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SHORT COMMUNICATION

A feasible method to extract DNA from the cambium of high-canopy trees: from harvest to assessment

Érica MANGARAVITE[1,2], Vanessa TERRA[3], Eric Koiti Okiyama HATTORI[4], Thaís Carolina da Silva DAL'SASSO[1], Leonardo Lopes BHERING[5], Luiz Orlando de OLIVEIRA[1,*]

[1] Universidade Federal de Viçosa, Departamento de Bioquímica, Laboratório de Biologia Molecular e Filogeografia, Av. P. H. Rolfs, S/N, Campus Universitário, 36570-900 Viçosa, Minas Gerais, Brazil

[2] Centro Universitário UNIFAMINAS, Colegiado de Biomedicina, Av. Cristiano Ferreira Varella, 655, Bairro Universitário, 36888-233 Muriaé, Minas Gerais, Brazil

[3] Universidade Federal de Uberlândia, Instituto de Ciências Agrárias, Campus Monte Carmelo, Rodovia LMG 746, Km 01, s/n, Bloco 1A, sala 309, 38500-000 Monte Carmelo, Minas Gerais, Brazil

[4] Universidade Federal dos Vales do Jequitinhonha e Mucuri, Instituto de Ciências Agrárias, Campus de Unaí, Av. Vereador João Narciso, 1380, Bairro Cachoeira, 38610-000 Unaí, Minas Gerais, Brazil

[5] Universidade Federal de Viçosa, Departamento de Biologia Geral, Laboratório de Biometria, Av. P. H. Rolfs, s/n, Campus Universitário, 36570-900 Viçosa, Minas Gerais, Brazil

[*] Corresponding author: luiz.ufv@hotmail.com; https://orcid.org/0000-0002-5578-2260

ABSTRACT

Many tropical trees have high canopies and their leaves are not accessible. Thus, the use of tissue from a more accessible organ (cambium) for DNA extraction may be an alternative for molecular studies. We adapted a feasible methodology for extracting genomic DNA from cambium tissue harvested in the field for the assessment with PCR. We tested three storage conditions (two buffers and a silica gel) and four periods of time after harvest. We used previously described protocols and tested them on three species that occur in Amazonian forests and other biomes: *Anadenanthera peregrina* var. *peregrina*, *Cedrela fissilis*, and *Ceiba speciosa*. Our protocol obtained suitable PCR-grade genomic DNA for DNA sequencing and microsatellite genotyping. We recommend the use of silica for long-term storage and the buffer with ascorbic acid for short-term storage.

KEYWORDS: ascorbic acid, dithiothreitol, DNA isolation

Um método viável para extrair DNA do câmbio de árvores de dossel alto: da coleta à aplicação

RESUMO

Muitas árvores tropicais possuem dossel alto e folhas não facilmente acessíveis. O uso de tecido de um órgão mais acessível (câmbio) para extração de DNA pode ser uma alternativa para estudos moleculares. Nós adaptamos uma metodologia viável para extrair DNA genômico de tecido cambial coletado no campo para avaliação com PCR. Testamos três condições de armazenamento (dois tampões e sílica gel) e quatro períodos após a coleta. Utilizamos protocolos descritos anteriormente e os testamos em três espécies encontradas em florestas amazônicas e outros biomas: *Anadenanthera peregrina* var. *peregrina*, *Cedrela fissilis* e *Ceiba speciosa*. Nosso protocolo foi eficaz na obtenção de DNA adequado para sequenciamento e genotipagem de microssatélites. Recomendamos o uso de sílica para armazenamento de longo prazo e o tampão com ácido ascórbico para curto prazo.

PALAVRAS-CHAVE: ácido ascórbico; ditiotrietol; isolamento de DNA

Molecular data has been helpful in revealing hidden aspects of the evolutionary history of plants (e.g., Hughes *et al.* 2013). The number of biodiversity studies using plant molecular data has increased over the last years (Vinson *et al.* 2018). Most of the traditional methods for DNA extraction from plants were designed to use leaves as the source of genomic DNA (e.g., Doyle 1990; Cota-Sánchez *et al.* 2006). However, many tropical trees have high canopies and their leaves are not readily accessible. Moreover, the vegetation of seasonally dry forests usually releases its leaves during the driest period (Oliveira-Filho and Fontes 2000). The use of commercial kits allows for the quick extraction of genomic DNA from leaves but adds cost to the analysis.

The use of an effective and inexpensive protocol that uses cambium tissue may be an alternative when leaves are not readily available. There are few studies that have used cambium

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tissue as a source of genomic DNA, but most of them adopted a time-consuming method using a commercial kit to achieve DNA extraction (e.g., Asif and Cannon 2005; Colpaert *et al.* 2005; Novaes *et al.* 2009; Lanes *et al.* 2013). Herein, we aimed to adapt a feasible methodology for extracting genomic DNA from cambium tissue, from the field sampling to the assessment of the DNA with PCR.

We sampled cambium tissue from three tree species that are native to the Amazon region and other Brazilian biomes: *Anadenanthera peregrin*a *(L.) Speg.* var. *peregrina* (Fabaceae), *Cedrela fissilis Vell.* (Meliaceae) and *Ceiba speciosa* (A. St.-Hil.) Ravenna (Malvaceae). Three trees of each species were sampled in Viçosa, Minas Gerais, Brazil (20º45'S, 42º52'W). To collect a piece of cambium, we used a puncher (diameter = 1 cm; thickness = 0.2 cm; length = 16 cm) and hammered it into the tree trunk until it reached the wood fibrous layer. The extracted sample was cleaned with a piece of tissue paper and washed with 100% ethanol.

We collected three samples from each tree (nine samples per species). The first sample was kept in a dithiothreitol transport buffer ("DTT" treatment). The second sample was kept in an ascorbic acid transport buffer ("AA" treatment). Both samples were kept in 15-mL Falcon tubes wrapped in aluminum foil. The third sample was kept in an air-sealed plastic bag containing approximately 50 g of silica gel beads ("SIL" treatment). The DTT and AA buffers consisted of 2/3 absolute ethanol and 1/3 1× CTAB buffer (Colpaert *et al.* 2005). Instead of using DTT (or β-mercaptoethanol) and AA together in the transport buffer, as Colpaert *et al.* (2005) did, we tested them separately. For the DTT buffer, we added 3 mM dithiothreitol; to the AA buffer, we added 0.3% (w v^{-1}) ascorbic acid. We did not use β-mercaptoethanol in these buffers. The DTT and AA buffers were kept at 4 ºC and the SIL was stored at room temperature (approximately 25 ºC).

Another novelty of our study was to test whether the storage conditions decreased the overall quality of the genomic DNA over time. Extractions were performed after four storage periods: "D0" (extraction performed on the same day of harvest); and "D7", "D14", and "D21" (extraction performed 7, 14, and 21 days after harvest, respectively). Extraction from the SIL samples was performed only after 14 and 21 days to allow for tissue dehydration. Prior to DNA extraction, the buffer-stored samples (DTT and AA) were washed with distilled water, sliced, and dried with tissue paper. The SIL samples were only sliced. Approximately 40 to 70 mg of each sample was placed into 2.0 mL microtubes together with two 3.2-mm chrome-steel beads per tube. The samples were then homogenized with a Bead Beater system (Mini-Beadbeater-24, BioSpec, Paulínia, SP, Brazil). We performed the genomic DNA extraction with three replicates following an eight-step protocol, based on Cota-Sánchez *et al.* (2006) (modified by Riahi *et al.* 2010), which was here applied for the first time for cambium tissue. The eight steps were applied as follows:

1. We preheated the CTAB buffer (2% CTAB, 100 mM Tris-HCl, pH 7.5, 1.4 M NaCl, 20 mM EDTA, pH 8.0; 4% PVP; adding 1% β-mercaptoethanol immediately prior to use) to 65 °C;

2. 800 µL of the hot CTAB buffer were added to the 2.0 mL microtube with the samples and beads;

3. The samples were pulverized with a bead beater: 3 min at 2,500 oscillations per minute for the silica-dried samples, and 5 min at 3,000 oscillations per minute for the buffer-stored samples. This step was repeated if the grinding was not complete after the first round;

4. The tubes were incubated at 65 °C for 15 min with occasional swirling;

5. The samples were cooled on ice for 2 min and 750 µL CIA solution (chloroform:isoamyl alcohol; 24:1) were added to each tube followed by invertion 50 times. The tubes were centrifuged for 15 min at 10,000 rpm (Eppendorf centrifuge 5424) and the supernatant was transfered to a new microtube and the CIA step was repeated;

6. The supernatant was transferred to a new microtube, 0.7-volume ice-cold isopropanol was added and followed by gentle invertion 10 times and centrifugation for 15 min at 10,000 rpm. The supernatant was discarded without disturbing the pellet;

7. The pellet was washed by adding 1 mL 70% ethanol. The microtube was centrifuged for 10 min at 10,000 rpm and the ethanol was discarded. This step was repeated with 1 mL absolute ethanol. The pellets were then dried at room temperature, with care not to overdry them;

8. Each pellet was resuspended in 30 µL TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0), containing RNAse A (10 mg mL^{-1}). The DNA sample was incubated at 37 °C for 30 min and then stored at 4 °C.

DNA quantification and quality control were performed with a NanoDrop Spectrophotometer (Thermo Scientific). We next tested whether the genomic DNA was suitable for PCR. The genomic DNA was amplified (the internal transcribed spacer, ITS) with the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White *et al.* 1990) and ITS Leu (5'-GTCCACTGAACCTTATCATTTAG-3', Baum *et al.* 1998). For the chloroplast gene, we amplified the intron of *trn*L (UAA) (CD) with the primer pair C (5'-CGAAATCGGTAGACGCTACG-3') and D (5'-GTTTACTTTTGGGCATGCTTCG-3') (*trn*L intron region, Taberlet *et al.* 1991). The PCRs were performed with three replicates, and the amplification (ITS or CD) was considered positive when a single, sharp band of the size expected was visible on a 1% (w v^{-1}) agarose gel. We calculated the percentage of positive amplifications from each

extraction period (D0, D7, D14, and D21) and each storage condition (DTT, AA, and SIL), for each species. We also used the microsatellite primers Acol15, Acol16 (Feres *et al.* 2012), Ced54, and Ced65 (Hernández *et al.* 2008) in order to test the suitability of the DNA. The PCR results were checked on 2% (wv^{-1}) agarose gels. ANOVA and Tukey tests, implemented in RBio version 119 (Bhering 2017), were used to analyze the differences in the DNA concentrations and the percentages of positive PCRs among treatments within species.

The two storage conditions (DTT and AA) allowed for PCR amplifications that were not possible previously for frozen bark samples of *A. peregrina* (Novaes *et al.* 2009) (Table 1). The SIL storage at room temperature (approximately 25 ºC instead of 4 ºC for the buffer samples) exhibited some DNA degradation, which was likely due to the temperature difference. However, several positive PCR amplifications were obtained (Table 2). The SIL storage resulted in a higher amount of DNA compared to previous studies. For samples of *A. peregrina* var. *peregrina* stored in SIL, our methodology recovered genomic DNA (496 ng mL^{-1}) with a yield higher than previously reported (364 ng mL^{-1}, Novaes *et al.* 2009). During field expeditions, silica gel is more amenable to handling than liquid buffers. Additionally, the use of silica gel requires less storage space in the laboratory when considering the size of the plastic bags and the tubes in the refrigerator. Despite some DNA degradation, we recommend SIL for long-term storage.

DNA extraction after different storage periods of cambium tissue has not been previously reported. There was no significant difference in the genomic DNA yield or quality among the four storage periods and none significantly impaired the use of the samples for PCR. This suggests that cambium tissue can be harvested and kept for some weeks prior to DNA extraction, which is frequently convenient in fieldwork schedules. We showed that buffers with either DTT or AA were suitable for extracting the DNA right after sample harvest. Additionally, the AA buffer was easier to prepare and carry into the field, and then be added to the remaining buffer ingredients as needed. The SIL-stored tissues were difficult to process during the first weeks. Nonetheless, the cambium tissues preserved in SIL storage were amenable to PCR four weeks after harvest (data not shown).

Anadenanthera peregrina exhibited higher genomic DNA yield than *C. speciosa* (Table 1). The high standard deviations might be due to the different sizes of the source tissue samples. Genomic DNA storage in DTT or AA exhibited sharp bands of a high-molecular mass, with little to no smears, suggesting high integrity. The A_{260}/A_{280} ratio values presented little variation among species. Most of the samples exhibited a A_{260}/A_{280} ratio greater than 1.8 (Figure 1), suggesting high purity. As a consequence, amplifications via PCR were successful. All tree samples were amplified for the

Table 1. Concentrations of genomic DNA (ng μL^{-1}) extracted from cambium samples of three tree species under different treatments (Treat) of storage time (0, 7, 14, 21 days) and storage media (DTT = dithiothreitol transport buffer, AA = ascorbic acid transport buffer, SIL = silica gel beads) per species . Values are averages and standard deviations of three replicates.

Treat	*Anadenanthera peregrina*	*Cedrela fissilis*	*Ceiba speciosa*
D0	328.1 ± 202.9	379.1 ± 183.8	155.8 ± 76.7
D7	682.1 ± 848.2	353.8 ± 367.3	188.0 ± 79.3
D14	395.8 ± 276.8	294.2 ± 287.6	187.0 ± 180.3
D21	322.5 ± 292.1	213.0 ± 189.5	136.4 ± 130.0
AA	420.9 ± 633.4	269.9 ± 184.8	158.1 ± 120.4
DTT	374.8 ± 226.0	353.3 ± 284.0	182.1 ± 133.6
SIL	496.1 ± 405.6	247.3 ± 352.9	148.6 ± 151.2
Average	431.5	301.5	165.1

Table 2. Percentage of positive amplifications per region (CD and ITS) of DNA extracted from cambium samples of three tree species under different treatments (Treat) of storage time (0, 7, 14, 21 days) and storage media (DTT = dithiothreitol transport buffer, AA = ascorbic acid transport buffer, SIL = silica gel beads).

	Treat	*Anadenanthera peregrina*	*Cedrela fissilis*	*Ceiba speciosa*
CD	D0	100.0	94.4	55.6
	D7	88.9	83.3	61.1
	D14	96.3	100.0	85.2
	D21	100.0	92.6	100.0
	AA	91.7	91.7	75.0
	DTT	100.0	94.4	83.3
	SIL	100.0	94.4	77.8
ITS	D0	88.9	94.4	33.3
	D7	83.3	83.3	27.8
	D14	92.6	70.4	81.5
	D21	96.3	88.9	81.5
	AA	83.3	86.1	61.1
	DTT	97.2	86.1	55.6
	SIL	94.4	72.2	72.2
Average		93.8	88.0	67.9

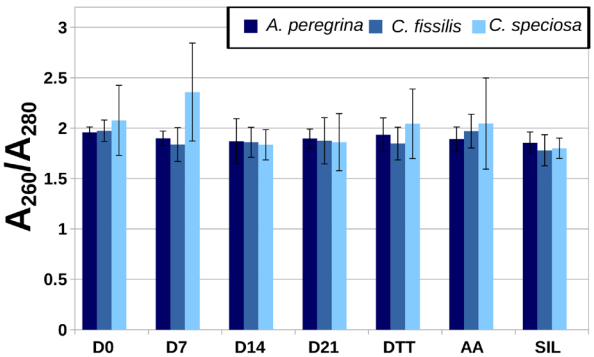

Figure 1. NanoDrop absorbances of the average A_{260}/A_{280} ratios for DNA extracted from cambium samples of three tree species under different treatments of storage time (0, 7, 14, 21 days) and storage media (DTT = dithiothreitol transport buffer, AA = ascorbic acid transport buffer, SIL = silica gel beads). The columns are averages of three replicates and the bar is the standard deviation. This figure is in color in the electronic version.

tested regions. *Anadenanthera peregrina* exhibited the highest percentage of positive amplifications (Table 2) for the CD and ITS regions, followed by *C. fissilis* and *C. speciosa*. Sharp bands characterized most of the amplifications. We checked the PCR products for microsatellites and the presence of bands indicated its suitability for genotyping.

To our knowledge, this is the first report on a multiplexing PCR system with microsatellites applied to cambium tissue DNA, with potential for application in studies of the genetic diversity of tree species. We recommend the use of silica for long-term storage and the ascorbic acid transport buffer for short-term storage.

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