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RESEARCH NOTE

Efficient protocols for the extraction of microbial DNA from the rhizosphere of hydrophilic forests in Chile

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Abstract

P. Cáceres, C. Cordero, G. González, K. Quiroz, J.C. Bobadilla, C. Bravo, P.D.S. Caligari, B. Carrasco, and R. García-Gonzáles. 2012. Efficient protocols for the extraction of microbial DNA from the rhizosphere of hydrophilic forests in Chile. Cien. Inv. Agr. 38(3): 585-592. A lysis buffer-based protocol (Protocol BA), a modified lysis buffer-based protocol (Protocol BA Mod) and a commercial extraction kit (Protocol PS Kit) (Power Soil, Mo bio Laboratories, CA USA) were each evaluated for their ability to produce high-quality DNA with yields sufficient to allow its use in biodiversity studies. Similarly, the effect of liquid nitrogen on the process of cell disruption in all of the protocols that were studied. DNA yields ranged from 12.4 ng g^{-1} of processed soil to 9620 ng g^{-1} using the modified lysis buffer and commercial extraction kit, respectively. The quality of the DNA was determined by the ability of the DNA to produce efficient and reproducible polymerase chain reaction (PCR) products, using primers for universal 16S and 18S ribosomal RNA regions from bacteria and fungi, respectively. High-quality DNA was obtained to run PCRs in all protocols, but the efficiency of the method depended on the dilution of the DNA prior to performing the PCR. The three extraction methods generated PCR products with 90% efficiency. The DNA produced with the commercial kit was able to produce the highest PCR efficiency (95%) when the 10^{-1} dilution was used. The method based on the use of lysis buffer produced the highest efficiency (90%) using a 10^{-2} dilution. Meanwhile, the modified lysis buffer-based protocol generated the highest efficiency of PCR products using the 10^{-3} dilution factor with 95% of efficiency. For the first time, reliable and efficient DNA isolation from the rhizosphere of hydrolic forest is documented, enabling a wide range of applications for this technique.

Key words: hydrophilic forests, 16S ribosomal RNA, PCR, rhizosphere, soil DNA extraction.

Introduction

Chile has just over 75 million hectares of national territory, of which 13.4 million are covered by native forest, 14.1 million comprise protected areas, and 2.1 million are occupied by artificial plantations. *Pinus radiata* represents 75% of the total planted forests, while a remaining 17% is planted with *Eucalyptus* spp. Only 5.9 million hectares out of the 13.4 million hectares of native forests are considered to be productive forests, according to the cadastre prepared by Coorporación Nacional Forestal (CONAF) and Comisión

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Nacional del Medio Ambiente (CONAMA). Forests that have greater coverage in the country are Siempreverde (4.3 million hectares), Lenga (3.4 thousand hectares), Coihue de Magallanes (1.8 thousand hectares) and Roble-Raulí-Coihue (1.3 million hectares) (CONAF, 1999).

The Chilean native forest holds a huge potential for social, cultural and economic development for the country. An estimated 20% of the native forests are owned by smallholders (Catalan *et al*., 2006). For this reason, sustainable forest management is a positive step towards the implementation of silvicultural methods tailored to each forest formation, also considering the needs of owners.

Spatial heterogeneity of soil is caused by the presence of plants in many types of terrestrial ecosystems (Zong *et al*., 2007). The soil is a very appropriate environment for the development of both eukaryotic (algae, fungi, protozoa) and prokaryotic (bacteria and archaea) microorganisms. These microorganisms are not randomly distributed but follow spatial patterns of aggregation at different scales (from nm to km) that overlap each other. This structure is due to the effect of different control factors and is fully dynamic, with the following being some of the more important variables: pH, water content, soil structure, presence of roots, climatic variations and biotic activity (Ettema *et al*., 2002).

The recent use of molecular techniques on soil samples has opened the way to the study of previously unexplored microbial communities (Eldor, 2007). Nucleic acid analysis has an advantage over other methods, as it reduces the time that is needed for samples to be processed, thus eliminating the requirement for the growth of microorganisms in nutrient media, a step that may make the outcomes non-representative of the actual situation in the soil (Eldor, 2007).

DNA extraction protocols often result in inadequate cell lysis and co-extraction of humic acids that, due to their similar physicochemical properties, prevent the correct hybridization of primers to template DNA, inhibit PCR amplification and restriction enzyme digestion. The same processes are also influenced by the phenolic groups of the humic acids, which denature proteins by bonding to amides. Furthermore, these molecules can be oxidized to form quinones that bind covalently to DNA (Lakay *et al*., 2007). The important factors to consider in regard to DNA extraction are as follows: (1) efficiency, which is accomplished by physical, chemical and enzymatic processes to ensure rupture of the resistant cell structures that are characteristic of some soil microorganisms or spores and (2) the removal of contaminants (*e.g.,* humic acids) that are extracted together with nucleic acids and interfere with subsequent molecular analysis (O'Donnell *et al*., 1999).

The cell disruption protocols can be classified into two categories: those in which the cells are lysed within the soil (direct extraction) and those in which the cells are removed from the soil mix and the extraction is performed on isolated cells (Courtois, 2001). The technique of direct lysis, which was used in this study, is the most widely used because it gives a higher DNA yield and has less bias with respect to the diversity of the microbial community (Miller *et al*., 1999). Direct extraction methods include different protocols such as grinding in liquid nitrogen, mixing along with homogenization using short homogenization times and heat-shock treatment in a microwave oven with different chemical extractants, such as the ionic detergent sodium dodecyl sulfate (SDS), which acts by dissolving the hydrophobic material in the cells. The material is then subjected to heat with chelating agents such as ethylenediamine-tetraacetic acid (EDTA) or Chelex 100 (Robe *et al*., 2003; Lakay *et al*., 2007).

The aim of this study was to establish a rapid, efficient and reliable method for the extraction of high-quality total genomic DNA from the rhizosphere of hydrophilic forests by evaluating three different methods for cell disruption and DNA extraction, as well as the use of liquid nitrogen

during the cell lysis step. As the efficient extraction of soil DNA from the rhizosphere of hydrophilic forests in Chile has never been reported before, this technique will open up new opportunities in metagenomics and other studies involving soil microorganisms associated with native trees.

Materials and methods

Soil sampling

Samples were derived from a fragment of degraded material collected from a hydrophilic forest that grows on a sandy and loamy soil, with granulated particles that were both medium and small in size according to Centro de Informaciones de Recursos Naturales (CIREN-CORFO, 1964). The study was conducted on a plot of humid forest placed around the Convento Viejo dam, near the city of Chimbarongo, Chile (34° 46' 40''; 71° 3' 45''). Sampling was performed from the rhizosphere by collecting the soil near the roots (a maximum of 5 cm from the root) and 10 cm from the soil surface. Rhizospheric soil samples were randomly taken from beneath five dominant tree species common in this type of Chilean forest: *Luma chequen* (Mol. A. Gray (Chequén), *Blepharocalyx cruckshanksii* (H. et A.) Nied. (Temu), *Myrceugenia exsucca* (DC.) Berg (Pitra), *Drimys winteri* J.R. Forst. & G. Forst (Canelo) and *Crinodendron patagua* Mol. (Patagua).

All the samples were sealed in plastic bags and transported on ice. In the laboratory, samples were stored at - 80 °C (Thermo, Model 702, Ohio, USA) until use.

DNA extraction

For DNA extraction, five soil samples for each treatment were collected in duplicate. The DNA extraction was performed on 500 mg of frozen soil samples, previously stored at -80 ºC. The effect of including liquid nitrogen while grinding the samples during the first steps of DNA extraction was evaluated by considering the DNA yield and its quality. Soil samples were macerated with or without liquid nitrogen. The liquid nitrogen-treated samples were macerated to obtain a homogeneous powder. Samples being macerated without liquid nitrogen were briefly frozen at -80 °C at five-min intervals during maceration until a homogeneous mix was obtained. After that, total rhizospheric DNA was obtained by using three different methods. Two of these methods (called BA and BA Mod for this study) were adapted from Edwards *et al.* (1991) as previously reported (Cordero *et al.,* 2011). The third extraction method was performed with the Power Soil extraction kit (MoBio Laboratories Inc., CA, USA) (called PS Kit for this study) according to the manufacturer's instructions.

Total DNA isolated by each protocol was checked by electrophoresis in agarose (1% w/v). The electrophoresis was performed at 110 V in 1X TAE running buffer. The concentration of DNA in each sample was measured directly in a spectrophotometer (Thermo Scientific, Nanodrop 2000, MA, USA) at 260 nm. The absorbance ratio at the 260 nm/ 280 nm wavelengths was scored for each sample.

Polymerase Chain Reaction (PCR). The quality of the DNA obtained was tested by its ability to produce polymerase chain reactions with primers for the 16S and 18S of bacterial and fungal ribosomal RNA (rRNA), respectively. The 16S rRNA was amplified using the primers 16SU1F1 (5'-ACTGCTTGCCTCCCGT-3´) and 16SR2 (5'-CTACCAGGGTATCTAACT-3´) (Paster *et al.*, 1994). Amplicons from the 18S rRNA from fungi were produced using the primers NS1 (5'-GTAGTCATATGCTTGTCTC) and NS2 (GGCTGCTGGCACCAGACTTGC) (White *et al.,* 1990). Mixes for both PCR reactions were prepared as follows: 2 µL of three dilution factors (10^{-1}, 10^{-2} or 10^{-3}) from the sample DNA extracted by each method; 1.5 mM of $MgCl_{2;}$ 2.5 U of Taq polymerase (Invitrogen, Sao Paulo, Brazil); 1.02

mM of each primer; 0.2 mM dNTP; 0.5 mg mL^{-1} of Bovine Serum Albumin (New England Biolabs Inc., IPSWICH, MA, USA); and PCR Buffer at 1X. The final volume of the PCR mix was 25 µL, completed with double-distilled water. Sterile distilled water was used as a negative control. PCR was run in an automatic thermocycler (Thermo Electron Corporation, PX2-220, MA, USA), using the following programs:

Bacterial 16S rRNA: A denaturation step at 94 °C for 4 min followed by 45 cycles of 94 °C for 50 sec, annealing at 59.3 °C for 50 sec, and elongation at 72 °C for 45 sec. A final elongation step was performed at 72 °C for 8 min.

Fungal18S rRNA: A denaturation step at 94 °C for 3 min followed by 45 cycles of 94 °C for 30 sec, annealing at 59 °C for 1 min and elongation at 72 °C for 2 min. A final elongation step was performed at 72 °C for 7 min.

All the amplicons generated were visualized after electrophoresis in an agarose gel (2% w/v) using 1X TAE buffer.

Statistical analysis. The homogeneity of the variances was determined through Bartlett's Test (P<0.05). For the processing and analysis of normally distributed data, an ANOVA and the multiple range test of Tukey Honestly Significant Difference (P<0.05) were carried out. Non-parametric data were analyzed by the Kruskal-Wallis test (P<0.05).

Results

Extraction of DNA

All of the tested protocols were able to isolate total DNA from the rhizospheric environment of the sampled trees. However, the three protocols produced different amounts of DNA. The BA Mod protocol yielded higher concentrations of total DNA than the BA and PS Kit protocols (Figure 1). Concentration of total DNA estimated by spectrophotometer ranged from 12.4 ng g^{-1} when the PS Kit was used to 9620 ng g^{-1} for the samples treated with the BA Mod protocol. The use of liquid nitrogen during sample maceration did not improve DNA yield for the BA and PS Kit protocols. However, using liquid nitrogen significantly increased the final concentration of DNA for the BA Mod protocol.

The absorbance ratio at the 260 nm/ 280 nm wavelengths, an indicator of DNA quality, showed an average of 1.5 for DNA obtained using the BA and BA Mod protocols. The PS Kit protocol produced an absorbance ratio of 4.20, which is very high and could be interpreted as incomplete purification of DNA samples. An insignificant effect on DNA quality was obtained with the use of liquid nitrogen during the maceration of the rhizospheric soil samples.

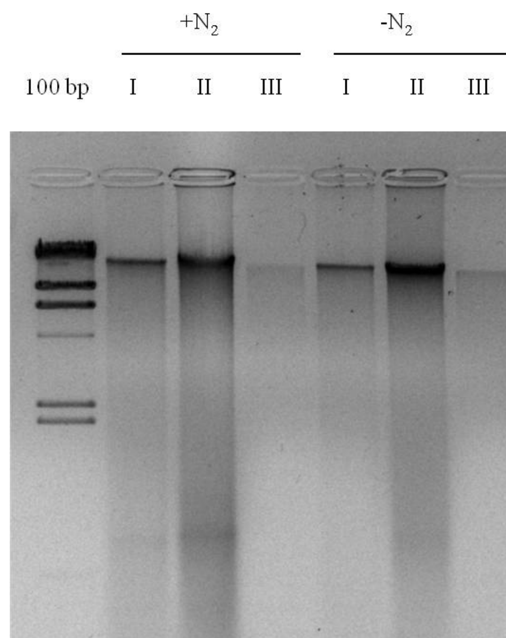

Figure 1. Extraction of DNA using different methods for one sample of soil. Lane 1: 100 bp DNA Ladder. Lanes 2, 3, 4: Extraction of DNA after grinding the soil samples with liquid nitrogen (I, BA protocol; II, BA mod protocol; III PS Kit protocol). Lanes 5, 6, 7: Extraction of DNA after grinding the soil samples without liquid nitrogen (I, BA protocol; II, BA mod protocol; III PS Kit protocol).

Amplification

The quality of the purified rhizospheric DNA samples was evaluated by considering their ability to produce polymerase chain reaction amplicons. The results shown in Table 1 reveal that PCR performed using DNA obtained with the BA protocol diluted at 10^{-2} and 10^{-3} as template, successfully produced amplicons for the 16S rDNA primers (90% and 80%, respectively) (Figure 2). However, the efficiency of amplicon production dropped to 25% when template DNA was diluted only by a factor of 10^{-1}. A similar effect was observed for the BA Mod protocol, in which the dilution factor of 10^{-3} of the template DNA increased the amplification efficiency to 90%. On the other hand, dilution of the DNA extracted with the PS Kit protocol below a 10^{-1} dilution factor reduced the PCR efficiency from 95% to 35% (10^{-2}) and zero (10^{-3}) (Table 1).

Considering the above results, template DNA was diluted using the best dilution factor obtained for each protocol, and 100% of amplicon production for the PCR of the 18S rDNA was obtained (Figure 3).

Table 1. Effect of the DNA extraction protocol and the dilution factor of the template DNA on the efficiency of the polymerase chain reaction (PCR). PCR was performed using the 16S rRNA primers 16SU1F1 (5'-ACTGCTTGCCTCCCGT-3´) and 16SR2 (5'-CTACCAGGGTATCTAACT-3´).

Protocol	Dilution factor	Polymerase Chain Reaction efficiency (%)
BA protocol	1/10	25.0 ab
	1/100	90.0 c
	1/1000	80.0 c
BA mod protocol	1/10	0.0 a
	1/100	10.0 a
	1/1000	95.0 c
Kit PS protocol	1/10	95.0 c
	1/100	35.0 b
	1/1000	0.0 a

Different letters indicate significant differences according to a Kruskal-Wallis test ($p<0.05$).

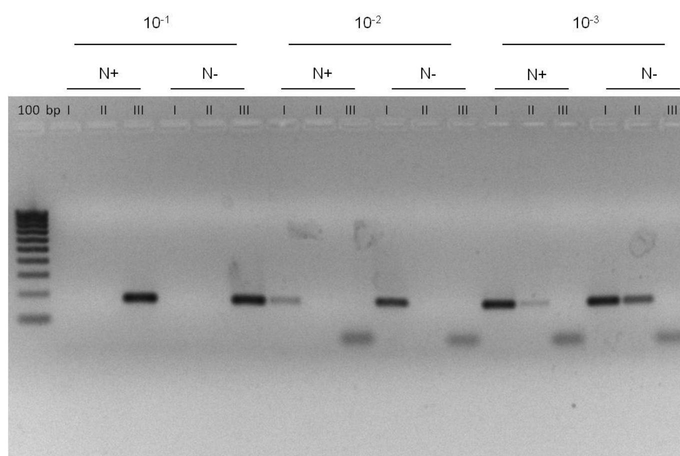

Figure 2. PCR products from rhizospheric DNA samples obtained with the different methods (I, BA protocol; II, BA mod protocol; III PS Kit protocol). PCR was performed using the primers 16S rRNA 16SU1F1 (5'-ACTGCTTGCCTCCCGT-3´) and 16SR2 (5'-CTACCAGGGTATCTAACT-3´), which amplify an internal region of the 16S rRNA from bacteria. N+, represents the use of liquid nitrogen during maceration of the samples; N-, represents the maceration of the samples without the use of liquid nitrogen.

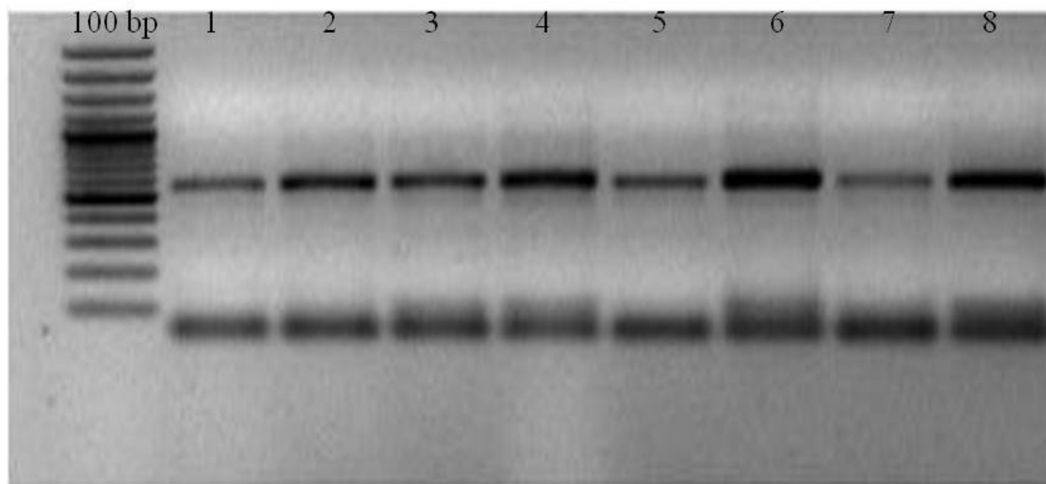

Figure 3. High efficiency of PCR reactions using a 10^{-2} dilution of the rhizospheric DNA samples obtained with the BA protocol as template. PCR was performed using the NS1 (5'-GTAGTCATATGCTTGTCTC) and NS2 (GGCTGCTGGCACCAGACTTGC) primers, which amplify an internal region of the 18S rRNA from fungi.

Discussion

The yield and quality of DNA are influenced by a number of factors that must be taken into consideration. The use of proteinase K at the end of the DNA extraction protocol has been demonstrated to produce high-quality DNA (Aras *et al.,* 2003). It has also been found that organic solvents, such as phenol and chloroform, can increase DNA yield and that the use of liquid nitrogen during the extraction can affect the DNA present both in the microbial community of the rhizosphere and already free in the soil (Sagova-Mareckova *et al.*, 2008).

Two of the tested protocols (BA and BA mod) showed an absorbance ratio for spectrophotometer readings at 260 nm/ 280 nm of 1.5, which is less than optimal for DNA samples (1.8), according to Sambrook *et al.* (1989). For DNA extraction protocols from soil samples based on the use of cetyl trimethyl ammonium bromide (CTAB) and polyvinylpolypyrrolidone (PVPP), the main problems of yield and quality of DNA have been associated with the failure to remove humic compounds, which are commonly present in the soil (Zhou *et al.,* 1996). Most likely, the rhizosphere environment of the Chilean humid forests sampled in this study harbors low molecular weight organic matter and humic acids, which may affect the efficiency of DNA extraction and its quality. The high content of organic matter, as well as iron oxide, produced DNA extracts with a dark color which have been noted to cause problems when DNA is used in PCR protocols (Thakuria *et al.*, 2008).

The PCR amplifications gave a suitable indicator of the quality of the DNA obtained by each protocol. It has been found that the enzyme Taq polymerase can be inhibited even when the source of DNA used as template is contaminated with less than 1 µg mL^{-1} of humic acid (Clegg *et al.*, 1997). In these cases, the dilution of the original samples is highly recommended prior to the preparation of the PCR mixes. Application of DNA dilution for the different protocols gave different results, but at least for the BA and BA mod protocols, the reduction of the pollutant concentration in each sample increased the PCR efficiency. In addition, the use of bovine serum albumin (BSA) in the reaction mix of the PCR is recommended to improve the amplification efficiency (La Montagne *et al.*, 2002), as this reagent reduces the inhibition of the Taq polymerase activity by organic and inorganic compounds (Mabru *et al.*, 2004).

It seems that DNA samples obtained by the BA mod protocol bring a higher amount of potential inhibitors, as PCR behaved better at higher dilutions, which is consistent with previous reports (Clegg *et al.*, 1997). On the other hand, the PS Kit produced a cleaner DNA sample, but yield was very low, which could be a disadvantage if larger studies involving the analysis of soil DNA have to be performed.

Finally, the use of the BA protocol for DNA isolation from the rhizospheric environment of Chilean hydrophilic forest appears to be the most suitable. The yield of DNA, as well as the 260 nm/280 nm absorbance ratio of the samples, is good enough to perform any genetic studies involving the soil DNA on a large scale. On the other hand, the use of liquid nitrogen is unnecessary as it did not give any worthwhile effects with this protocol.

Resumen

P. Cáceres, C. Cordero, G. González, K. Quiroz, J.C. Bobadilla, C. Bravo, P.D.S. Caligari, B. Carrasco y R. García-Gonzáles. 2012. Protocolos para la extracción eficiente de ADN microbiano a partir de la rizosfera del bosque hidrófilo en Chile. Cien. Inv. Agr. 39(3): 585-592. Se evaluó el uso de Buffer de lisis (Protocolo BA), Buffer de lisis modificado (Protocolo BA Mod) y el uso de kit comercial de extracción (Protocolo PS Kit) (Power Soil, Mo bio Laboratories, CA USA) y su efectividad para producir ADN de calidad biológica con rendimientos que permitan su uso en estudios de biodiversidad. De igual forma, se evaluó el efecto del nitrógeno líquido en el proceso de ruptura celular en todos los protocolos estudiados. Se obtuvieron rendimientos de ADN desde 12,4 hasta 9620 ng g^{-1} de suelo procesado utilizando los métodos basados en el buffer de lisis modificado y kit de extracción comercial. La calidad biológica del ADN se determinó mediante la habilidad del ADN para producir eficientes y reproducibles Reacciones en Cadena de la Polimerasa utilizando partidores del ADN ribosomal 16 S y 18 S de bacterias y hongos, respectivamente. Se obtuvo ADN de calidad biológica para PCR en todos los protocolos, pero la eficiencia del método dependió de la dilución del ADN previo al desarrollo de las PCRs. Los tres métodos de extracción generaron productos de PCR por encima del 90% de las muestras analizadas. Se observó que el factor de dilución del ADN afecta la eficiencia de la técnica de PCR en los tres protocolos evaluados pues para la extracción con Kit comercial, la mayor eficiencia en el PCR se obtuvo cuando la dilución 10^{-1} fue utilizada. El método basado en el buffer de lisis produjo la mayor eficiencia (90%) utilizando la dilución 10^{-2}. Por otro lado, el protocolo buffer de lisis modificado generó la más alta eficiencia utilizando la dilución 10^{-3} con un 95% de eficiencia. Los métodos evaluados permiten, por primera vez, la obtención eficiente y confiable de ADN de alta calidad a partir la rizósfera del bosque hidrófilo chileno.

Palabras clave: bosques húmedos, 16 S ribosomal, extracción ADN de suelo, PCR, rizósfera.

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