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Full Length Research Paper

Genomic DNA isolation from aromatic and medicinal plants growing in Turkey

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Herbal and aromatic plants are attracting more attention among contemporary plant researchers because some human diseases resulting from bacterial antibiotic resistances have gained worldwide concern. A number of methods are available and are being developed for the isolation of nucleic acids from plants. Because plants contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all plants can ever exist. Therefore, we developed 4 modified new methods that produced good quality DNA from these plants. This article deals with modern approaches in determining genetic variability, in which three categories of genetic markers are applied by morphological, biochemical and molecular. Furthermore, the aim is to assess the available genetic diversity for each species; to provide more accurate and detailed information than is available using classical phenotypic data in this subject. Various types of plant materials and a number of different protocols for the isolation of DNA were tested in order to obtain good quality DNA for PCR reactions. Ten populations of different aromatic and medicinal plants from Turkey were tested in the study. The number of plants examined for each population varied from two to five. When fresh or frozen leaves of plants collected in autumn were used for the isolation of DNA, no positive result in PCR reaction was obtained regardless of the isolation protocol being used. Four different DNA methods were compared for the isolation of DNA from the different plant homogenates, namely the CTAB, Plant Genomic DNA Purification Kit, and EZ1 Nucleic acid isolation methods and DNA extraction with phenol purification and liquid nitrogen method.

Key Words: Genomic DNA extraction, PCR, aromatic plants, medicinal plants.

INTRODUCTION

The use of medicinal and aromatic plants (MAPs) has a long history in the world. According to the World Health Organization, 20,000 species are used for medicinal and aromatic purposes. Today, 4000 drugs are widely used and 10% of them are commercially exploited or produced. Around 500 plant species are used for medicinal and aromatic purposes in Turkey (Başer, 1998). However, all these species are neglected or underutilized and only a few are cultivated or subjected to research. The Aegean Agricultural Research Institute (AARI) is one of the institutions working on and giving importance to MAPs to improve commercial varieties, find out new crops and assess agronomic requirements of related plant species. Activities on MAPs at AARI were started in the 1970s. The first studies were on identification, collection and conservation of MAPs. The most economically important species were given research priorities. Turkey is one of the significant and unique countries in the world from the point of view of plant genetic resources and plant diversity. On the other hand, in Turkey there are agricultural opportunities to increase canole (*Brasica napus* L. Summer and winter) production by expanding into the new reclaimed regions. Furthermore, purslane (*Portulaca oleracea* L.), as most people in modern societies do not limit their diet to a few cultivated vegetables and purslane offers better nourishment than the major cultivated vegetables. *Echinacea* species used medicinally are Echina-

Abbreviations: CTAB, Hexadecyltrimethylammonium Bromide; PCR, Polymerase Chain Reaction; MAP, Medicinal and Aromatic Plant.

cea purpurea, Echinacea angustifolia and *Echinacea pallida*. In various pharmacological tests measuring the effects of *E. purpurea* come out slightly ahead of other species and are the most scientifically proven. *Ginkgo biloba* leaf is the most widely used to treat the symptoms of illness. Flaxseed (*Linum usitatissimum* L.) is a rich source of alpha-linolenic acid (ALA), one of the new plant sources of all-important omega-3 polyunsaturated fatty acids associated with improved cardiovascular health, cancer prevention etc.

Current trends in support to environmental friendly agriculture and greater participation of local people in the sustainable conservation and use of natural resources are leading towards innovative approaches to enhance the use of medicinal and aromatic plants, more participatory in nature and hence more focused on local needs. The increasing number of organizations involved in medicinal and aromatic plants, the access to new tools for biodiversity prospecting, characterization and data analyses along with the change of traditional conser-vation systems towards more use-oriented initiatives should be seen as opportunities for revising research goals and partnerships, create greater synergies at the national level and a more conducive policy environment. Current trends in support of environmental friendly agriculture and greater participation of local people in the sustainable conservation and use of natural resources are leading towards innovative approaches to enhance the use of medicinal and aromatic plants, more participatory in nature and hence more focused on local needs. The increasing number of organizations involved in medicinal and aromatic plants, the access to new tools for biodiversity prospecting, characterization and data analyses along with the change of traditional conser-vation systems towards more use-oriented initiatives should be seen as opportunities for revising research goals and partnerships, create greater synergies at the national level and a more conducive policy environment.

Morphological markers (such as plant height, leaf shape, colour, etc.) are among the oldest markers used in the evaluation of genetic variability. However, they are not sufficiently specific and informative because different gene expression in different environments causes wide variability of phenotypic characters in individuals. Similarly, biochemical markers (such as albumin content, isoenzymes or essential oil contents in an individual organism) can be considered to be non-specific due to the wide variability of biochemical characters, which are strongly influenced by an individual's environment. The development of recombinant DNA in the 1980s enabled the development and use of molecular markers, thus providing a modern tool for determining genetic variability. Molecular markers show variability among individuals at the DNA level, which is not influenced by the environment. Different genetic markers (e.g. RFLP, AFLP, RAPD, SSR, SCAR) have different properties, (dominant and co-dominant markers, different coverage of the genome) and different advantages and disadvantages (e.g specificity, cost, ease of analytical interpre-tation of the resulting data). However, they are highly informative about genetic variability among individuals, populations and cultivars. Their use is universal for all organisms. Molecular markers can be considered to be essential tools in cultivar identification (DNA typing), assessment of genetic variability and relationships, management of genetic resources and biodiversity, studies of phylo-genetic relationships and in genome mapping. The main aim of the management of genetic resources is to ensure that as much as possible of the existing genetic diversity of species is conserved. The effectiveness of this depends to a large extent on the genetic information available on the germplasm under study. Molecular mar-kers provide genetic information of direct value for *ex situ* and *in situ* conservation. For *ex situ* conservation, the acquisition of data on the diversity of collections is important. Molecular markers can be used (i) to identify valuable genetic variation that is under-represented in a collection sample; (ii) to identify duplicate accessions and to monitor changes in genetic structure as accessions are generated; (iii) to assess the available genetic diversity for each species; (iv) to provide more accurate and detailed information than is available using classical phenotypic data; (v) to identify traits and types quickly. On a more fundamental level, molecular marker infor-mation may lead to the further identification of useful genes contained in a collection. Molecular data on diversity may provide essential information to develop core collections that accurately represent the entire colle-ction. For *ex situ* conservation, molecular markers may be used in: the determination of identity and similarity of accessions or individuals; the measurement of the structure of diversity among individuals, accessions, populations and species; and the detection of particular allele or nucleotide sequences in a taxon, genebank accession or *in situ* population.

MATERIALS and METHODS

Plant material

In this study, we used medicinal and aromatic plants. The plant list shown in the Table 1 and marked with (+++) excellent amplification, (++); very good amplification, (+); good amplification. Ten populations of different aromatic and medicinal plants from Turkey conserved in the Turkey national genebank for medicinal and aromatic plants were included in the study. The number of plants tested per population varied from two to five.

DNA isolation and PCR optimization

For the isolation of DNA, different types of plant material (fresh, frozen, young, old, lyophilized leaves, leaves dried at 40°C, plants grown *in vitro*) were taken and different protocols were tested: two modified CTAB protocols (Liber et.al., 2006), SDS protocol (Beye and Raeder 1993) and a commercial kit (Dneasy Plant Mini Kit, Qiagen). PCR amplifications were carried out in 25 µl mixture with DNA content and Mg^{2}+ concentrations being optimized. Different types of *Taq* DNA polymerases were also tested. Twenty-five arbitrary 10-mer primers (Operon Technologies) were applied. A

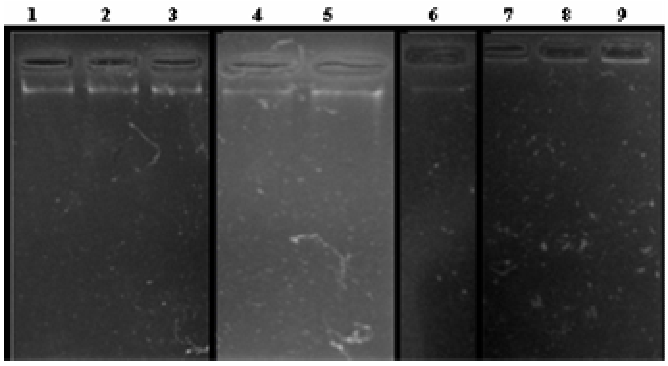

Figure 1. Genomic DNA s were loaded in a 1% agarose gel and separated by electrophoresis for 90 min at 100 V, then visua-lised by ethidium bromide staining with transillumination. Lane 1-3. Genomic DNAs isolated from Soybean (*Glycine max.* L.) with Bio Robot EZ-1. Lanes 4-5. Genomic DNAs isolated from Echinacea (*E. purpurea* L.) with manual DNA isolation method. Lane 6. Genomic DNA isolated from Daphnae (*Daphne alpina* L.) with manual DNA isolation method. Lanes 7-8. Genomic DNAs isolated from Canola summer (*Brasica napus* L.) and lane 9 Canola (*B. napus* L.) winter with manual DNA isolation method.

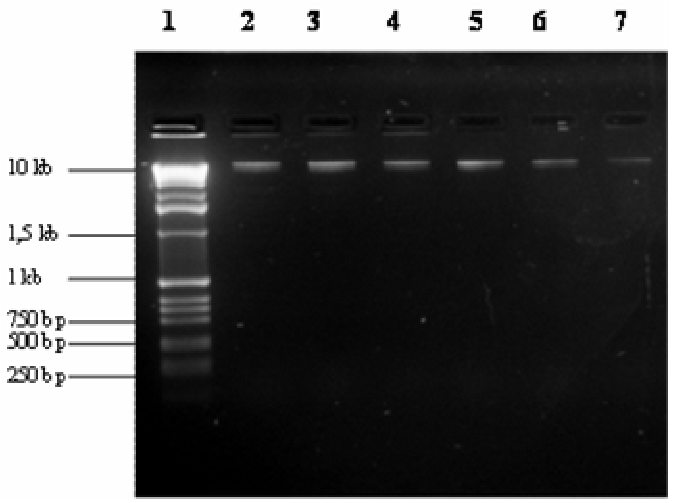

Figure 2. Screening of agarose gel electrophoresis of plants DNA using with EZ1 Nucleic acid isolation method and digested with restriction endonuclease (EcoRI/HindIII). Lane 1, 1 kb ladder size standard marker; lane 2, Canola *(Brassica napus* L.) summer; lane 3, Canola winter; lane 4, Canola (Manisa); lane 5, Purslane *(Portulaca olerecea* L.) *(*Adana); lane 6, Linseed *(Linum usitatissimum* L.) (Adana); lane 7, Linseed (Antalya).

preliminary study of genetic variability using RAPD molecular markers was made. For data analysis and the construction of a dendrogram, NTSYS-pc software was used (Rohlf 1993). UPGMA cluster was performed using the RAPD data.

Genomic DNA isolation from plant tissue

Fresh, seed, plantlets and as well as herbarium specimens were used in this study for DNA extraction. Herbarium samples were collected by the author in the field gathered in herbaria. Samples analysed included dried soybean seeds were used directly for DNA extraction as they were found to yield DNAs comparable in quality and quantity to that obtained using EZ1 Nucleic acid isolation analyser (QIAGEN, 2007) and by the using of the CTAB method, Plant Genomic DNA Purification Kit method, and DNA extraction with phenol purification and liquid nitrogen method (Sambrook et al., 1989). A soybean seed bulk sample was ground to fine powder. This experiment was repeated twice under repeatability conditions resulting in all DNA samples. Plant seeds were used directly for DNA extraction as they were found to yield DNAs comparable in quality and quantity to that obtained using beans ground in liquid nitrogen. A soybean seed bulk sample was ground to fine powder. DNA extractions were performed on 1 g flour sub-samples. This experiment was repeated twice under repeatability conditions resulting in all DNA samples. Furthermore, soybean seeds were germinated then these plantlets was ground to powder by liquid nitrogen treatment. Seeds were placed in each 2.0 ml deep-well of a 96-well plate containing a 4 mm stainless steel grinding ball and soaked for 12 h in distilled water prior to processing. Using a grinding ball dispenser a grinding ball was also placed on top of each seed. The plate was sealed with a fitted Teflon®/silicone mat and placed in the Geno/Grinder. A piece of adsorbent paper was placed on top of the plate and the plate was locked into the grinder. The seeds were disrupted for 2.5 min at 1500 rpm. The deep well plate with lid was then centrifuged at 1500 rpm to pellet lysate and condense liquid from the rim and walls of the well. Without centrifugation, the probability of well to well cross contamination of genetic material is greatly increased. Once centrifuged, the lid is carefully removed. Four different DNA methods were compared for the isolation of DNA from the different plant homogenates, namely the CTAB, Plant Genomic DNA Purification Kit, and EZ1 Nucleic acid isolation methods and DNA extraction with phenol purification and liquid nitrogen method (Figure 1).

Agarose gel electrophoresis

Genomic DNA products were electrophoresed on 1% agarose gel using 0.5x TAE buffer (10 mM Tris HCl and 1 mM EDTA pH. 8.0) and visualized by ethidium bromide staining. The patterns were photographed and stored as digital pictures in gel documentation system. The DNA samples of every plants were confirmed by repeating each experiment three times.

Genomic DNA restriction digest

Protocol 1 contain 2 µl 10 X Buffer, 0,5 µl Lambda EcoRI/Hind III, 0,2 µl 100 x BSA,13 µl water and 2 µl genomic DNA. 55°C and 2 h incubation respectively in lane 2 and 3 identified with protocol 1.

Protocol 2 contain 2.5 µl 10X Buffer, 0.6 µl Lambda EcoRI/Hind III, 1 µl RNase, 16.9 µl water and 4 µl genomic DNA. 37°C and 3 h incubation respectively in lane 4 and 5 identified with protocol 2. After digestion, the reaction mixture was electrophorosed through 0.8% agarose in 50xTAE buffer. The sample was also tested for nuclease activity (Figure 2).

PCR optimization of plant samples

In this study, DNA isolation and PCR optimization conditions were suitably provided for each plant samples Optimization of RAPD reaction for *E. purpurea* DNA. For the optimization of RAPD reaction using DNA extracted from *E. purpurea* different decamer primers, from Operon Technologies Inc. were used. The initial protocol of the RAPD reaction was the one recommended by the manufacturer of the polymerase. For a reaction volume of 25 ml, the follow-

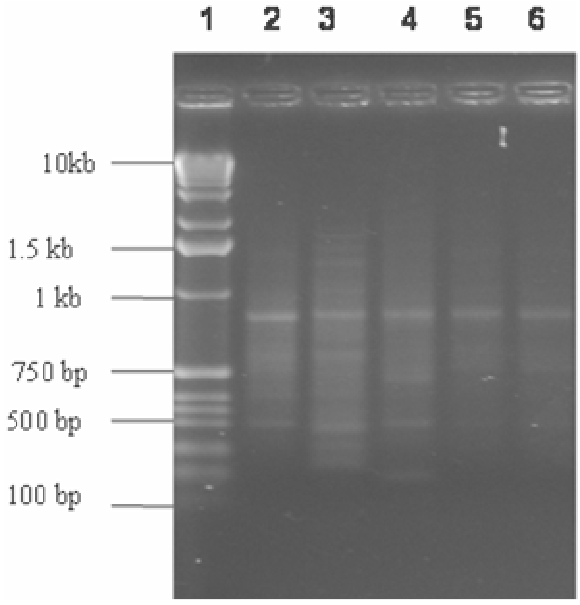

Figure 3. Screening of agarose gel electrophoresis of RAPD-PCR products amplified with OPA3 in Echinaceae genotypes growing in different regions of Turkey. Lane 1, 100 bp ladder size standard marker; lanes 2 and 6, PCR product of Echinaceae growing in Karadeniz region in Turkey after manual DNA isolation; lane 3, PCR product of Echinaceae growing in Ege region or İzmir origin after genomic DNA isolated by Bio Robot; lanes 4 and 5, PCR product of Echinaceae growing in Marmara region or İstanbul origin after genomic DNA isolation by Bio Robot.

ing components were used: A reaction tube contained 25 ng of DNA, 0.6 U of *Taq* DNA polymerase enzyme, 100 m M of each dNTP, 1X *Taq* DNA polymerase buffer with 1.5 mM MgCl2, and 10 pmol decanucleotide primer. Amplifications were carried out in a DNA thermal cycler (Eppendorf, mastercycler gradient) using the following parameters: 94°C for 5 min; 45 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were subjected to agarose gel (1.5% [w/v]) electrophoresis in 0.5X TBE buffer, along with 1 kb and 1000bp DNA ladders (MBI, Fermentase) as size markers. DNA was stained with ethidium bromide and photographed under UV light. Five Random primers (OPA1, OPA2, OPA3, OPA4, OPA6) (Qiagen Operon Technologies Inc) were screened by RAPD for identification of specific marker. Amplification of DNA from the six *Echinacea* samples was carried out using a single primer. The amplicon, which was monomorphic to all the *Echinacea* species was identified. All PCR reactions were run in triplicate, and only reproducible and clear bands were scored. For example, display of RAPD-PCR products amplified with OPA3 primer of *E. purpurea* (Figure 3).

RESULTS and DISCUSSION

Various types of plant material and a number of different protocols for the isolation of DNA were tested in order to obtain good quality DNA for PCR reactions. When fresh or frozen leaves of plants collected in autumn were used for the isolation of DNA, no positive result in PCR reaction was obtained regardless of the isolation protocol being used. This was probably due to the accumulation of large amounts of secondary metabolites in old plant material, as previously reported (Khanuja et al., 1999). DNA purified with DNeasy Plant Mini Kit (Qiagen) from plants grown *in vitro* gave the best results in PCR reactions. A positive PCR reaction was also obtained when DNA was isolated from leaves taken from the greenhouse or dried at 40°C. It was found that the age and quality of plant material were of major importance for a successful PCR amplification. The results of PCR reactions with different types of plant material used and different isolation protocols applied are presented in Table 1.

Molecular markers can be considered to be essential tools in cultivar identification (DNA typing), assessment of genetic variability and relationships, management of genetic resources and biodiversity, studies of phylo-genetic relationships and in genome mapping. The main aim of the management of genetic resources is to ensure that as much as possible of the existing genetic diversity of species is conserved. The effectiveness of this depends to a large extent on the genetic information available on the germplasm under study. Because of a limited number of samples from individual populations and a small number of polymorphic RAPD profiles generated after electrophoresis of PCR products, the results of statistical analysis showed that samples belonging to the same populations, as well as outgroups on the dend-rogram, did not usually cluster together. There are many plant species in the world with medicinal properties. However, almost all MAPs are neglected or under-utilized and insufficiently documented. Cultivation of MAPs is limited and most are wildly crafted, causing degradation of natural habitats. Therefore, a need for conservation is arising for many species. The number of research activi-ties, scientists studying MAPs and species investigated should be increased to produce information and better understanding to conserve MAPs. An international net-work storing information on MAPs should be established for better use of MAPs and sharing related infor-mation. In the present study a protocol for DNA isolation was developed and PCR reactions were optimized. In further investigations, genetic analysis will be performed on a larger number of samples from each population of MAPs and possible correlations between genetic varia-bility, morphological and biochemical properties will be assessed. Classical methods of estimating genetic diver-sity among groups of plants have relied upon morpho-logical or chemical characters, but these characters can be influenced by environmental factors. By looking directly at the genetic material itself, molecular markers represent a powerful and potentially rapid method for the characterization of diversity per se within the *in situ* and *ex situ* conservation (Ford-Lloyd, 2001). However,molecular studies dealing with medicinal and aromatic plants (MAPS) (MAPs) are rare in comparison with other cultivated plants. This is probably due to the presence of large amounts of secondary metabolites and essential oils in MAP tissues, which inhibit DNA amplification in PCR re-

Table 1. Plant materials, isolation protocols and resulting PCR amplification using in this study.

Plant material	Source		Isolation protocols	PCR amplification
	In vitro grown plant	Grown plant		
Brasica napus L. collected in summer	graminated seed or plantlet	dried seed	CTAB method EZ1 nucleic acid isolation equipment	++
Brasica napus L. collected in winter	graminated seed or plantlet	dried seed	CTAB method EZ1 nucleic acid isolation equipment	++
Echinaceae purpurea L.		fresh plant or leaves from greenhouse	CTAB method	+
Ginkgo biloba		fresh plant	CTAB method CsCl method Sorbitol method	+++
Daphne alpina L.		fresh plant or leaves from greenhouse	CTAB method Sorbitol method	++
Linum usitatissimum L.	graminated seed or plantlet	dried seed	EZ1 nucleic acid isolation equipment	+
Rhizoma aronis L.		dried seed	CTAB method	+
Glycine max L.	graminated seed or plantlet	dried seed	EZ1 nucleic acid isolation equipment	+
Portulaca oleraceae L.	graminated seed or plantlet	dried seed	CTAB method	+
Rosmarinus officinalis L.		Herbarium plant	EZ1 nucleic acid isolation equipment	+

+++ = Excellent amplification; ++ = very good amplification; + = good amplification.

reaction (Khanuja et al., 1999; Mizukami and Okabe, 1999). There have been only two molecular studies on the genus *Origanum* reported until now. A molecular systematic study of the family Lamiaceae using *rbcL* gene sequences was made by Kaufmann and Wink (1994) with two *Origanum* species (*O. vulgare* and *O. laevigatum*) and *Majorana hortensis,* among others. Some authors have treated *M. hortensis* as a member of the *Origanum* genus, but the results of this study show that it differs significantly from the two *Origanum* species and was therefore suggested to be a separate species (Kintzios, 2002).

We successfully isolated DNA from these plants using the 4 protocols described above. High quality DNA was isolated using the protocols which is suited to produce usable DNA from these sources. These methods produced RNA-free DNA from 50 mg per gram or 1 g of fresh and dried tissue from the plant sources.

The aim of the present study was to develop the efficient protocol for DNA isolation and to optimize the PCR reactions for further evaluation of genetic diversity of aromatic and medicinal plants growing in Turkey. To our knowledge, this work represents novel methods that do not require ultracentrifugation or reaction dilutions and UV spectrophotometric analysis of DNA to isolate DNA from a variety of herbal or aromatic plants growing in Turkey. Because we found the methods described in this paper functional for plants that were otherwise recalcitrant to DNA isolation, we believe that these methods will be of help for molecular biological studies of many other aromatic and herbal plants.

UV spectrophotometric analysis of DNA

We routinely work with DNA, RNA, and proteins and have devised some simple, fast spectrophotometric assays for these molecules. The purpose of this exercise is to use the UV absorbance of biological samples to obtain qualitative and quantitative information about those samples. The concentration of an RNA or DNA sample can be

checked by the use of UV spectrophotometry. Both RNA and DNA absorb UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5 ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. Using a 1-cm light path, the extinction coefficient for nucleotides at this wavelength is 20. Based on this extinction coefficient, the absorbance at 260 nm in a 1-cm quartz cuvette of a 50 µg/ml solution of double stranded DNA or a 40 µg/ml solution of single stranded RNA is equal to 1. The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85. We obtained the concentration purity of DNA from aromatic and medicinal plants by our modified protocols that do not require ultracentrifugation or reaction dilutions. It is therefore easy to separate repetitive DNA by ultracentrifugation in herbal medicine but do not use and UV spectrophotometric analysis of DNA.

Data analysis

Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). The genetic similarity among accessions was determined by Nei's genetic distance. A dendrogram was constructed based on the matrix of distance using Unweighted Pair Group Method with Arithmetic averages. Scores entered in matrix were analyzed using TAXAN version 4.0 software based on the degree of bands sharing. Similarity matrix was generated using Dice coefficient as SI = 2Nab/Na+Nb where Na = total number of bands present in lane a, Nb = total of bands in lane b, Nab number of bands common to lanes a and b. The dice values were then used to perform the UPGMA analysis.

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