described by Khanuja *et al*. (1999), and Murray and Thompson (1980). Major problems encountered were low DNA yield or poor PCR amplification reactions.

Khanuja *et al*. 1999 described isolation of DNA from dry and fresh samples of several genera. They evaluated the procedures described by Doyle and Doyle (1987), Dellaporta *et al*. (1983), and Porebski *et al*. (1997). The protocol described by Khanuja *et al.*(1999) used three ml of extraction buffer. In our protocol, we used 10 ml of extraction buffer. Moreover, the procedure also eliminates the necessity of NaCl, and addition of 3M sodium acetate (pH 5.5) for sedimentation of DNA. The problem arising from the presence of high levels of proteins was overcome by using phenol:chloroform:isoamylalcohol. The standard way to remove proteins from nucleic acid solutions is to extract first with phenol:chloroform:isoamylalcohol and then with chloroform. This procedure takes advantage of the fact that de-proteinization is more efficient when two different organic solvents are used instead of one.

The protocol described by Murray and Thompson (1980) involved only addition of β-mercaptoethanol. It has been known that Polysaccharides and secondary metabolites which are abundant in mint leaves are bound by PVP. The addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols from *Mentha* species (Neal Stewart and Via, 1993).

The above protocol invariably achieved good yield of high quality DNA from fresh tissues of above plants. By using the above protocol a fairly high quality DNA was obtained for each plant (Figure 1a). The amount of DNA recovered per gram of plant leaves was sufficiently

high. Table 1 showed yield of DNA isolated from various samples of different species of mint plants using the CTAB procedure modified in this study.

The utility of the isolated DNA for use in PCR amplification for RAPD profiling was demonstrated with several random primers and with DNA preparation of all the plant species tested (Figure1b). The method described here is, therefore, rapid, simple and efficient for the isolation of DNA from plants that possess a wide range of activities that can interfere with DNA extraction and analysis. This procedure can be directly applied to *Mentha* species and closely related genera which produced essential oils and secondary metabolites.

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Table 1: Yield of DNA isolated from various leaf samples of four *Mentha* species using the CTAB procedure as modified by this study

Plant(family)	Number of accession	Range of DNA yield µg/g tissue
M. longifolia	3	100.5 - 173.3
M. piperita	4	32.1 - 272.4
M.spicata	9	43.7 – 175
M.suaveolens	1	135.5

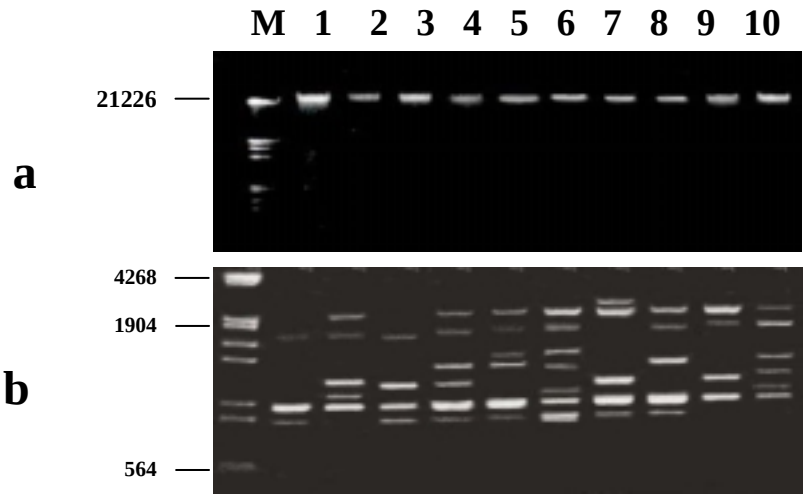

Figure 1: Isolated DNA from 10 accessions of *Mentha* species electrophoresed by 0.8% agarose gel (Figure 1a), Figure 1b, shows PCR profiles of the DNA samples amplified with primer 5'- CACAggCggA-3' oligo-primer.