Rapid and High Quality DNA Isolation from *Origanum onites* for RAPD and ISSR Analysis

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Origanum onites is an economically important medicinal plant with high essential oil content. Lack of an appropriate DNA isolation procedure is a limiting factor for any molecular study of this plant. We have used a protocol for genomic DNA isolation based on a hexadecyltrimethylammonium bromide (CTAB) method described for other plant species. The method involves mortar grinding of leaf tissue, modified CTAB extraction using high salt concentrations and polyvinyl pyrrolidone, and successive isoamyl alcohol/chloroform extractions. The yield was approx. 20 μg DNA per 200 mg of initial fresh plant material. The genomic DNA obtained by this method was suitable to be used in restriction digests, inter simple sequence repeat (ISSR) and randomly amplified polymorphic DNA (RAPD) reactions. This extraction method should facilitate the molecular analysis of *Origanum* chemotypes.

Key words: CTAB DNA Extraction, *Origanum onites*, RAPD, ISSR

Introduction

The genus *Origanum* (Labiatae) is an annual, perennial and shrubby herb growing on stony slopes and in rocky mountains up to 4000 m altitude (Ietswaart, 1982). This genus comprises 38 species native to the Mediterranean, Euro-Siberian and Irano-Siberian regions (Aligiannis *et al.*, 2001), but most *Origanum* species (ca. 75%) are found exclusively in the East Mediterranean subregion (Gounaris *et al.*, 2002).

Origanum onites L. (Turkish oregano) is a perennial species with woody stems; it can be distinguished from other *Origanum* species by the form of inflorescences (spikes arranged in false corymbs) and one-lipped calyces. The plant is around 65 cm tall with many branches and grows on stony hills and rocky slopes at an altitude up to 1400 m (Ietswaart, 1982). *O. onites* grows naturally in Agean and Mediterranean regions of Turkey; it is also widely cultivated due to its economical importance. *O. onites* is one of the main medicinal and aromatic plants exported from Turkey, the annual quantity being up to 7 million kg (Başer, 2001). It is known to have antiseptic, antibacterial and antispasmodic characteristics due to its volatile oils and terpenic materials (Başer *et al.*, 1993; Aligiannis *et al.*, 2001; Lambert *et al.*, 2001; Manohar *et al.*, 2001; Daferera *et al.*, 2000, 2003). Crop improvement and agronomy studies were undertaken with this species to obtain different chemotypes rich in carvacrol or thymol.

The growing number of DNA isolation protocols for specific plant species suggests that extraction of DNA is not always simple due to high amounts of polysaccharides or presence of secondary metabolites such as alkaloids, flavanoids, phenols and terpenes (Doulis *et al.*, 2000).

Many earlier studies discuss problems faced during genomic DNA isolation from medicinal plants (Khanuja *et al.*, 1999), especially in case of *Eugenia jambolana* (Manoj *et al.*, 2007) and *Terminalia arjuna* (Sarwat *et al.*, 2006).

Currently, no study has been reported on the molecular level of the genus *Origanum.* In order to begin an extensive study on chemotype identification, improving DNA isolation methods and optimizing PCR conditions, for example for randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses, are necessary. In order to isolate good quality DNA from *O. onites*, we have used a standard hexadecyltrimethylammonium bromide (CTAB) technique described by Doyle and Doyle (1987) through which we obtained clean DNA that was consistently amplifiable by polymerase chain reaction (PCR) using the ISSR (Fisher *et al.*, 1996) and RAPD techniques (Williams *et al.*, 1990) and suitable for restriction enzyme digestion.

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Materials and Methods

Plant material

O. onites plants were kindly provided by Atatürk Research Institute of Garden Cultivars, Medicinal and Aromatic Plants Division, Yalova, Turkey. Leaf tissues were collected, frozen in liquid nitrogen and stored at −20 °C for future use.

DNA isolation

Approx. 200 mg of leaf tissue were ground in liquid nitrogen to a fine powder and transferred to a 2 ml Eppendorf tube. About 900 µl freshly prepared and preheated (62 °C) extraction buffer [100 mM Tris-HCl (Tris [hydroxymethyl] aminomethane-hydrochloride), pH 8.0, 25 mM EDTA (ethylenediaminetetraacetic acid), 1.4 mM NaCl (sodium chloride), 2% CTAB (w/v), 1% PVP (polyvinylpyrrolidone) (w/v) and 0.4% β-mercaptoethanol added to buffer prior to use] were added. The tube was inverted several times and incubated at 62 °C for 1 h with regular inversion every 10 min. After incubation an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and mixed gently by inversions for 10 min. The mixture was centrifuged at $5300 \times g$ for 10 min. The upper aqueous layer was transferred to a new tube and 2/3 volume of isopropanol (−20 °C) was added. The mixture was incubated at −20 °C for 2 h and centrifuged at $9300 \times g$ for 10 min. The supernatant was discarded and the pellet was washed twice with 300 µl 70% ethanol (−20 °C). The resultant pellet was air-dried and dissolved in 50 µl DNase RNase-free water. In order to remove RNA, the DNA sample was treated with 4 µl RNase A (50 mg/ml) at 37 °C overnight. The quantity and purity of the DNA sample was determined by using a Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA).

DNA amplification

Amplification of RAPD and ISSR fragments from genomic DNA was carried out in a total reaction volume of 25 µl containing 10 ng of genomic DNA, 1X *Taq* polymerase reaction buffer, 2 mM $MgCl_2$, 0.1 mM each of dNTPs (dATP, dCTP, dGTP, and dTTP), 0.2 mM primer and 1 U of *Taq* DNA polymerase (Fermentas, Maryland, USA). Amplifications were performed in a Progene thermocycler (Techne Inc., Burlington, USA) programmed as follows: 3 min denaturation at 94 °C and 40 cycles of 1 min each denaturation at 94 °C, 1 min annealing at 36 °C for RAPD or 50 °C for ISSR amplification, and a 2 min extension at 72 °C, followed by a final extension at 72 °C for 7 min. Amplification products were separated on 1.4% agarose gel containing ethidium bromide (0.5 µg/ml). Gels were visualized under UV light and digitally photographed with an UVIpro gel documentation system (UVItec, Cambridge, UK).

Restriction digestion

Approx. 10 µg of genomic DNA were digested overnight at 37 °C with 10 units of restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III) and buffer following the manufacturer's recommendation (Fermentas). The digested DNA samples were electrophoresed on 0.8% agarose gel at 5 V/cm and photographed by using an UVIpro gel documentation system (UVItec).

Results and Discussion

Molecular techniques require isolation of high quality genomic DNA suitable for both PCR and restriction enzyme digestion. Optimization of the DNA extraction procedure, therefore, is an important issue for many researchers especially those working with economically important crop plants.

The isolation of good quality DNA from *Origanum* is complicated due to its high content of phenolic substances and polysaccharides. It is known that phenolics become oxidized during tissue homogenization and covalently bind to the protein and nucleic acids. This irreversible binding causes the formation of a gelatinous DNA which is unsuitable for PCR and restriction enzyme digestion analyses (Katterman and Shattuck, 1983; Porebski *et al.*, 1997). Polysaccharides are also important contaminants in DNA samples and interfere with the PCR by inhibiting the *Taq* polymerase activity (Fang *et al.*, 1992). One way to avoid the problems with polyphenols is to freeze the tissue during or before homogenization (Katterman and Shattuck, 1983). Alternatively, PVP or other antioxidants can be included during the DNA extraction procedure.

The DNA isolated from various plant species by using the original CTAB method of Doyle and Doyle (1987) is usually found to be contaminated with high levels of proteins and polysaccharides (Manoj *et al.*, 2007; Sarwat *et al.*, 2006; Remya *et al.*, 2004). Therefore, an extra step of column puri-

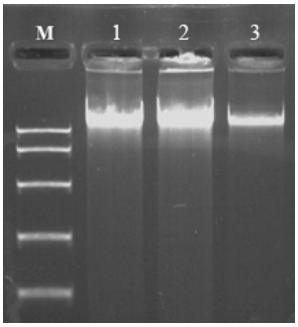

Fig. 1. Electrophoretic analysis of total DNA isolated from *Origanum onites* by the CTAB method. Lane M, 1 kb DNA ladder (Fermentas); lanes 1–3, *Origanum onites* DNA samples.

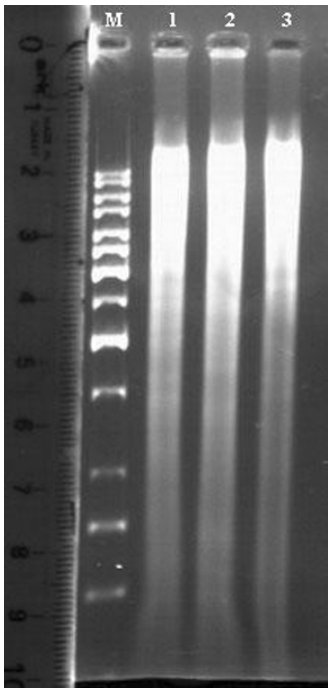

Fig. 2. Restriction digestion analysis of *Origanum onites* DNA. Lane M, 1 kb DNA ladder (Fermentas); lanes 1–3, DNA digested with *Bam*HI, *Eco*RI and *Hind*III, respectively.

fication is often suggested to overcome this problem.

In this study, however, we obtained a high yield of good quality DNA from an aromatic, phenolics-rich plant, *Origanum onites*, using the standard CTAB method. This method is very simple, quick, and inexpensive and efficiently removes polysaccharides and provides high quality genomic DNA from fresh or frozen *Origanum onites* leaves. In a single day, one person can complete the DNA isolation from more than 30 different leaf samples, and isolated DNA can be stored at −20 °C for a long period. The method yields large amounts of DNA (18–21 μg/200 mg fresh weight leaves), enough to conduct numerous PCR amplifications or two restriction digestion for Southern blot analysis.

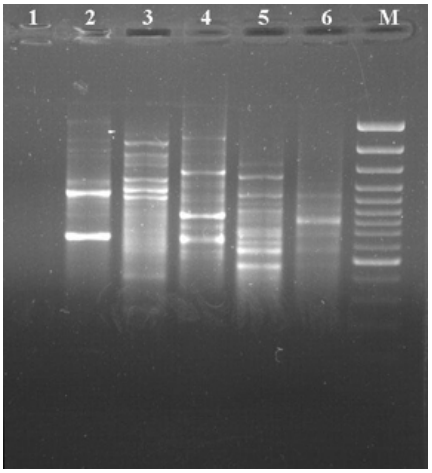

Fig. 3. Representative gel fractionation of RAPD-PCR amplification products of *Origanum onites* genomic DNA (5 ng). Amplification products were fractionated in an 1.4% agarose gel. Lane M, molecular marker (100 bp DNA ladder, Fermentas); lane 1, negative control; lanes 2–4, RAPD-PCR products amplified using RAPD primers P1 (5′-GTGACGCCGC-3′), P3 (5′-GAGGC GGCGA-3′), P14 (5′-CAGCACTGAC-3′); lanes 5, 6, ISSR products amplified using ISSR primer 1, GAG-$(CAC)_5$, and ISSR primer 2, $(CAG)_5$, respectively.

The purity of the isolated DNA was confirmed by an A260/280 ratio of 1.85–2.00 for all samples tested (data not shown) and indicated that the DNA samples were practically free from polysaccharides and other contaminants. DNA samples were run on agarose gel to check their quality. A perfect migration pattern of a single band was observed without any sign of DNA degradation (Fig. 1).

DNA isolated from *O. onites* was digested with *Bam*HI, *Eco*RI, *Hind*III restriction enzymes (Fermentas) to check for the effective removal of polysaccharides from the DNA sample (Fig. 2). The DNA samples were digested successfully. Effective digestion of DNA by these enzymes, particularly by *Hind*III, would be regarded as indicator of ab-

sence of polysaccharides (Do and Adams, 1991) and suitability for Southern analysis.

The DNA quality was also checked by PCR. RAPD and ISSR primers were used to amplify *Origanum* DNA, and results showed the distinct amplification of genomic DNA at the molecular weight range of 400 bp to 2.5 kb (Fig. 3). The optimization of RAPD and ISSR analyses would be useful for identifying different chemotypes of *Origanum onites* and other *Origanum* species. Currently, we are using this methodology to characterize and differentiate the important chemotypes of *Origanum onites* and *O. vulgare* growing in Turkey.

In conclusion, the CTAB DNA isolation procedure (Doyle and Doyle, 1987) is a simple and consistent DNA isolation method for *Origanum* that yields a large amount of pure and intact DNA suitable for restriction digestion and PCR without any ultracentrifugation or column purification steps. This method can be used for diverse medicinal and aromatic plants, which produce essential oils and secondary metabolites such as alkaloids, flavanoids, phenols, terpenes and quinones.

Aligiannis N., Kalpoutzakis E., Mitaku S., and Chinou B. I. (2001), Composition and antimicrobial activity of the essential oils of two *Origanum* species. J. Agric. Food Chem. **49**, 4168–4170.

Başer K. H. C. (2001), Her derde deva bir bitki: Kekik. Bilim ve Teknik Dergisi **5**, 74–77.

Başer K. H. C., Özek T., Tümen G., and Sezik E. (1993), Composition of the essential oils of Turkish *Origanum* species with commercial importance. J. Essent. Oil Res. **5**, 619–623.

Daferera D. J., Ziogas B. N., and Polissiou M. G. (2000), GC-MS analysis of essential oils from Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. J. Agric. Food Chem. **48**, 2576–2581.

Daferera D. J., Basil N., Ziogas N., and Polissiou M. G. (2003), The effectiveness of plant essential oils on *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. Crop Prot. **22**, 39–44.

Do N. and Adams R. P. (1991), A simple technique of removing plant polysaccharides contaminants from DNA. Biotechniques **10**, 162–166.

Doulis A. G., Harfouche A. L., and Aravanopoulos F. A. (2000), Rapid, high quality DNA isolation from cypress (*Cupressus sempervirens L.*) needles and optimization of the RAPD's marker technique. Plant Mol. Biol. Rep. **17**, 1–14.

Doyle J. J. and Doyle J. L. (1987), A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochem. Bull. **19**, 11–15.

Fang G., Hammer S., and Groumet R. (1992), A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biofeedback **13**, 52–54.

Fisher P. J., Gardner R. C., and Richardson T. E. (1996), Single locus microsatellites isolated using 5′ anchored PCR. Nucleic Acids Res. **24**, 4369–4371.

Gounaris Y., Skoula M., Fournaraki C., Drakakaki G., and Makris A. (2002), Comparison of essential oils and genetic relationship of *Origanum intercedens* to its parental taxa in the island of Crete. Biochem. Syst. Ecol. **30**, 249–258.

Ietswaart J. H. (1982), *Origanum* L. In: Flora of Turkey and East Aegean Islands, Vol. 7 (Davis P. H., ed.). Edinburgh University Press, Edinburgh, pp. 297–313.

Katterman F. R. H. and Shattuck V. I. (1983), An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. Prep. Biochem. **13**, 347–359.

Khanuja S. P. S., Shasany A. K., Darokar M. P., and Kumar S. (1999), Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oil. Plant Mol. Biol. Rep. **17**, 74.

Lambert R. J. W., Skandamis P. N., Coote P. J., and Nychas G. J. E. (2001), A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. **91**, 453–462.

Manohar V., Ingram C., Gray J., Talpur N. A., Echard B. W., Bagchi D., and Preuss G. (2001), Antifungal activities of *Origanum* oil against *Candida albicans*. Mol. Cell. Biochem. **228**, 111–117.

Manoj K., Tushar B., and Sushama C. (2007), Isolation and purification of genomic DNA from black plum (*Eugenia jambolana* Lam.) for analytical applications. Int. J. Biotech. Biochem. **3**, 49–55.

Porebski S., Bailey L. G., and Baum B. R. (1997), Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. **15**, 8–15.

Remya R., Syamkumar S., and Sasikumar B. (2004), Isolation and amplification of DNA from turmeric powder. Br. Food J. **106**, 673–678.

Sarwat M., Negi M. S., Lakshmikumaran M., Tyagi A. G., Das S., and Srivastava P. S. (2006), A standardized protocol for genomic DNA isolation from *Terminalia arjuna* for genetic diversity analysis. Electron. J. Biotechnol. **9**, 86–91.

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A., and Tingey S. V. (1990), DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**, 6531–6535.