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Evaluation of Efficiency of Different DNA Extraction Protocols and Molecular Identification of Commercial Soybean (*Glycine max. , L.*) Growing in Turkey

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Abstract

Four DNA extraction methods were used to obtain DNA from herbarium specimens of soybean and fresh soybean seeds, and germinated soybean seeds in this study. The quality of DNA obtained was estimated by using a spectrophotometer to measure the A260/280 absorbance ratio. Four different DNA extraction methods were compared for the isolation of DNA from the soybean homogenates, namely the CTAB extraction method, Plant Genomic DNA Purification Kit method, EZ1 Nucleic acid isolation method, and DNA extraction with phenol purification and liquid nitrogen. The main goal of study is to evaluate various methods of DNA isolation in terms of DNA yield and amplification quality. To preserve DNA well, it is necessary to dry plants as fast as possible. Extraction results depend on how the plant material is prepared, and the type of chemicals or DNA isolation protocols used. Obtaining high quality DNA depends on the isolation technique used. Several methods that are useful for dry plant tissue from herbarium specimens have been described [9, 16, 19]. Four extraction protocols were compared using fresh material and dry herbarium specimens and seeds. Plant Genomic DNA Purification Kit method; the concentration of DNA in the second elution extract was very low, thus repeated elution was not applied. DNA extraction is done with phenol purification and liquid nitrogen. This technique is not quite suitable for fresh plant leaves. It is usually used for herbarium samples of at least 0.2 g CTAB extraction method. This protocol is quite suitable for DNA isolation from the fresh plant material or germinated seed plantlets. The total DNA was isolated from approximately 0.5-1 g of fresh leaves (conserved in CTAB) of the same collection of sample. EZ1 Nucleic acid isolation tehnique is quite useful for high yield and quality of DNA isolation from dried soybean seeds. In this methods, no further purification was needed for molecular analysis.

Key Words: Genomic DNA extraction, soybean (*Glycine max*. L.)

Abbreviations: CTAB, Hexadecyltrimethylammonium bromide

INTRODUCTION

The genus Glycine Wild is divided into two subgenera (species), *G.max.* and *soja.* The subgenus Soja (Moench) includes the cultivated Soybean, *G. max* (L.) Merrill, and the wild soybean, *G. soja* Sieb. & Zucc. both species are annual. *Glycine max* and *G. soja* form the primary gene pool for the cultivated soybean. Evidence from several sources, including morphology, cytogenetics, seed proteins, phytoalexins, restriction endonuclease fragment analysis of mitochondrial DNA, ribosomal RNA, and chloroplast DNA supports the hypothesis that *G. soja* is the wild ancestor of the soybean [9, 11, 12]. The soybean *Glycine max* (L.) Merr. is a "world commodity" grown in more than one hundred countries. Numerous foods, feed, fiber, medicinal, and endustrial products are produced using soybean. Today, soybean is a key source of protein and edible oil. It contains about 40% protein and 20% oil in the seed and, in the international trade markets, is ranked number one in oil production (48%) among the major oil seed crops. The soybean's valuable characteristics have propelled it into the agricultural mix in many parts of the world. In 2004, soybeans accounted for approximately 35% of the total harvested area worldwide of annual and perennial oil crops according to the Food and Agriculture Organization of the United Nations (FAO). The United States is the leader in soybean production, followed by Brazil, China, Argentina, India and Turkey. Despite its economic importance, the genetic base of soybean cultivars is extremely narrow. By using recombinant DNA technology has been improved to be useful tool for crop breeding. Recently, many molecular markers associated with disease resistance genes have been identified in soybean [18]. Since a large number of materials are handled in plant breeding, it is necessary to develop a simple, rapid, useful method for DNA extraction in order to conduct molecular marker-assisted selection. Herbarium collections are a potentially important source of material for phylogenetic and crop improvement studies. With the expansion of molecular techniques, historical collections have become relatively widely used. The specimens are useful especially for very rare or endemic plants because sometimes more plants are in the herbarium than in nature. This article should serve as a tool for projects involving DNA extraction from herbarium specimens and fresh soybean seeds and germinated plantlets. The problem of DNA extraction is crucial for further analyses of herbarium samples. The satisfactory quality of DNA is essential for success of the whole molecular study. In some species, separating DNA from naturally occurring plant cell contaminants, such as polysaccharides and phenolic compounds, is difficult. Furthermore, isolating intact DNA from dry plant tissues is more difficult, and a smear appearance is usually visible on the gel. Current studies indicate

that extraction of DNA is not always routine and simple, and conventional methods are not necessarily reproducible for all species, especially for dry material [1, 2, 17] . Simple extraction protocols suggested by Wang et. al. (1993) did not yield any DNA from dry materials of *Hesperis* specimens, and other published protocols include enzymatic digestion of proteins and the use of detergents such as CTAB [2], SDS [1], and polyvinylpyrrolidone (PVP) [6]. The objectives of this study are to establish a DNA isolation protocol suitable for dry material of soybean, to make comparisons with some other published protocols, and to obtain a RAPD optimization with the isolated DNA. In this paper, we thus describe a new DNA extraction method using fresh material and dry herbarium specimens and seeds. These methods enable to extract DNA from a large number of samples rapidly and efficiently.

MATERIALS and METHODS

Soybean samples

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. Soybean (*Glycine max. L.*) seeds or grains samples provided by Karadeniz Agricultural Research Institute. 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 [10] . A soybean seed bulk sample was ground to fine powder. 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.

DNA isolation from Soybean seeds

Soybean (*Glycine max. L.*) 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 gram 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. Soybeans 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 hours 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 soybean 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.

CTAB method for DNA purification method

The ground soy was diluted in 500 µl CTAB buffer (20 g CTAB/liter, 1.4 M NaCl, 0.1 M Tris/HCl, 20 mM EDTA) (MBI, Fermentase). This mixture was incubated for 30 minutes at 65°C. It was then centrifuged for 10 minutes at 9000 rpm. The upper layer was extracted with an equal volume of chloroform. After mixing for 30 seconds, the mixture was centrifuged for 10 minutes at 9000 rpm. The supernatant was transferred to a new tube, and two volumes of CTAB precipitation solution (5 g CTAB/liter, 0.04M NaCl) were added. The mixture was incubated for 60 minutes at room temperature and then centrifuged for 5 minutes at 9000 rpm. The supernatant was removed, and the precipitate was dissolved in 1.2M NaCl. This solution was chloroform extracted. The upper layer (aqueous phase) was transferred to a new tube and 0.7 volumes of isopropanol was added [8]. The solution was then frozen for 20 minutes at -80°C. The sample was thawed at room temperature and centrifuged. The resulting DNA pellet was washed with ice-cold 70% ethanol and centrifuged. The ethanol was discarded, and residual ethanol was removed with a DNA SpeedVac. The DNA pellet was resuspended in TE buffer.

Plant genomic DNA purification kit method

After grinding, the soybean was mixed with 600 µl Nuclei Lysis Solution and incubated for 15 minute at 65° C. RNase was added followed by a 15 minute incubation at 37° C and a 5 minute cooling to room temperature. Protein precipitation solution was added followed by centrifugation to pellet precipitated proteins. The supernatant containing the DNA was transferred to another microfuge tube containing isopropanol. The sample was mixed gently by inversion and then centrifuged at 9000 rpm. The supernatant was removed and the pellet was washed with 70% ethanol. The tube was centrifuged and the ethanol was decanted. Excess ethanol was removed by inverting the tube onto absorbent paper and air-drying for 15 minutes. The DNA was resuspended in 50 µl DNA rehydration solution during incubation for 1 hour at 65°C.

DNA Isolation using EZ1 Nucleic acid isolation analyser

Most samples were extracted with the DNeasy Plant Mini Kit (QIAGEN, ATQ, Biotechnology), but modified Doyle and Doyle (1987) and Struwe et. al. (1998) methods were also employed. Extractions of herbarium material were done with a modification of the QIAGEN protocols and included the addition of 570 mg (30µl) of PCR grade proteinase K (MBI, Fermentase), 6.5% (30µl) ß-mercaptoethanol (BME) and incubation at 42 °C for 12-24 hours on a rocking platform. Then DNA isolation was done using tissue kit with Nucleic acid isolation equipment (QIAGEN, Bio Robot EZ1) from dry seed.

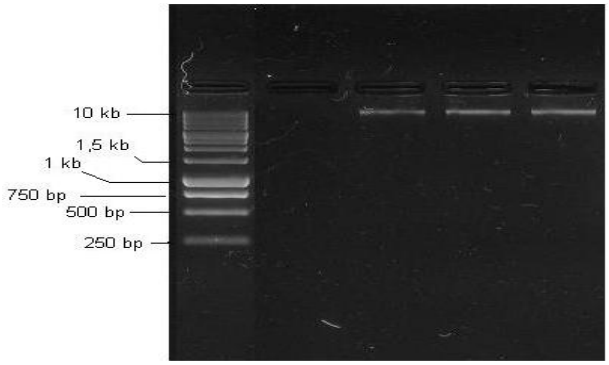

Figure 1. Screening of agarose gel electrophorosis of isolated DNA from soybean.

Genomic DNAs were loaded in a %0.7 agarose gel and separeted by electrophoresis for 90 min at 50 V, then visualised by ethidium bromide staining with transillumination. Respectively, Lane 1-5; Lane 1, 1 kb ladder size standart. Lane 2 negative control water balnk (none DNA). Lane 3, 4 and 5 genomic DNA isolated from soybean with Bio-Robot EZ1. None of the DNA samples showed indications of significiant smearing.

DNA extraction with phenol purification and liquid nitrogen method

Phenol was used for purification. After a 15 min incubation in the thermoblock, a mixture of phenol and chloroform (1:1) was added and mixed. In this protocol, we were applied to a very small amount of plant tissue [2].

Electrophoretic and Spectral Analysis

Agarose Gel Electrophoresis

Five microliter of each DNA was analyzed on a 1% agarose gel (TAE buffer), including a molecular weight marker (Figure 1) and then stained with ethidium bromide (0.5 µg/ml) for 30 min and then agarose gel washed in double-distilled and UV-irradiated H_2O.

Analysis of DNA fragmentation was performed by ethidium-bromide stained agarose gel electrophoresis. The ethidium bromide luminescence from the CCD camera is integrated for 1-2 s into the computer memory directly from the gel on the UV Transilluminator using Gel Doc. 1000 system (Bio Rad). One of the most common methods for nucleic acid detection is the measurement of solution absorbance at 260 nm (A260) due to the fact that nucleic acids have an absorption maximum at this UV wavelength. Although a relatively simple and time-honored method, A260 suffers from low sensitivity and interference from nucleotides and single-stranded nucleic acids. Furthermore, compounds commonly used in the preparation of nucleic acids absorb at 260 nm leading to abnormally high quantitation levels. However, these interference and preparation compounds also absorb at 280 nm leading to the calculation of DNA purity by performing ratio absorbance measurements at A260/ A280 [1, 3].

Polyacrylamide Gel Electrophoresis

A discontinuous borate/formate buffer system is presented for horizontal polyacrylamide gel electrophoresis of DNA fragments. The resolution potential of the system could be altered by changing the total monomer concentration (5-9 %T), as well as the concentration of formate in the gel (40-120 mM), the leading ion of the buffer system. The separation of DNA fragments would be improved by increasing the migration distance from 22 to 28 cm. This discontinuous polyacrylamide gel electrophoresis system proved highly reproducible [13]. In this study we compared the various electrophoretic methods for DNA fingerprint of soybean genotypes. It is reported that polyacrylamide gel electrophoresis and agarose gel electrophoresis is preferred because of the high similarities of their band patterns, especially when used for differentiation of DNA band profile of soybean genotypes (Figure 2).

Restriction Digestion

Restriction digestion of DNA protocols

Protocol 1 containing; 2 µl 10XBuffer, 0,5 µl Lambda EcoRI/HindIII, 0,2 µl 100 x BSA, 13 µl water and 2 µl genomic DNA. 55°C and 2 hours incubation respectively in lane 2 and 3 identified with protocol 1.

Protocol 2 containing; 2,5 µl 10XBuffer, 0,6µl Lambda EcoRI/HindIII, 1 µl RNase, 16,9 µl water and 4 µl genomic DNA. 37 °C and 3 hours 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 3).

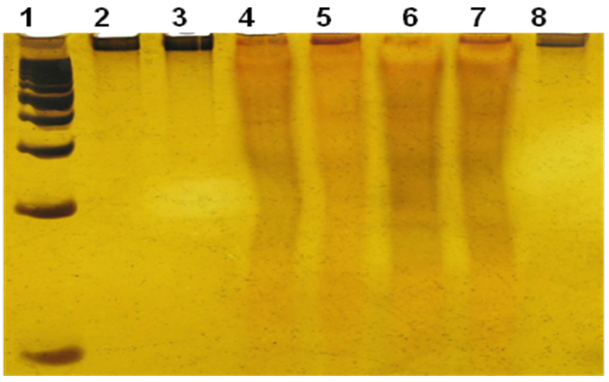

Figure 2. Polyacrilamide gel electrophoresis of Soybean DNA.

Lane 1, 100 bp ladder size standard. Lane 2, genomic DNA isolated from Soybean with Bio Robot EZ-1. Lane 3-4, genomic DNA isolated from Soybean with Bio Robot EZ-1. Lane 5-8, genomic DNA isolated from Soybean with manuel isolation method.

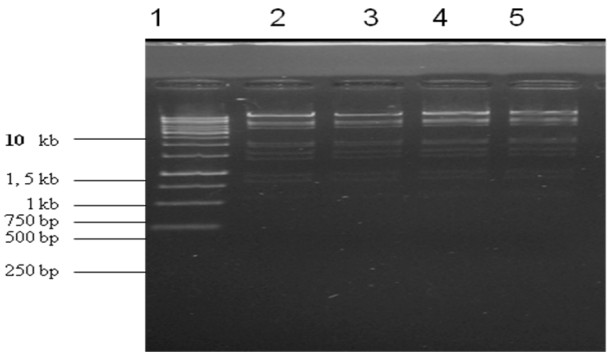

Figure 3. Screenning of agarose gel electrophoresis of soybean DNA digested with restriction endonuclease (EcoRI/Hind III).

Lane 1, 1 kb ladder size standard marker. Lane 2 and 4, digested with restriction endonuclease of DNA isolated from Soybean with EZ1 Nucleic acid isolation method. Lane 3 and 5, digested with restriction endonuclease of DNA isolated from Soybean with manuel DNA isolation method.

RESULT AND DISCUSSION

Soybean is a valuable agricultural commodity due to its unique chemical composition. The soybean seed consists of lipids, proteins, carbohydrates and minerals. Other valuable components found in soybeans include phospholipids, vitamins and minerals. On average, oil and protein together consitute about 60% of dry soybeans. The remaining dry matter is componsed mainly of carbohydrates (35%) and ash (5%) [5]. Nineteen amino acids make up the protein content of soybean flour, including the essential amino acids, lycine, isoleucine, leucine and valine. A typical soybean plant cell contains at least 10,000 different enzymes [7]. As long as conditions are favorable, enzymes will catalyze repeatedly their respective chemical reactions.

Extraction (isolation) of DNA nuclear, mitochondrial, and/or chloroplast DNA from sample to be studied is the first step for all molecular marker types. DNA can be extracted either from fresh, lyophilized, preserved or dried samples but fresh material is ideal for obtaining good quality DNA. There are many alternative protocols for DNA extraction and the choice of a protocol depends on the quality and quantity of DNA needed, nature of samples, and the presence of natural substances that may interfere with the extraction and subsequent analysis. DNA extraction protocols vary from simple and quick ones [4] that yields low quality DNA but nevertheless good enough for routine analyses to the laborious and time-consuming standard methods [1] that usually produce high quality and quantity of DNA. The isolation method used for DNA extraction from soybean homogenate affects the quantity and quality of the genomic DNA isolated. Electrophoretic "gels" are composed of either agarose or polyacrylamide. These two substrates differ in resolving power, and also in the difficulty of setting them up - agarose gels are used much more commonly except for small fragments of DNA. Polyacrylamide gels are also widely used for electrophoresis of proteins. Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders). Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins [15]. Agarose gel electrophoresis and polyacrylamide gels of the genomic DNA (Figure 3 and 4) illustrates a significant difference in yield and fragment size of the DNA.

Based on the fluorescence of the DNA smear in the ethidium bromide stained gel, the CTAB method produced the greatest yield, but fragment size is extremely small as compared to the other DNA preparations and measured against the molecular weight markers. Depending upon the application of the DNA, lower yields of large sized fragments may be preferred.

We successfully experimented with mature leaves of field and greenhouse grown plants. However, for breeding applications, leaf tissue is preferred because its collection is the least destructive. Fresh leaf tissue was collected and placed on ice. Tissue can be used immediately or stored at -80 ^{o}C. Freeze-dried tissue can be used, but is not recommended because DNA yields are reduced. Fresh tissue (1 g) was used for the isolation. The tissue was frozen in liquid nitrogen and ground in a mortar and pestle. We examined rapid and simple genomic DNA extraction method to detect material soybeans. Genomic DNA was extracted from soybean CTAB, and Plant Genomic DNA Purification Kit Method, DNA extraction with phenol purification and liquid nitrogen method and EZ1 Nucleic acid isolation methods. Genomic DNA solutions from soybean by both extraction methods were of adequate purity and yield for applying PCR, resulting that amplifications of the expected 100 bp fragment using the soybean specific primer pair were detected. When genomic DNA was extracted from soybean the DNA solutions by CTAB method were of a sufficient purity (A_{260}/A_{280}=1.7–2.0). In addition, the amplification products from the genomic DNA solutions by CTAB method were not detected. Thus, these results suggested that the desirable genomic DNA was not extracted from soybean by CTAB method, although it was possible to extract it by EZ1 nucleic acid DNA isolation method were of a sufficient purity (A_{260}/A_{280}=1.7–1.8). Genomic DNA Purification Kit, and EZ1 Nucleic acid isolation methods were found that of material soybeans can be extracted by rapid and simple methods. Therefore, the soybean DNA extraction procedure is sufficiently efficient and yields adequate amounts of genomic DNA with a sufficient level of repeatability. A DNA extraction method has been developed and applied successfully to the detection soybeans. This study has shown that DNA can be extracted efficiently from processed samples using different protocols. The study reported confirm that the extraction method, produces DNA of suitable quantity and quality for subsequent PCR based detection applications. The procedures are reliable and reproducible, typically displaying a success rate of over 90%. In summary, we developed a fast and reliable genomic DNA extraction protocol for soybean genotypes.

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