

Efficient Strategies for Elimination of Phenolic Compounds During DNA Extraction from Roots of *Pistacia vera* L.

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ABSTRACT

Optimization of DNA extraction protocols for plant tissues and including endophytic microorganisms is a critical step of advanced plant-microbe interaction in agricultural studies. Pistachio (*Pistacia vera* L.) root tissue contains high levels of polyphenols have been known as major extract contaminants and inhibitors of enzymatic activities during amplification. The present study aimed to develop reliable strategies to purify DNA from Pistachio root samples. Inhibiting substances were removed from DNA through a process including extraction with hot detergent contains SDS-Tris-EDTA, $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ as chemical coagulating factor and CTAB-NaCl. Following typically organic extraction/alcohol precipitation, denaturing agarose electrophoresis performed to purify probable remain contaminants. The purified DNA was enough free of polyphenols based upon loss of color and spectral quality ($260/230 > 1.6$) and efficiently amplified during polymerase chain reaction particularly in the present of GC-clamp primers. This method proved well with detection of *Glomus* sp. (arbuscular mycorrhiza fungi) associated with *Pistacia vera* L. using denaturing gradient gel electrophoresis (DGGE).

Keywords: Arbuscular Mycorrhiza (AM); chemical coagulation; DNA extraction; polyphenols; *Pistacia vera* L.

INTRODUCTION

Preparation of high quality genomic DNA from agricultural plants is a critical stage of most genomic analyses studies toward plant genetic improvement and understanding plant-microbe interaction. Plant roots have been considered as the settlement of soil endophytic microorganisms some of which enhance nutrients availability and plant growth, improve the plant ability to tolerate abiotic (drought, salinity, etc.)

and biotic stress (plant pathogens) nevertheless, most of the endophyte-plant relationships are not well understood (Hardoim, van Overbeek, & van Elsas, 2008; Porras-Alfaro et al., 2008; Reinhold-Hurek & Hurek, 2011; Bulgarelli, Schlaeppi, Spaepen, Ver Loren van Themaat, & Schulze-Lefert, 2013; Nair & Padmavathy, 2014; Tkacz & Poole, 2015). Direct isolation of DNA from various part of plant tissues is a preliminary step for studying many associating and symbiosis relationship especially which types are not culturable in experimental culture media (Stewart, 2012).

Pistachio (*Pistacia vera* L.) is the key of horticultural plant in arid regions of Iran which currently includes 10 percent of non-petroleum export value however, excessive soil salinity as a current major ecological and agronomical problem has significantly reduced productivity of pistachio trees. Several studies have investigated the role of endophytic microorganisms -for instance arbuscular mycorrhiza (AM)- in protection of plants against salt stress by various mechanisms (Marulanda, Azcón, & Ruiz-Lozano, 2003; Marulanda, Porcel, Barea, & Azcón, 2007; Wu, Zou, Xia, & Wang, 2007; Wang & Liu 2001).

In order to study the colonized-mycorrhiza fungi with Pistachio roots, the first step was isolation of DNA from root tissues. AM fungi is being an obligate symbiont that cannot be cultured in the absence of a suitable host therefore, direct extraction of DNA from root tissue and analysis of fungal ribosomal DNA sequence was the reliable way to study AM communities. In this study DNA extraction from Pistachio roots via commercial DNA extraction kits resulted in dark color DNA with low spectral quality (Table 1). The problem was relevant to dark brown-colored compounds in root cells, called polyphenols substances that have a similar size and charge with DNA, tending to co-precipitate with extracted DNA,

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interfering with downstream enzymatic applications (Schrader, Schielke, Ellerbroek, & John, 2012; Borse, Joshi, & Chaphalkar, 2011; Healey, Furtado, Cooper, & Henry, 2014). Once the plant cells are broken apart, polyphenols become exposed to oxygen and reacted with polyphenol oxidases. Polyphenol oxidation products covalently bind to the phosphate backbone of nucleic acids, making them forcefully impossible to be removed (Manoj, Tushar, & Sushama, 2007; Zhang & Stewart, 2000; Borse, Joshi, & Chaphalkar, 2011). In order to nucleic acid extraction, selecting very young leaves or cotyledons has been recommended to reduce trouble of polyphenols however, for some studies like gene expression in a certain part of the plant or endophytic investigation, the conditions are not ideal furthermore considering age and type of the plant tissue (roots, leaves or stems), the content of polyphenol compounds/secondary metabolite (as DNA contaminants) will be various. In that case, the purity of extracted DNA is out of the power of commercial DNA extraction kits which are usually mentioned in the user instruction booklet as troubleshooting. Under these circumstances, it should be developed a particular strategy or improve available isolation protocols for elimination of polyphenol compounds from DNA. Common procedures involve using antioxidants (Ascorbic acid) and certain polymers (polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP) for removing phenolic compounds in leaf tissues (Peterson, Boehm, & Stack, 1997; Porebski, Bailey, & Baum, 1997; Khanuja, Shasany, Darokar, & Kumar, 1999; Carrier et al., 2011; Sahu, Thangaraj, & Kathiresan, 2012). PVPP-40 has addressed to remove polyphenols along with high concentration of NaCl, resulting in polyphenol-free DNA. Ascorbic acid was also being used for isolation of nucleic acids in polyphenol rich plants (Bielski, 1982). In the current study with regard to high concentration of polyphenol compounds in the root of Pistachio, it was suggested a reliable DNA extraction and purification method to eliminate polyphenol so that being well-suited for sensitive downstream reactions.

MATERIALS AND METHODS

Preparing Soil Samples

Pistachio (*Pistacia vera* L.) roots were collected from Pistachio Orchard in Rafsanjan (Kerman province, south-eastern of Iran). After

removing soil particles, roots were sterilized by 10 % sodium hypochlorite for 10 min and grinded in the liquid nitrogen.

DNA Extraction and Purification

Two grams of grinded root was mixed with 4 ml of pre-warmed extraction buffer (65 °C) included 100 mM Tris-HCl (pH 8.5-9), 25mM sodium EDTA (pH 8.5), 2 % SDS and 50-mM $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (adjusted pH to 8.5 with 1 M NaOH) and incubated for 2 hours at 65 °C, inverting every 15 minutes through incubation. Then, the mixture was centrifuged at 1500 xg for 5 minutes and 2 ml of pre-warmed (37 °C) 5M NaCl-5% CTAB (Cetyl trimethyl ammonium bromide) was added to the supernatant and incubated for 10 minutes at 65 °C. The temperature was necessary to assure high yields of DNA, due to lower solubility of CTAB salts below 50 °C (Abu Almakarem, Heilman, Conger, Shtarkman, & Rogers, 2012). Incubated mixture was extracted two times with equal volume of chloroform-isoamyl alcohol (24:1), followed by centrifugation at 2500 xg, for 15 minutes at room temperature. Upper phase was carefully recovered and precipitated with 0.6 volumes of isopropanol and incubated at -20 °C for at least 2 hours (or one overnight). The DNA was precipitated by centrifugation at 26000 xg for 15 minutes at 4 °C. The DNA pellet was washed using a washing solution (7 vol absolute ethanol, 2 vol ddH₂O and 1 vol ammonium acetate 3M) and centrifuged at 26000 xg for 15 minutes. DNA pellet was diluted in sterile ddH₂O and purified by loading in 2 % agarose gel containing 1X Tris-acetate-EDTA (TAE) and equal volume formamide. Following electrophoresis and staining with GelRed, the bands containing the large molecular weight DNA were excised then transferred to a sterile tube and agarose solubilizing buffer was added (agarose Gel DNA Extraction Kit (Roche) regarding to the kit instructions). Melted agarose mixed by binding buffer and precipitated by washing buffer. By discarding solution via centrifuge at 16000 xg for 30 seconds, DNA pellet resolved in sterile water.

For extracting DNA using plant DNA extraction kit, 100 mg grinded root mixed with lysis and protein precipitation buffers. Lysate was centrifuged to remove residual debris. The clear supernatant was mixed with binding buffer (to prepare optimal binding to the silica membrane) and washed two times with washing buffer. DNA was eluted in water for subsequent analysis and processing.

Polymerase Chain Reaction and DGGE Fingerprinting

The amplification potential of the extracted DNA has been evaluated using PCR with universal primers including, 18S rDNA universal primers for plant (F: 5'-GTACAAAGGGCAGGGACGTA-3' and R: 5'-GGAAGGCTGAGGCAATAACA-3' (Rajaei, Niknam, Seyed, Ebrahimzadeh, & Razavi, 2009)) and 18S rDNA primers for AM fungi (NS31-GC: 5'-GC(2)C(3)G(4)CG(2)C(4)G(3)CG(1)G(3)CG(1)G(4)CACG(1)G(4)TTGGAGGGCAGTCTGGTGCC(1)-3' and Glo1: 5'-GCCTGCTTTAAACACTCTA-3' (Liang et al., 2008)). PCR amplifications were performed using Bio-Rad thermal cycler as following; 2 minutes at 94 °C for initial denaturation, 30 cycles with denaturation for 45 seconds at 94 °C, annealing for 45 seconds at 60 °C, and extension for 45 minutes at 72 °C. A final extension step at 72 °C for 15 minutes was conducted to allow complete extension. PCR products were visualized by running the agarose electrophoresis. Denaturing gradient gel electrophoresis was performed for 25 µl of NS31-GC and Glo1 PCR products on a Bio-Rad DCode system (Bio-Rad, Mississauga, Ont.) described by Lawrence et al. (2004).

Hybridization

Extracted DNAs were denatured and then spotted onto a Hybond nylon membrane (Roche). The membranes were hybridized with DNA probes under the high-stringency prehybridization,

hybridization and washing conditions at 65 °C. The probes were labeled with the digoxigenin (DIG), and detected using the DIG DNA Labeling and Detection Kit (Roche), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The first step of DNA extraction was to break up the tissues and cells to access DNA. Woody Pistachio roots seemed tougher and hearty during the bead beating and they were ground using liquid nitrogen and a mortar and pestle instead. Following breaking the cells via grinding in the liquid nitrogen, products of polyphenols oxidation which had a high affinity for the nucleic acid, covalently bound to DNA in the extract mixture; adding the alcohol gave a brown color and viscous feature to the extract mixture and finally precipitated DNA. Polyphenols are most common PCR inhibitors which require the extensive clean-up steps to be used in amplification process (Moazzam Jazi, Rajaei, & Seyed, 2015). In the present study, $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ as a major component for phenolic compounds precipitation was added to the extraction buffer in order to chemical coagulation of phenolic compounds during lysis step. Fig. 1 shows the color of extracted and precipitated DNA using current protocol (A) and DNA extraction kit (B). The clear DNA was obtained using chemical coagulation method (Fig. 1A), while DNA was dark brown when the extraction was conducted using plant tissue extraction kit (Fig. 1B).



Fig. 1. The appearance (color) of precipitated root DNAs which were extracted using chemical coagulation (A) and DNA extraction kit (B)

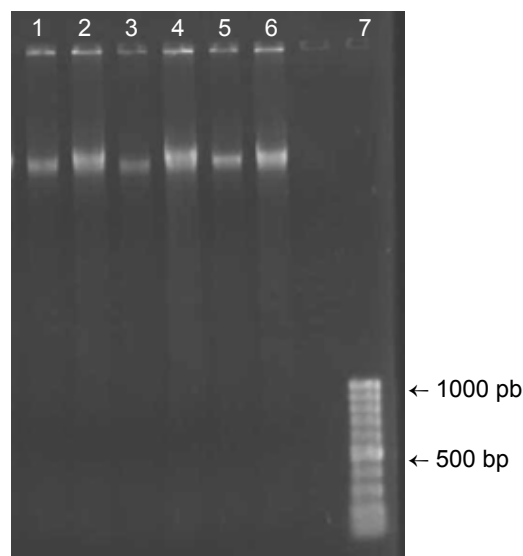


Fig. 2. Agarose electrophoresis of extracted DNA from roots of *Pistacia vera* L. under 100 (1, 3 and 5) and 50 mM (2, 4 and 6) concentration of $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in extraction buffer

Table 1. The quality and quantity of extracted DNA from root of *Pistacia vera* L. (using the Nanodrop)

Method	Root sample	DNA ($\mu\text{g}/\mu\text{l}$) ^a	260/280	260/230
Present	PR1	88.2 ± 0.19	1.31 ± 0.025	1.65 ± 0.045
	PR2	163.2 ± 0.17	1.56 ± 0.020	1.55 ± 0.038
	PR3	48.6 ± 0.27	1.44 ± 0.042	1.80 ± 0.026
	PR4	293.4 ± 0.35	1.62 ± 0.086	1.64 ± 0.074
	PR5	49.8 ± 0.20	1.73 ± 0.044	1.57 ± 0.069
DNA extraction kits	PR1	44.2 ± 0.13	1.62 ± 0.087	0.10 ± 0.016 ^b
	PR2	80.5 ± 0.10	1.83 ± 0.039	-
	PR3	12.7 ± 0.05	1.53 ± 0.011	0.25 ± 0.021
	PR4	179.1 ± 0.07	1.75 ± 0.045	0.02 ± 0.040
	PR5	26.5 ± 0.09	1.81 ± 0.020	0.15 ± 0.036

Remarks: ^a Mean ± standard error (n=3); ^b Dilution ratio 1:50

Direct application of solid $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in the extraction buffer (final concentration of 50 mM) remarkably improved the quality and quantity of extracted DNA (Table 1). The quantity of isolated DNA with this modification was approximately twice as that of obtained using 100mM solution of $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (Fig. 2) which could be attributed to the chaotropic effect and DNA loss in higher concentrations of $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (Braid, Daniels, & Kitts, 2003; Bakken & Rostegård, 2006; Gadkar & Fillion, 2013). Addition of solid $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ directly to the lysis buffer also led to pH decline to 4; therefore, following addition of $\text{AINH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ adjusting pH to 9 was indispensable.

Agarose gel pattern of root genomic DNA

(stained with Ethidium bromide) obtained by proposed method has been shown in Fig. 3. In this protocol, chloroform and isoamyl alcohol (24:1, v/v) was used for the denaturation of contaminating proteins. Phenol is a very hazardous chemical which usually used for removing proteins (Liao et al., 2004). Phenol-based method intensified production of brown color in DNA pellet (Chang, Puryear, & Cairney, 1993; Moazzam Jazi, Rajaei, & Seyedi, 2015), therefore it was important to ignore the application of phenol.

The quality and quantity of the extracted DNA was determined using a Nano Drop spectrophotometer and shown in Table 1. The yield of extracted-purified DNA using proposed protocol varied from 48.6 to 293.4 $\mu\text{g}/\mu\text{l}$. Since root

samples were prepared from mature trees in the field, they were very woody and tough containing very small quantities of DNA because of lignified cells dominance in wood. The present protocol could successfully extract DNA from samples however, the aim of this study was not focusing on the yield of DNA, in fact the main objective was removing polyphenolic compounds which precipitated concomitant with DNA through nucleic acid extraction. Polyphenolic contamination of DNA was determined by A260/A230 ratio, the ratio closed to 2 or > 2 showed a very low or no contamination in DNA (Kasem, Rice, & Henry, 2008; Rodrigues et al., 2007). The A260/A230 ratio of extracted DNAs by introduced method varied between 1.55-1.80 whereas the ratio for extracted DNAs using commercial kit was very low between 0.02-0.25, indicating the presence of organic contaminants (Table 1). Healey, Furtado, Cooper, & Henry (2014) tried to raise the quality of extracted DNA from

recalcitrant plant species (*Corymbia* and *Coffea*) by adding β -mercaptoethanol to a CTAB based method and the centrifugation step after 65 °C incubation. In this investigation the high concentration of phenolic compounds accumulated in pistachio roots was eliminated using $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ during DNA extraction. Similarly, Braid, Daniels, & Kitts (2003) reported that adding $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ to the DNA extraction buffer significantly declined the concentration of humic inhibitors with a minimal loss in the quantity of soil DNA.

Prepared DNA by the present method and DNA extraction kit were amplified using a standard PCR protocol. Fig. 4 shows agarose electrophoresis of PCR products includes 18S rDNA fragments. Amplification of the 18S rDNA ribosomal subunit of plants was not possible in the presence of extracted DNA template via DNA extraction kit while the sharp PCR bands were gained when the DNA had been extracted using the chemical coagulating method.

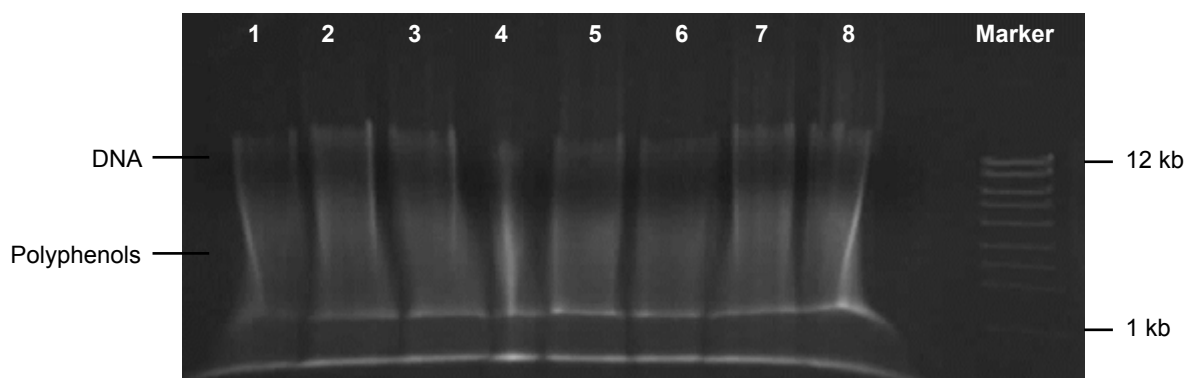


Fig. 3. Agarose electrophoresis of extracted DNA from roots of *Pistacia vera* L. (1-8) using plant-tissue DNA extraction kit

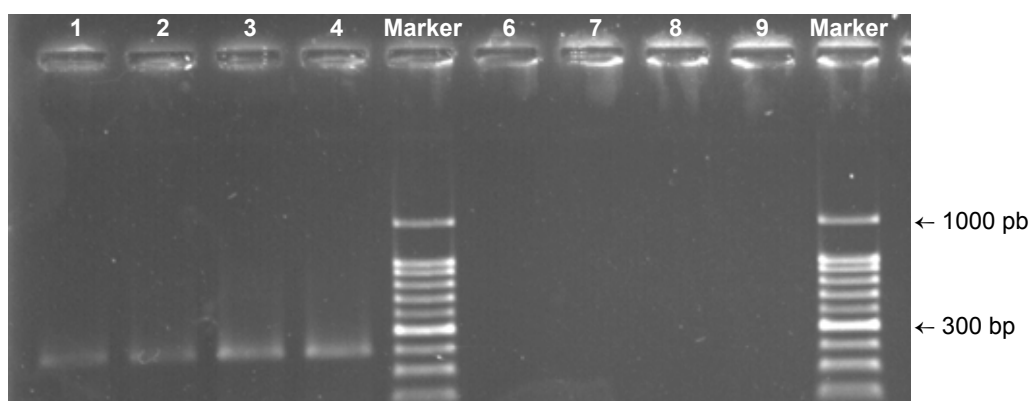


Fig. 4. Agarose electrophoresis of PCR products (300 bp) extracted DNA as template and 18S primers for plant. PCR products resulted from isolated DNA using the coagulating extracted method and plant-tissue extraction kit were illustrated in the lanes of 1-4 and lanes of 6-9, respectively

According to the DGGE image in Fig. 5, fingerprints were obtained by separation of PCR products (which were produced in the presence of NS31-GC and Glo1 primers, Fig. 6) on denaturing gradient gel (gradient range of 35-55 %). PCR-DGGE produced high number of distinct and sharp bands, demonstrating the current method appears to be an efficient protocol for studying biodiversity of AM fungi which colonized pistachio trees. Fungi were characterized from excised DGGE bands, which

mainly belonged to the *Glomus* genus according to the basic local alignment search tool (BLAST) (<https://www.ncbi.nlm.nih.gov>) (Fig. 5).

Extracted DNAs from pistachio roots were hybridized using an oligonucleotide probe complementary to a highly conserved sequence in the region between NS31 and Glo1 with dot blot technique. Location of spotted DNA onto a Hybond nylon membrane was clear without any pollution; no significant cross-hybridization was observed (Fig. 7).

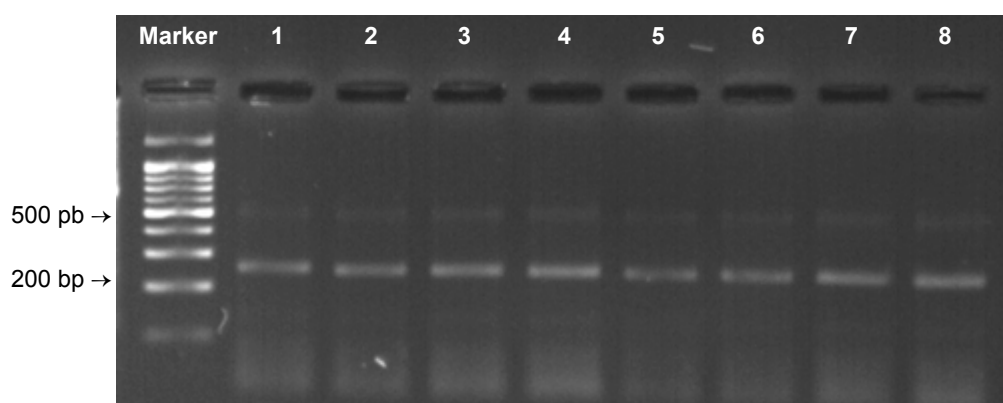


Fig. 5. Agarose electrophoresis of PCR products (280 pb in lane 1-8) amplified using NS31-GC and Glo1 primers (280 bp) specific of arbuscular mycorrhiza and extracted DNA from pistachio roots as template

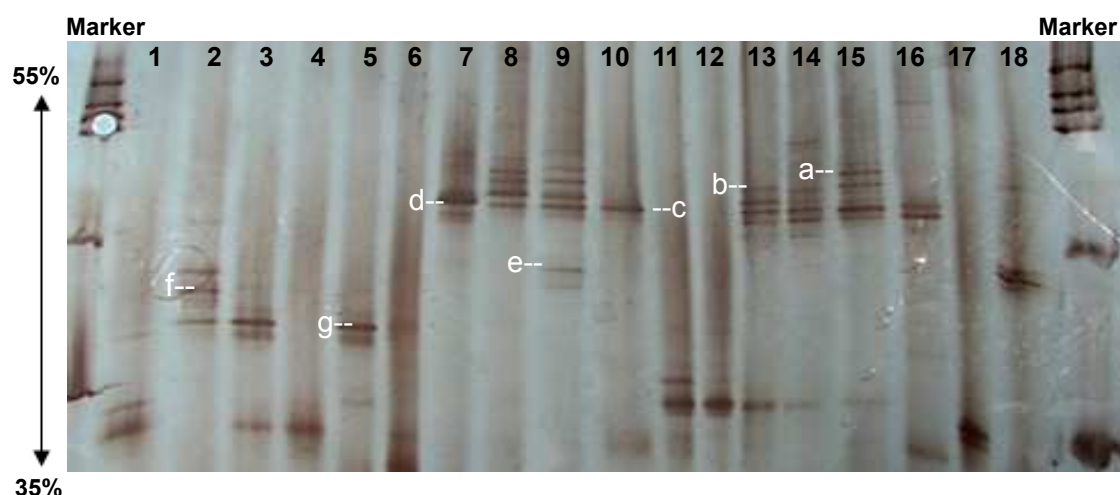


Fig. 6. Silver-stained band pattern of DGGE analysis for 18S rDNA fragments of arbuscular mycorrhiza were amplified in PCR-DGGE using pistachio roots DNA as the template and NS31-GC and Glo1 primers. Each lane belonged to the individual root. DGGE gel composed of 6% acrylamide in a denaturing gradient, from 55 to 35%. a, b, c, d, e, f and g bands were cloned and sequenced. a: uncultured *Glomus* (KT033907), b: uncultured *Xylariales* (KT033908), c: uncultured *Glomus* (KT033909), d: uncultured *Glomus* (KT033910), e: uncultured *Glomus* (KT033911), f: uncultured *Glomus* (KT033912), g: uncultured *Glomus* (KT033913)

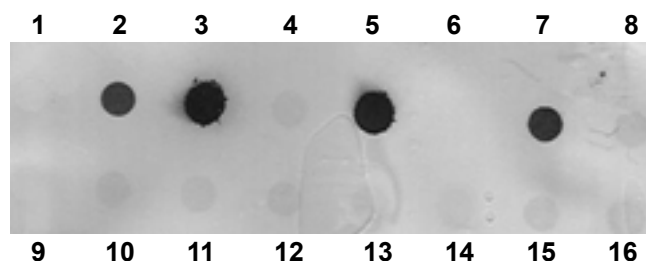


Fig. 7. DNA-DNA hybridization of extracted DNA from *Pistacia vera* L. roots using synthetic probe NS31/Glo1 specific of arbuscular mycorrhiza genome (spot 2 and 7) and synthetic probe 18S specific of plant genome (lanes 3 and 5). Spots 1, 4, 6, 8 -16 show control wells without DNA sample

Proposed post purification step, denaturing agarose electrophoresis using formamide, appropriately removed other residual-PCR inhibitors. Considering beneficial denaturing activity of formamide through agarose electrophoresis, residual-PCR inhibitors were detached from DNA. Moreover, PCR inhibitor including polyphenols traveled faster through the gel compared to DNA according to Fig. 2. Newman, Feminella, & Liles (2010) embedded the extracted genomic DNA in agarose plugs and incubated in a formamide-NaCl solution to remove contaminants, however, in the present study electrophoresis of DNA was more time consuming than incubation. Depending upon concentration of $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in lysis buffer, the co-purification of PCR inhibitors were reduced; nonetheless, upper concentrations of $\text{AlNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (above 50mM) noticeably decreased amount of DNA yield (Fig. 2). Recovery of agarose gel-embedded DNA also leveled up quality of DNA in polyphenolics-rich samples. Combination of two steps promoted the quality of highly contaminated DNA. Lack of smears and the appearance of sharp bands indicated that DNA degradation or shearing had not taken place (Fig. 2).

CONCLUSION

Acquiring the high quality DNA from plant tissues is the prerequisite key of plant microbe interaction studies. As the biochemical profiles of plant tissues and species considerably vary, it is almost impossible to rely on a universal isolation protocol/kit. The present study provided a reliable and simple technique for isolation of intact and high quality DNA from polyphenolic-rich pistachio roots. Inhibiting substances were eliminated from DNA through processes, including the chemical coagulating and denaturing agarose electrophoresis purifying. Based upon the color of purified DNA and

260/230 ratio >1.5, DNA was polyphenols-free while the 260/230 ratio of prepared DNA using commercial extraction kits was nearly zero. Regarding to the results, extracted DNA from the studied procedure was quite appropriate for PCR amplification and hybridization as well. Extracted DNA was too proper for studying biodiversity of plant endophytes, particularly the mycorrhiza fungi that cannot be cultured in the routine laboratory media (without host). Furthermore, using the current protocol and subsequent molecular biology techniques, *Glomus* sp. was reported as the most important symbiont of pistachio root. Overall, the research proposed that the current procedure can be considered for extracting DNA from other plants containing high levels of polyphenol.

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