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Robust CTAB-activated charcoal protocol for plant DNA extraction

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ABSTRACT

DNA extracted from plants rich in polyphenols and/or polysaccharides is often problematic when subjected to polymerase chain reaction, especially when mature tissues are used for DNA extraction. In order to overcome the problems associated with poor-quality DNA extracted from such plant samples, a protocol has been developed, availing on a high salt concentration and on the combination of polyvinylpyrrolidone and activated charcoal in the extraction buffer, in order to prevent the solubilization of polysaccharides and polyphenols in the DNA extract. Mild temperature conditions during extraction and precipitation were also recognized as important parameters. Besides DNA purity, mild precipitation conditions were found to be beneficial in obtaining less low-molecular mass nucleic acids in the final DNA extract. The homogenization step and the amount of sample extracted were also found to be crucial in keeping the extraction procedure robust.

Key words: activated charcoal, DNA extraction, PCR amplification, polyvinylpyrrolidone

IZVLEČEK

ROBUSTEN PROTOKOL ZA EKSTRAKCIJO RASTLINSKE DNK S POMOČJO CTAB IN AKTIVNEGA OGLJA

DNK, ekstrahirana iz rastlin z visoko vsebnostjo polifenolov in/ali polisaharidov, je pogosto problematična z vidika uporabe le-te v polimerazno verižni reakciji, še posebej če je bila DNK ekstrahirana iz zrelih tkiv. V izogib težavam, povezanih s slabo kakovostjo ekstrahirane DNK, smo razvili ekstrakcijski protokol, ki temelji na ekstrakcijskem pufru z visoko vsebnostjo soli ter kombinirane uporabe polivinilpirolidona in aktivnega oglja. Taka sestava ekstrakcijskega pufra preprečuje sočasno raztapljanje polisaharidov in polifenolov z DNK. Kot odločilen dejavnik pri postopku izolacije DNK smo identificirali tudi blage temperaturne razmere v

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stopnjah ekstrakcije in obarjanja. Poleg ugodnega vpliva na čistost, so blage razmere obarjanja pomebne tudi zaradi vpliva na manjšo vsebnost nukleinskih kislin z nižjimi molekulskimi masami v končnem DNK ekstraktu. Ugotovili smo tudi, da na robustnost postopka pomembno vplivata tako količina ekstrahiranega vzorca kot njegova homogenizacija.

Ključne besede: aktivno oglje, ekstrakcija DNK, amplifikacija s polimerazno verižno reakcijo, polivinilpirolidon

1 INTRODUCTION

DNA extraction from plants is preferentially performed from young tissues due to the lower content of polysaccharides, polyphenols and other secondary metabolites which coprecipitate with DNA in the extraction procedure, inhibit DNA digestion and PCR (Zhang and McStewart, 2000), presumably by irreversible interactions with DNA (Dabo et al., 1993). It has been shown that DNA extracts from tissues past the budding stage are problematic and also unstable under long-term storage (Lodhi et al., 1994). However, in cases when only older tissues are available, a proper procedure for selective extraction of DNA is needed. Hexadecyltrimethylammonium bromide (CTAB) is a frequently used surfactant in DNA extraction and several modifications of the CTAB protocol originally published by Doyle and Doyle (1987) have been made. Modifications were focused on the use of polyvinylpyrrolidone (PVP) or activated charcoal or combination of both (Maliyakal, 1992; Bi et al., 1996; Porebski and Bailey, 1997; Peterson and Boehm, 1997; Kim et al., 1997; Martellossi et al., 2005) in order to remove polyphenolics from further extraction steps. For polysaccharide removal, precipitation by high salt concentrations proved to be effective (Fang et al., 1992), or eventually, pectinase could also be used (Rogstad et al., 2001).

The presented protocol was primarily developed for DNA extraction from maturing leaf tissue of fennel (*Foeniculum vulgare*), known for its high essential oil and polyphenolic content (Oktay et al., 2003; Parejo et al., 2004; Krizman et al., 2006). Maturing fennel leaf tissue, with a considerable amount of secondary metabolites, was used as starting material for genetic studies since the same samples were used for chemotypic studies as well. The protocol proved to be useful also for DNA extraction from samples of other recalcitrant plant species such as oregano (*Origanum vulgare*) leaves, hemp (*Cannabis sativa*) seeds, hop (*Humulus lupulus*) dried cones and coffee (*Coffea arabica*) green beans. The extraction procedure is based on activated charcoal and PVP for binding of polyphenolics during extraction and on mild extraction and precipitation conditions, promoting high-molecular weight DNA isolation without interfering contaminants. During protocol development, we have also focused on keeping the protocol as simple as possible, minimizing the number of steps involved.

2 MATERIALS AND METHODS

2.1 Plant material

Fennel leaves and fruits were collected during the flowering period (July 2005) and the ripening period (October 2005), respectively, in Slovene Istria region. Oregano leaves were obtained from the experimental fields (yield 2005) of Biotechnical Faculty, University of Ljubljana (Slovenia). Dried hop cones were obtained from the experimental fields (yield 2005) of Institute of Hop Research and Brewing Žalec (Slovenia). Hemp seeds were from a commercial lot of Hungarian cultivar Unico. Samples of coffee beans were kindly donated by Dr. Furio Suggi Liverani and Dr. Lorenzo Del Terra from Illycaffè S.p.A., Triest, Italy.

2.2 Reagents and chemicals

- Extraction buffer: 100 mM Tris-HCl (pH 8), 2.0 M NaCl, 20 mM EDTA (pH 8), 2 % (w/v) CTAB, 1 % (w/v) PVP (PVP K10, MW 10.000). Before use, suspend 0.5 % (w/v) of activated charcoal in the extraction buffer and use it within 3 days. Agitate the suspension before pipetting.
- Chloroform-isoamylalcohol: 4% (v/v) isoamylalcohol in chloroform
- Wash solution: 15 mM ammonium acetate in 75 % (v/v) ethanol
- TE buffer: 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)
- Isopropanol

2.3 DNA extraction protocol

- Finely homogenize 5 mg to 50 mg of plant tissue (depending on the species, the tissue and its maturity) in a mortar with 1.5 mL of extraction buffer. Transfer the mixture into a microcentrifuge tube. When expecting low DNA yields, increase the sample amount, but proportionally increase the volume of extraction buffer as well. Incubate the mixture at 55 °C for 30 min with frequent agitation, avoiding the suspension to settle. Cool down to room temperature.
  Note: Avoid tissue freeze-grinding, milling or another type of homogenization before adding the extraction buffer, since it increases the chances of DNA contamination, unless the tissue is physically though and with a low amount of water (e.g. seeds, beans etc.). When dealing with though tissues (e.g. coffee beans), it is advisable to presoak the ground tissue (only the finest fraction) in the extraction buffer for 2 hours at room temperature before extraction and avoiding further homogenization with the extraction buffer in a mortar.
- Centrifuge at 16000 g for 10 min at room temperature. Transfer the supernatant to a new tube.
- Add 1 volume of chloroform-isoamylalcohol to the supernatant and vortex thoroughly. Centrifuge at 16000 g for 10 min at room temperature. Transfer the aqueous (upper) phase to a new tube. Repeat the chloroform-isoamylalcohol extraction once or more, if cloudiness in the solution persists.
- Transfer the supernatant to a new tube, add 0.45 volume of isopropanol and mix by inversion. Incubate at 25 °C for 1 hour. Centrifuge at 700 g for 10 min at room temperature.
- Discard the supernatant. Wash the pellet by adding 1 mL of wash buffer and vortex. Centrifuge at 900 g for 10 min at room temperature.
- Discard the supernatant and air dry the pellet at room temperature, but do not overdry.
- Dissolve the pellet in 25 μL of TE buffer. If there are some impurities left, centrifuge at 16000 g for 10 min at room temperature and transfer the supernatant to a new tube. Store the DNA solution at 4 °C for short-term or at –20 °C for long-term storage. If needed, treat the DNA solution with RNase before use.

3 RESULTS AND DISCUSSION

The extracted DNA was quantified by a fluorometric assay using a Hoefer DyNA Quant™ 200 fluorometer and a DNA-specific dye, Hoechst H33258 (Hoefer, San

Francisco, CA, USA), according to manufacturer's instructions. For each type of plant sample, six replicates were subjected to fluorometric measurements. The average results are shown in Table 1. Suitability of the isolated DNA was assessed by PCR amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (White et al., 1990) by using a TGradient thermal cycler (Biometra, Göttingen, Germany) with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (MWG-Biotech, Ebersberg, Germany). Each PCR 25 μL reaction mixture consisted of 2.5 μL 10x PCR buffer (Promega, Madison, WI, USA), 0.8 mM dNTPs, 1 μM of each primer, 0.5 units of Taq polymerase (Promega) and 35 ng of template DNA. After the initial denaturation at 94 °C for 5 min, 35 PCR cycles were performed with 35 sec at 93 °C, 55 sec at 53 °C and 45 sec at 72 °C. The DNA extracts as well as the amplification products were run in 0.8 % and 1.1 % agarose gels, respectively, stained with ethidium bromide and visualized under UV light. For comparison, fennel samples were also extracted, according to the commonly used CTAB protocol (Doyle and Doyle, 1987), and tentatively amplified (Figure 1).

Table 1. Comparison of DNA yield between different plant samples by using the presented extraction protocol. DNA yield is expressed as micrograms per gram of sample.

Species	Tissue	Sample amount extracted (mg)	Extraction volume (mL)	DNA yield (μg/g of sample)
Foeniculum vulgare	leaves	25	1.5	211.3
Foeniculum vulgare	fruits	8	1.5	171.8
Origanum vulgare	leaves	25	1.5	47.4
Cannabis sativa	seeds	10	1.5	166.3
Humulus lupulus*	dried cones	10	1.5	173.0
Coffea arabica*	green beans	90	15	7.2

* The ground tissue was presoaked for 2 hours in the extraction buffer before incubation, without further homogenization.

In this protocol we combined the individual characteristics of previously published ones as well as accentuated them. As already observed, the incorporation of activated charcoal in the extraction mixture before sample incubation greatly increases the chances for DNA to be amplifiable (Bi et al., 1996), most likely by preventing irreversible interactions of DNA with polyphenolics, since cytosol-borne compounds should come in contact with activated charcoal earlier than DNA. We also believe that PVP has a synergistic effect in binding polyphenolics on activated charcoal. As it has been already used as a polyphenolics-binding agent (Maliyakal, 1992; Bi et al., 1996; Porebski and Bailey, 1997; Peterson and Boehm, 1997; Kim et al., 1997), PVP should have strong interactions with activated charcoal due to the sp^2-electronic configuration of carbon rings of the latter. Using the same principle as in the precipitation of polysaccharides under high salt concentration (Fang et al., 1992), we adopted a high salt concentration (2 M NaCl) in preventing or diminishing the dissolution of polysaccharides during the extraction step. The precipitation step is also crucial in obtaining high quality DNA. In agreement with Michiels et al. (2003), we also observed the importance of a high precipitation temperature, 25 °C instead of 4 °C or even –20 °C. However, we further reduced the precipitation time and the amount of isopropanol added. As it is evident from Figure 1 , lanes 2 (G) and 3 (G),

the fennel DNA extract according to our protocol shows less presence of low-molecular mass nucleic acids. A feasible explanation for this occurrence is in the mild precipitation conditions, which diminish the possibility of shorter nucleic acid molecules to precipitate. A final note should be addressed to the plant tissue amount involved in the extraction. Although the expected DNA yield from a smaller sample amount should be lower, the possibilities for contaminants to coprecipitate with DNA are also lower, due to the fact that their saturation concentration during precipitation is less likely reached or exceeded.

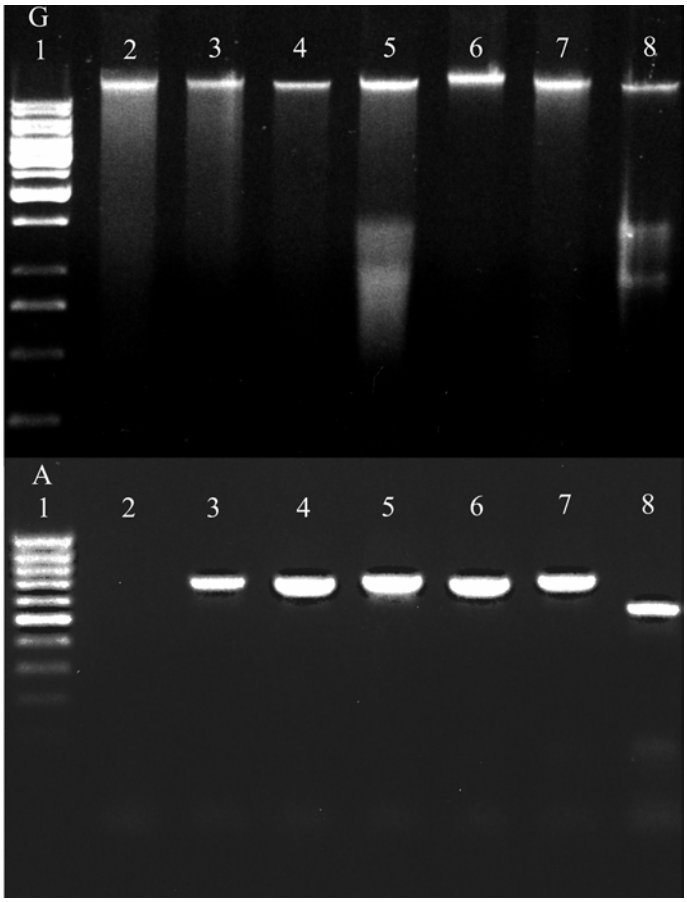

Figure 1. Gel electrophoresis (0.8 % agarose) of DNA extracts from plant species in study without RNase treatment (G) and gel electrophoresis (1.1 % agarose) of their internal transcribed spacer region PCR products (A). Lane 1 (G), 1 kb DNA ladder; lane 1 (A), 100 bp DNA ladder; lane 2, DNA extract of *Foeniculum vulgare* leaves (G) using a previously published extraction protocol (Doyle and Doyle, 1987) and its tentative PCR product (A); lane 3, DNA extract of *Foeniculum vulgare* leaves (G) and its PCR product (A); lane 4, DNA extract of *Foeniculum vulgare* fruits (G) and its PCR product (A); lane 5, DNA extract of *Origanum vulgare* leaves (G) and its PCR product (A); lane 6, DNA extract of *Cannabis sativa* seeds (G) and its PCR product (A); lane 7, DNA extract of *Humulus lupulus* dried cones (G) and its PCR product (A); lane 8, DNA extract of *Coffea arabica* green beans (G) and its PCR product (A).

4 CONCLUSIONS

Using our protocol, 0.5-10 μg of DNA per tube are typically obtained, sufficient for several runs of PCR-based assays. Although the protocol does not excel in DNA yield, it provides a rather simple and robust option in extracting DNA from problematic plant samples, owing to mild extraction and precipitation conditions. However, when larger DNA quantities are required, a scale-up of the protocol is not an issue.

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5 REFERENCES

Bi I.V., Harvengt L., Chandelier A., Mergeai G., du Jardin P. 1996. Improved RAPD amplification of recalcitrant plant DNA by the use of activated charcoal during DNA extraction. Plant Breeding 115: 205-206.

Dabo S.M., Mitchell E.D., Melcher U. 1993. A method for the isolation of nuclear DNA from cotton (*Gossypium*) leaves. Analytical Biochemistry 210: 34-38.

Doyle J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bullettin 19: 11-15.

Fang G., Hammar S., Grumet R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques 13: 52-54.

Kim C.S., Lee C.H., Shin J.S., Chung Y.S., Hyung N.I. 1997. A simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. Nucleic Acids Research 25: 1085-1086.

Krizman M., Baricevic D., Prosek M. 2006. Fast quantitative determination of volatile constituents in fennel by headspace-gas chromatography. Analytica Chimica Acta 557: 267-271.

Lodhi M.A., Ye G.-N., Weeden N.F., Reisch B.I. 1994. A simple and efficient method for DNA extractions from grapevine cultivars and *Vitis* species. Plant Molecular Biology Reporter 12: 613.

Maliyakal E.J. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Research 20: 2381.

Martellossi C., Taylor E.J., Lee D., Graziosi G., Donini P. 2005. DNA extraction and analysis from processed coffee beans. Journal of Agricultural and Food Chemistry 53: 8432-8436.

Michiels A., Van den Ende W., Tucker M., Van Riet L., Van Laere A. 2003. Extraction of high-quality genomic DNA from latex-containing plants. Analytical Biochemistry 315: 85-89.

Oktay M., Gülçin İ., Küfrevioğlu İ. 2003. Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebensmittel-Wissenschaft und-Technologie 36: 263-271.

Parejo I., Jaregui O., Sánchez-Rabaneda F., Viladomat F., Bastida J., Codina C. 2004. Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. Journal of Agricultural and Food Chemistry 52: 3679-3687.

Peterson D.G., Boehm K.S. 1997. Isolation of milligram quantities of nuclear DNA from tomato (*Licopersicon esculentum*), a plant containing high levels of polyphenolic compounds. Plant Molecular Biology Reporter 15: 148-153.

Porebski S., Bailey L.G. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Molecular Biology Reporter 15: 8-15.

Rogstad S.H., Keane B., Howes Keiffer C., Hebard F., Sisco P. 2001. DNA extraction from plants: the use of pectinase. Plant Molecular Biology Reporter 19: 353-359.

White T.J., Bruns T., Lee S., Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Snisky J.J., White T.J. (Eds.), PCR Protocols, p. 315-322, Academic Press, London.

Zhang J., McStewart J.D. 2000. Economical and rapid method for extracting cotton genomic DNA. Journal of Cotton Science 4: 193-201.