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RESEARCH ARTICLE

PROTOCOL ADAPTATION FOR BRAZIL NUT (*Bertholletia excelsa* Bonpl.) DNA EXTRACTION

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ABSTRACT

Brazil nut (*Bertholletia excelsa* Bonpl.) is an Amazonian Forest species, which is acknowledged for the commercialization of its almonds. It is an endangered species, fact that reinforces the need of conducting conservation studies. Molecular markers are tools that may be used in conservation studies and the primordial stage preceding the use of molecular markers lies on DNA extraction. A well-designed protocol allows isolating DNA in sufficient amount and quality to conduct molecular studies. Thus, the aim of the current study is to adapt a total DNA extraction protocol to be applied to Brazil nut plants. Tests were conducted at different CTAB (2% and 4%) and β-Mercaptoethanol (0.2%, 0.8% and 1.4%) concentrations; washes using chloroform: isoamyl alcohol (CIA) were performed (one or two washes). The extraction products were confirmed in 0.8% agarose gel and quantified in spectrophotometer (in ng μL-1). Plant tissue (vascular cambium and leaf) was collected from 30 Brazil nut individuals in order to confirm the efficiency of the protocol adapted to the species. Samples were subjected to PCR amplification using 10 microsatellite markers. CTAB and β-Mercaptoethanol at concentrations 4% and 0.2%, respectively, along with one wash using CIA, showed the best results when the quality of the extracted material was assessed through its absorbance ratios. The vascular cambium oxidized more easily than the leaf during the DNA isolation process. Thus, it is essential taking some precautions at the time to handle it during collection and storage to assure process efficiency.

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INTRODUCTION

Bertholletia excelsa Bonpl., also known as Brazil nut, belongs to family Lecythidaceae and is an important Amazon region extractive exploitation species, whose seeds present high nutritional and commercial value. The income generated through the trading of Brazil nuts benefits many families; thus, extractivism is considered an Amazon-conservation strategic activity, because it combines economic forest use and biodiversity conservation, besides enabling the survival and settlement of the families (Wadt, 2008; Santana *et al.,* 2017). The Amazon region stands out for the diversity of plant species showing potential to be used in genetic breeding programs. However, the lack of knowledge, besides issues such as deforestation, agricultural frontier advancements and burnings lead to the risk of losing natural populations that have not yet been studied (Maués and Oliveira, 2010). Molecular markers are a way to study genetic diversity and support the strategic planning aimed at conserving these populations. Polymerase Chain Reaction (PCR) markers are available for the most diverse plant species (Caixeta *et al.,* 2013). Microsatellites, also called SSR (Simple Sequence Repeats) and STR (Short Tandem Repeats), stand out among these markers, because they are abundant and well-distributed in eukaryotes, highly polymorphic in repetitive regions, and result from mutagenic events (Borém and Fritche-Neto, 2013). Twenty-four (24) SSR molecular markers were already developed to be applied to the Brazil nut species; 12 were developed by Reis *et al.* (2009) and the other 12, by Sujii *et al.* (2013). The stage preceding studies on molecular markers, as well as other molecular biology studies, lies on DNA extraction. Silva and Coelho (2005)

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pointed out that it is essential developing specific, fast and robust DNA extraction protocols to be applied to studies involving a large number of individuals. Some DNA extraction process stages are important to assure material in sufficient amount and quality, for molecular analyses. Polysaccharides, phenols and secondary compounds may make the material unfeasible, since they affect Taq DNA polymerase enzyme efficiency (Silva and Coelho, 2005). Many DNA extraction protocols available in the literature are generalized and do not make species-specific assessments. Most protocols are based on the study by Doyle and Doyle (1987), who investigated DNA extraction from leaf tissue. However, each species has its own peculiarities; therefore, testing different reagent concentrations, as well as different reagents, may help improving extracted DNA quality and amount, less time spent in the process and, consequently, lower cost in the analyses. There is no Brazil nut-specific DNA extraction protocol assessing different reagent concentrations; thus, protocols based on the proposal by Doyle and Dolye (1987) have been used so far. Silva and Wadt (2008) just assessed the efficiency of tissue maceration in extraction processes using automated equipment. In addition, it is worth investigating the efficiency of the extraction from different plant tissues. Since Brazil nut trees may grow over 40 meters tall, collecting leaves from adult native specimens is not feasible; thus, the vascular cambium becomes the best alternative. Given the economic and environmental importance of Brazil nut trees for the Amazon region, and due to the need of conducting genetic breeding and conservation studies on the species, the aim of the current study was to adapt a DNA extraction protocol to be applied to Brazil nut species. The protocol was based on different plant materials, such as leaves and vascular cambium, in order to obtain quality DNA to be used in analyses involving molecular markers such as microsatellites, as well as in other molecular analyses.

MATERIALS AND METHODS

Plant material

Two plant tissues, leaf and vascular cambium, collected from a Brazil nut individual located at Embrapa Agrossilvipastoril, Sinop, MT, Brazil, were used in the DNA extraction protocol adaptation. Vascular cambium samples were collected with the aid of chisel and hammer. The material was placed in 2-mL microtubes and stored in transport buffer containing 1.14 mM ascorbic acid, 2% CTAB, 1% PVP, 0.2% β-Mercaptoethanol and absolute ethanol. Leaf samples in intermediate maturation stage were wrapped in aluminum foil in order to be transported and stored. The materials (vascular cambium and leaf) were transported in a Styrofoam box containing ice and stored in a freezer at -20°C to be used in the following day. The samples were used for DNA extraction through different protocols assessed in the current study. A test comprising 30 genotypes at different development stages was performed after the most appropriate DNA extraction protocol to be applied to the Brazil nut species was identified. Fifteen (15) genotypes derived from adult native forest trees, and the other 15 came from seedlings grown under greenhouse conditions. The vascular cambium was collected from native forest individuals, whereas leaves (maximum height: 15 cm; maximum age: 3 months) were collected from seedlings of greenhouse individuals. The collected tissues were stored as previously mentioned; however, the vascular cambium was stored in transport buffer for 1 year, whereas the leaves were stored for 1 month.

Assessed protocols

The basic protocol adopted in the current study was the CTAB method described by Doyle and Doyle (1987), who used 2% CTAB, 0.2% β-Mercaptoethanol and 1 wash with chloroform:isoamyl-alcohol (24:1) (CIA) for DNA extraction. Modifications were carried out in the original proposal and, then, tested to adapt the Doyle and Doyle protocol to DNA extraction from different Brazil nut tissues. The fore, 12 protocol proposals were developed based on the following combinations: 2 CTAB (2% and 4%), 3 β-Mercaptoethanol concentrations (0.2%, 0.8% and 1.4%) and different washes (1 and 2 washes) with chloroform:isoamyl-alcohol (24:1) (CIA); thus, totaling 12 samples for each plant tissue (leaf and vascular cambium) (Table 1).

Table 1. CTAB and β-Mercaptoethanol reagent concentrations and different CIA washes used to test species *Bertholletia excelsa*. Numbers from 1 to 12 refer to leaf samples; numbers from 13 to 24 refer to vascular cambium samples

% CTAB	% β-Mercaptoethanol	CIA washes	Samples
2	0.2	1	1 and 13
		2	2 and 14
	0.8	1	3 and 15
		2	4 and 16
	1.4	1	5 and 17
		2	6 and 18
4	0.2	1	7 and 19
		2	8 and 20
	0.8	1	9 and 21
		2	10 and 22
	1.4	1	11 and 23
		2	12 and 24

Sixty milligrams (60 mg) of leaf and vascular cambium tissue were weighed for each sample. The material was placed in 2-mL microtubes containing 2 tungsten beads (diameter: 3 mm) for future tissue maceration. Each tube was added with 700 µL extraction buffer (1M Tris-HCl, pH 8; 5M NaCl, 0.5M EDTA, pH 8; 1% PVP, 2% or 4% CTAB) preheated in water bath (65°C) containing different β-Mercaptoethanol concentrations. The samples were placed in TissueLyser® (Qiagen) and macerated for 1 minute at frequency 30Hz, according to Silva and Wadt (2008). The samples were kept in water bath at 65°C, for 30 minutes. Subsequently, they were removed from the water bath; each tube was added with 600 µl CIA (24:1) and homogenized for 1 minute in a vortex-type stirrer. The samples were centrifuged at 12,000 rpm for 15 minutes. Two (2) phases were identified at this stage in each tube. The supernatant (the upper phase) was transferred to a new microtube (± 500 µL per sample). The process was repeated, but with the addition of 600 µl CIA (24: 1), centrifugation and supernatant transfer to conduct the test with 2 CIA washes. After the deproteinization stage, 500 µl cold isopropanol was added to the microtube containing the supernatant. The tubes were carefully inverted and stored in a freezer (-20°C) for 3 hours. The samples were centrifuged at 12,000 rpm for 15 minutes after incubation. Pellet formation took place in all samples. Then, the supernatant was discarded. The pellet was washed 2 times in 300 µl of 70% ethanol and 1 time in absolute ethanol. The samples were centrifuged at 12,000 rpm for 3 minutes, after each wash. The pellet was left to dry at room temperature (± 15 minutes) in exhaust hood after the

ethanol was discarded. Finally, the samples were resuspended in 40 µL TE solution (Tris-EDTA) containing RNAse (20 mg mL^{-1}) and taken to water bath for 1 hour.

Quantifying the extracted DNA

Samples were subjected to the agarose gel electrophoresis technique (0.8%), and their concentrations (ng μL^{-1}) and the 260/280 absorbance ratio were quantified in the NanoDrop™ spectrophotometer (Thermo Scientific) in order to assess the quality and amount of the extracted DNA.

Microsatellite molecular markers

DNA extracted from the 24 samples tested in different protocols was subjected to amplification analysis through the PCR technique, using the BET 15 microsatellite molecular marker developed by Sujii *et al.* (2013) for application in the Brazil nut species. Tests comprising 30 different genotypes were performed after the most appropriate DNA extraction protocol for application in Brazil nut species was identify. The extracted DNA was subjected to PCR amplification using 10 microsatellite primers (BET 12, BET 14, BET 15, BET 16, BEX 02, BEX 09, BEX 22, BEX 27, BEX 33 and BEX 37) developed by Reis *et al.* (2009) and Sujii *et al.* (2013) for Brazil nuts. The reactions contained 3 µL DNA at concentration 3 ng μL^{-1}; the protocol for DNA amplification through PCR was used according to Cabral *et al.* (2017). The amplification program consisted of an initial denaturation cycle at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, at 56°C for 1 minute, at 72°C for 1 minute, and by a final extension cycle at 72°C for 7 minutes. The amplification product was confirmed through the 3% agarose gel electrophoresis technique, which was applied to the samples tested for protocol adaptation purposes. Capillary electrophoresis was performed in an ABI 3730 sequencer (Applied Biosystems) to confirm the amplification product from the samples referring to the 30 collected individuals.
Allele sizes were detected in base pairs using the GeneMapper 4.1® software (Applied Biosystems) and rounded in the Allelobin software (Idury and Cardon 1997).

RESULTS

All the 24 combinations assessed through different CTAB and β-Mercaptoethanol concentrations and CIA washes, as well as the 2 plant tissues used in the current study, allowed extracting DNA from *B. excelsa*, as shown in Figure 1. The extracted DNA was easily visualized in agarose gel and did not present smearing, i.e., it did not show signs of contamination (Figure 1). Absorbance ratios (260/280) ranged from 1.10 to 1.98 (Table 2). The DNA extracted from the vascular cambium was the one that presented values lower than 1.80. There was increase in the amount of DNA extracted from Brazil nut samples when the CTAB concentration increased from 2% to 4%. Although the increased β-Mercaptoethanol concentrations enabled extracting more DNA, it negatively affected the quality of its absorbance ratios. The use of 0.2% β-Mercaptoethanol allowed isolating DNA presenting enough quality (absorbance ratio) at sufficient amount, from both the leaf and the vascular cambium, to conduct a satisfactory analysis (Table 2). The analyses applied to CIA washes did not show significant differences between them (Table 2). The variables presenting greater influence were different CTAB and β-Mercaptoethanol concentrations.

The DNA extracted from the 24 samples tested at different reagent concentrations was subjected to PCR amplification through the BET 15 microsatellite molecular marker (Sujii *et al.,* 2013). The expected fragment amplification was recorded in all the tests.

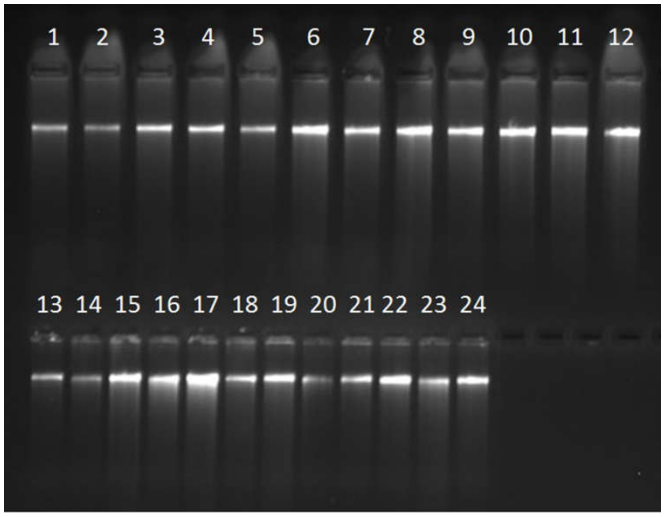

Fig. 1. Agarose gels (0.8%) containing *Bertholletia excelsa* DNA extracted in different tests. Numbers from 1 to 12 refer to leaf samples; numbers from 13 to 24 refer to vascular cambium samples

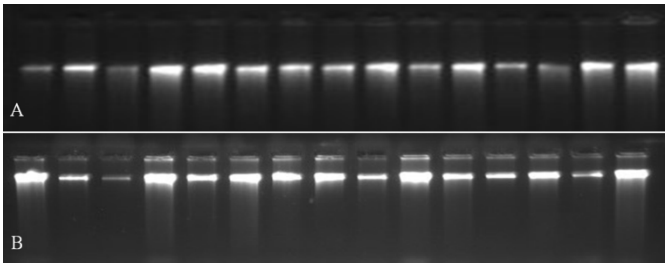

Fig. 2. Total DNA extraction from the vascular cambium of *Bertholletia excelsa* genotypes, collected in natural population (A) as well as from the leaf tissue, collected in greenhouse (B), according to the improved protocol. 1µL of total extracted DNA was used in the gel

Results showed that the best reagent combination to be used in Brazil nut DNA extraction was 4% CTAB, 0.2% β-Mercaptoethanol and 1 CIA wash. This combination will be referred to as "improved protocol" in order to facilitate the discussion presented below. The improved protocol was applied to 30 different genotypes collected from Brazil nut trees to confirm DNA extraction. Table 3 shows the DNA quantification results of 30 different individuals. Leaves were collected from 15 individuals - vascular cambia were collected from the other 15 - for DNA extraction purposes. The DNA extracted from the leaves presented good quality (260/280 absorbance ratio) and amount (Table 3; Figure 2). Despite the good DNA amount collected through the extraction performed in the vascular cambium, it was observed that the storage time affected the isolation of good-quality DNA, as it was checked through the 260/280 absorbance ratio (Table 3; Figure 2). The vascular cambium was stored for 1 year, and it may have affected the quality of the material. The DNA extracted from 30 Brazil nut genotypes was analyzed through 10 microsatellite molecular markers and all the used primers resulted in amplified fragments. The 10 herein used markers amplified 31 fragments, in total.

Table 2. Extracted DNA concentrations and respective absorbance ratios (260/280) according to CTAB, β-Mercaptoethanol concentrations and CIA washes, in *Bertholletia excelsa*. Numbers from 1 to 12 refer to leaf tissue (L), and numbers from 13 to 24 refer to vascular cambium (VC)

CTAB	β-Mercaptoethanol	CIA washes	Plant tissue	DNA ng μl^{-1}	260/280
2	0.2	1	1(L)	119.8	1.81
2	0.2	1	13(VC)	114.8	1.99
2	0.2	2	2(L)	122.6	1.93
2	0.2	2	14(VC)	104.3	1.8
2	0.8	1	3(L)	136	1.88
2	0.8	1	15(VC)	350.9	1.73
2	0.8	2	4(L)	121.1	1.96
2	0.8	2	16(VC)	164.6	1.18
2	1.4	1	5(L)	107.4	1.95
2	1.4	1	17(VC)	475.3	1.72
2	1.4	2	6(L)	201.1	1.94
2	1.4	2	18(VC)	224.4	1.1
4	0.2	1	7(L)	179.1	1.96
4	0.2	1	19(VC)	146.7	1.93
4	0.2	2	8(L)	246.8	1.8
4	0.2	2	20(VC)	108.5	1.81
4	0.8	1	9(L)	168.3	1.98
4	0.8	1	21(VC)	209.8	1.51
4	0.8	2	10(L)	147.7	1.83
4	0.8	2	22(VC)	321.3	1.7
4	1.4	1	11(L)	375.6	1.51
4	1.4	1	23(VC)	403.5	1.51
4	1.4	2	12(L)	511.5	1.37
4	1.4	2	24(VC)	565.6	1.7

Table 3. Absorbance ratio (260/280) and concentration of DNA extracted from the leaf and vascular cambium of 30 *Bertholletia excelsa* individuals, according to the improved protocol

Leaf			Vascular Cambium		
Samples	DNA ng μl^{-1}	260/280	Samples	DNA ng μl^{-1}	260/280
1	848.9	1.8	1	1218.4	0.98
2	264.6	1.85	2	1480.5	1.01
4	83.5	1.65	4	234.1	1.02
4	970.1	1.92	4	1371.4	1.04
5	865.6	1.92	5	6207.5	1.08
6	594.3	1.7	6	274.8	1.27
7	422.4	1.74	7	175.4	1.34
8	328	1.92	8	217.2	0.94
9	376.1	1.84	9	236.2	1.63
10	1139.9	1.87	10	1242.6	1.01
11	529.5	1.87	11	346.6	1.09
12	513.2	1.94	12	11539.1	1.08
13	471.9	1.87	13	660.4	1.07
14	1151.6	1.96	14	2585.7	1.02
15	1011.4	1.92	15	9905.8	1.09

DISCUSSION

The total DNA extraction is the stage preceding molecular analyses done for several purposes such as species conservation studies. Thus, particularities inherent to each species require defining the appropriate reactant concentrations during the process. The CTAB method proposed by Doyle and Doyle (1987) is one of the most successful techniques applied to different plant species for total DNA extraction. It is a detergent able to solubilize cell membranes and, along with the DNA, it forms a complex, which facilitates DNA precipitation (Weising *et al.,* 1995; Romano and Brasileiro, 1999). To adapt the Doyle and Doyle protocol for DNA extraction from different Brazil nut tissues, different CTAB and β-Mercaptoethanol concentrations and CIA washes were used to obtain DNA with quality and amount enough to enable different molecular analyses conducted after the extraction process. Absorbance ratios (260/280) ranging from 1.10 to 1.98 were recorded in the 24 samples (vascular cambium and leaf) tested at different reagent concentrations (Table 2). According to Sambrook *et al.* (1989), absorbance ratios above 1.8 indicate optimal DNA; values lower than 1.80 indicate material contaminated with proteins. Romano (1998) pointed out that values above 2.00 also indicate contamination, although with phenol. The plant tissue "vascular cambium" presented absorbance ratio (260/280) lower than 1.8 (Table 2), because it oxidized more easily than leaves. Some precautions may help reducing the oxidation process during material handling. The vascular cambium should be rapidly collected from the plant, as well as immediately placed in the transport buffer and kept at low temperature. After the material is removed from the transport buffer in the laboratory, it should be placed in extraction buffer as soon as possible. Danner *et al.* (2011) tested the DNA extraction protocol applied to Brazilian grape trees and recorded rapid vascular cambium oxidation when it was not correctly stored. Polyphenolic compounds oxidize when they bind to proteins and nucleic acids during the tissue homogenization phase in the DNA extraction process (Loomis, 1974; Porebski *et al.,* 1997). Neutralizing the action of these contaminants using antioxidants such as β-Mercaptoethanol, ascorbic acid, BSA and PVP (Polyvinylpyrrolidone) helps preventing quality loss issues in extracted DNA (Clark, 1997; Dehestani and Tabar, 2007). According to the assessments carried out in the current study,

increased β-Mercaptoethanol concentrations allowed extracting more DNA; however, by comparing the absorbance ratios, it was possible seeing that such increase negatively affected the quality of the DNA. There was increase in the amount of DNA extracted from the Brazil nut species when CTAB concentrations increased from 2% to 4%. Iglis *et al.* (2016) tested the protocol by Doyle and Doyle (1987) by applying 3% CTAB and 1% β-Mercaptoethanol to leaves and vascular cambia from different plant species (*Eucalyptus spp., Anacardium occidentale, Pereskia aculeata, Manihot esculenta, Arachis spp*.). They also conducted a prewash using sorbitol buffer. Results showed that CTAB concentration changes and the prewash using sorbitol provide good results in species contaminated with polysaccharides and polyphenols. Genetic studies about the Brazil nut tree (Coelho, 2013; Prado and Carvalho, 2009; Sujii *et al.,* 2013; Sujii *et al.,* 2015; Vieira, 2012; Wadt *et al.,* 2015) were based on the CTAB protocol described by Doyle and Doyle (1987) and Doyle and Doyle (1990), who used CTAB concentration 2%. The study by Serra *et al.* (2006) was based on the protocol described by Nienhuis *et al.* (1995), who also used 2% CTAB. Vieira (2014) also used the CTAB protocol described by Doyle and Doyle (1987), although with modifications in the CTAB (3%), β-Mercaptoethanol (3%) and PVP (2%) concentrations, as well as in storage time in water bath (5 minutes). The modification conducted in the protocol allowed extracting total DNA from 86 different Brazil nut genotypes collected in Alta Floresta, MT.

The most suitable concentrations at each of the herein tested reagents were 4% CTAB, 0.2% β-Mercaptoethanol and 1 CIA wash. This protocol was called "improved protocol" and it allowed isolating DNA from 30 genotypes of the herein investigated species. In addition to DNA isolation, the analyses using 10 microsatellite markers resulted in amplified fragments, and it confirmed the success of the improved DNA extraction protocol. It is worth highlighting that the quality of extracted DNA affects the efficiency of the Taq DNA polymerase enzyme when the used material is contaminated with polysaccharides, phenols and secondary compounds (Silva and Coelho, 2005). However, although the DNA extraction from the vascular cambium showed low absorbance ratios (Table 3), it was possible conducting DNA amplification through PCR by using all the markers applied to the 30 Brazil nut individuals. Since the vascular cambium oxidizes very easily, it is essential taking additional precautions not only in the collection, which should be done rapidly, but also in the material storage time. The vascular cambium used in the current study was stored for 1 year, and it may have affected the quality of the material. Extracting the DNA soon after the vascular cambium collection would be most appropriate for this plant tissue. These results may be checked by comparing the DNA extraction conducted to generate the improved protocol (Table 2), since the tests were carried out the day after the material was collected. Considering the foregoing, it is possible saying that the DNA extraction protocols applied to the Brazil nut were not specifically developed for the specie, and did not test different reagent concentrations, although they enabled DNA extraction. However, due to the cost of the molecular analyses conducted after the DNA extraction stage, it is necessary obtaining higher quality and yield in the extracted DNA to improve the process. Adapting the protocol may help reducing the laboratory time and the number of reagents used in the process; consequently, it may help reducing the analysis costs.

Conclusion

It was possible isolating DNA in all the herein analyzed tests. However, the best results for the amount and quality of extracted DNA were recorded at concentrations 4% CTAB, 0.2% β-Mercaptoethanol and 1 CIA wash.

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Conflict of interest

The authors declare that they have no conflict of interest.

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