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OPTIMIZED METHOD FOR DNA EXTRACTION AND PCR AMPLIFICATION IN AROEIRA TREE

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Abstract

Molecular markers are important tools in the characterization of plant genetic diversity and can provide support for conservation strategies for endangered populations. The different molecular techniques involve the evaluation of many individuals; therefore, it is crucial to have fast, efficient, and inexpensive methods for DNA extraction. Given the importance of the Aroeira (*Myracrodruon urundeuva* Fr. All.) it is pertinent to optimize a protocol that allows the obtainment of intact and pure DNA, aiming to assist conservation strategies for this species that is threatened with extinction. Thus, this study aimed to compare five DNA extraction methods: Dellaporta et al. (1983), Doyle and Doyle (1987) modified, Ferreira and Grattapaglia (1995), Romano and Brasileiro (2015), and Khanuja et al. (1999) and optimize the most efficient protocol for *M. urundeuva*. The modified DNA extraction protocol proposed by Doyle and Doyle (1987), using 100 mg of leaf tissue and 6 µl of β-mercaptoethanol was the protocol that presented the sharpest bands after DNA electrophoresis and after the reactions of amplification employing Polymerase Chain Reaction (PCR). Therefore, it is suggested to use the protocol described by Doyle and Doyle (1987) modified for the extraction of DNA from young *M. urundeuva* leaves to carry out techniques involving molecular markers.

Keywords: CTAB. DNA isolation. *Myracrodruon urundeuva.*

1. Introduction

The Aroeira (*Myracrodruon urundeuva* Fr. All.) is a tree belonging to the Anacardiaceae family, which occurs naturally from Brazil (Ceará) to Argentina and Paraguay, being found in vegetation formations of Caatinga, Cerrado, and rain forests (Lorenzi and Matos 2002). It has pharmacological potential because the extracts from its leaves have antioxidant and antimicrobial activity. Its bark has a large amount of tannins with anti-inflammatory, astringent, anti-allergic, and healing properties (Souza et al. 2007). Due to its economic importance, *M. urundeuva* had an exacerbated extractive exploitation in

Brazil. It is considered a vulnerable species in the official list of species of the Brazilian flora threatened with extinction. Thus, natural populations of *M. urundeuva* are threatened, with the risk of genetic erosion occurring.

Knowledge of genetic diversity is essential in genetic conservation programs, and genetic diversity can be accessed through molecular markers, which reveal DNA polymorphism and do not suffer environmental influence (Ferreira and Grattapaglia 1995). The efficient isolation of plant DNA allows the realization of DNA amplification, digestion, and cloning reactions, enabling the analysis of the plant genome structure. Molecular data enable the characterization of genetic diversity and are useful for programs for the conservation of genetic resources (Romano and Brasileiro 2015).

There are commercial kits that are a fast and efficient alternative for DNA extraction in plant species, but they present a higher cost when compared to conventional extraction protocols (Sousa et al. 2014). The large number of conventional DNA extraction protocols is explained by the need to select the most appropriate protocol for the biochemical composition of the species that will be analyzed in molecular studies. Later, in many cases, it is necessary to optimize the DNA isolation method (Tiwari et al. 2012). Both methodologies may not present satisfactory results, due to the possibility of DNA interaction with molecules of the plant cells. However, in the utilization of conventional protocols of extraction, there is the possibility of optimization to minimize the complex formation of DNA with these compounds, besides being a more economical method when compared to commercial kits (Raimundo et al. 2018). Thus, there are a great diversity of protocols for the extraction of genomic DNA that were efficient for vegetable species (Viana et al. 2015; Almeida et al. 2017; Furtado Filho et al. 2021; Silva et al. 2021).

In the specific case of *M. urundeuva*, there are aromatic substances in its leaves, which can make it difficult to obtain pure DNA. In aromatic plants, there are reports of problems in the isolation of DNA due to the presence of essential oils, with the need for modifications in the protocols (Khanuja et al. 1999; Sousa et al. 2022). Therefore, the present study aimed to identify, among five DNA extraction protocols, Dellaporta et al. (1983), Doyle and Doyle (1987), Ferreira and Grattapaglia (1995), Romano and Brasileiro (2015), and Khanuja et al. (1999), the most efficient for *M. urundeuva* concerning the quantity and quality of the genetic material and to carry out the necessary modifications for the effective isolation of the DNA. The results obtained here are important for future molecular studies to characterize the genetic diversity in Aroeira and may help to study the conservation of this endangered species.

2. Material and Methods

Samples of young leaves of *M. urundeuva* were collected at the Center for Agricultural Sciences of the Federal University of Piauí (UFPI), transported in a Styrofoam box with ice, and storing them at a temperature of -20 ºC, until the start of DNA extractions. In general, the use of young leaves is recommended, as DNA with greater purity can be obtained due to the low concentrations of phenolic compounds in young leaves (Azêvedo et al. 2019).

Five DNA extraction methods were tested: Dellaporta et al. (1983), Doyle and Doyle (1987), Ferreira and Grattapaglia (1995), Romano and Brasileiro (2015), and Khanuja et al. (1999). Among these protocols, the one proposed by Doyle and Doyle (1987) showed the best result. After that, some modifications were made to the method described by Doyle and Doyle (1987) to obtain DNA with a minimum of contamination. Modifications were made in the amounts of leaf tissue and the volume of β-mercaptoethanol used, as follows: 1) 100 mg of leaf tissue and 6 µl of β-mercaptoethanol; 2) 100 mg of leaf tissue and 8 µl of β-mercaptoethanol; 3) 170 mg of leaf tissue and 6 µl of β-mercaptoethanol. After DNA extraction, the DNA pellet was diluted in 100 µL of Tris-Ethylenediaminetetraacetic acid (EDTA) buffer solution, TE buffer (1 mM EDTA, 10 mM TrisHCl, pH 8.0).

The extracted DNA samples were quantified on a 0.8% agarose gel, with 0.5X Tris-Borate-EDTA (TBE), using 5 µL of each sample and the unfragmented DNA marker (λ uncut DNA) at a concentration of 100 ng/µL and separated by electrophoresis conducted at 90 V for 1.5 hours. The gel was stained with ethidium bromide and photographed under ultraviolet light. The DNA samples that generated the sharpest bands were selected for amplification utilizing PCR, to assess the quality of the extracted DNA. The genomic DNA regions obtained through the extraction were amplified using a thermocycler Veriti™ 96

Well Thermal Cycler (Applied Biosystems) programmed for the following conditions: 60 seconds at 94 ºC for initial denaturation, followed by 40 repetitions of a cycle of 45 seconds at 94 °C and 45 seconds for annealing, extension at 72 °C for 120 seconds, a final step at 72 °C for 6 minutes and then stored at 4 °C. Amplification reactions contained: 0.1 µl of Taq polymerase (Quatro G Biotecnologia):1 unit/µl, 1.0 µl of amplification buffer (100 Mm Tris HCL, pH 8.4; 500 Mm KCL, pH 8.5), 1.0 µl of a dNTPs solution (0.8 mM), 0.5 µl of UBC 812 Inter-simple sequence repeats (ISSR) primer (5'-3' GAG AGA GAG AGA GAG AA) and 6.5 µl of ultrapure water. The amplification products were separated on a 1.5% agarose gel with 0.5X TBE buffer at 80 V for 3h. The gels were stained with GelRedTM 10.000X (Uniscience), visualized under ultraviolet light, and photographed.

3. Results

The DNA samples analyzed on 0.8% agarose gel electrophoresis indicated that the protocols of Doyle and Doyle (1987) modified, Ferreira and Grattapaglia (1995) and Khanuja et al. (1999) obtained better amounts of DNA, observed through well-defined bands indicating satisfactory amounts of DNA (Figure 1).

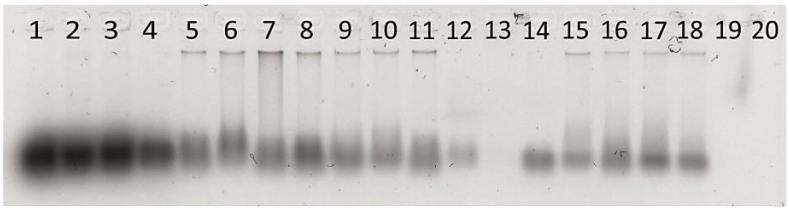

Figure 1. 0.8% agarose gel electrophoresis in samples of *M. urundeuva*, representing the protocols: Dellaporta et al. (1983) - 1 to 4; Khanuja et al. (1999) - 5 to 8; Doyle and Doyle (1987) modified - 9 to 12; Romano and Brasileiro (1999) - 13 to 16; Ferreira and Grattapaglia (1998) - 17 to 20.

Spectrophotometry was performed on a spectrophotometer (NanoDrop® 2000-2000C), to indicate the purity of the extracted DNA. The results were analyzed by absorbance between DNA (360 nm), proteins (280 nm), and polysaccharides (230 nm). DNA is considered of low quality when the 260/280 index is less than 1.6 and excellent when it is greater than 1.8. For the 260/230 index, the value below 1.6 is considered of low quality, and excellent when it is greater than 1.9. The protocols Romano and Brasileiro (2015) and Dellaporta et al. (1983) showed A260/280 indexes of 1.3, indicating protein contamination. The A260/230 index was also below the standard 0.84 and 0.94, respectively. Despite the protocol by Dellaporta et al. (1983) presenting a high concentration of DNA (384.3 ng/µL), there were no good results in relation to the concentrations of A260/280 and A260/230, that is, the DNA was contaminated, therefore, both protocols are not suitable for DNA extraction in *M. urundeuva* (Table 1).

Table 1. Spectrophotometry concentrations by Nanodrop® 2000-2000c for the protocols tested.

Protocols	Concentration ng/µL	A260/280	A260/230
Dellaporta et al. (1983)	384,3	1,3	0,84
Khanuja et al. (1999)	346,9	1,8	2,7
Doyle e Doyle (1987) modificado	361,4	1,9	1,7
Romano e Brasileiro (1999)	147,9	1,3	0,94
Ferreira e Grattapaglia (1998)	133,0	1,8	4,2

Samples from the protocol by Khanuja et al. (1999) showed DNA concentrations of 346.9 ng/µL, DNA and protein ratio (A260/280) of 1.8, considered good, and DNA and secondary compounds ratio (A260/230) of 2.7. considered good (Table 1). The protocol by Ferreira and Grattapaglia (1995) presented a DNA concentration of 133 ng/µL. Despite being the lowest concentration obtained among the other protocols, the data suggest pure DNA, with an A20/280 ratio of 1.8 and A260/230 of 4.2.

The modified Doyle and Doyle (1987) protocol showed the best results using Nanodrop among the five protocols evaluated, showing good results in electrophoresis and spectrophotometry, with a DNA concentration of 361.4 ng/µL, A260/280 of 1.9, indicating a high degree of purity and an A260/230 ratio of

1.7, considered reasonable (Table 1). Therefore, the modified Doyle and Doyle (1987) protocol was the most efficient for DNA extraction in Aroeira.

The modified DNA extraction protocol proposed by Doyle and Doyle (1987) was the only one that showed clear bands after DNA electrophoresis. Subsequently, this protocol was optimized with modifications in the amounts of leaf tissue and β-mercaptoethanol, aiming at greater efficiency in the isolation of DNA free of impurities and in sufficient quantity for molecular studies. The samples extracted using the modified Doyle and Doyle (1987) protocol, D1 and D2, contained smaller amounts of leaf tissue and showed the best results when observed in the 0.8% agarose gel (Figure 2). The mean DNA concentrations in these two samples were 7 and 6 ng/µL, respectively. Samples with the highest amount of leaf tissue, D3, did not show visible bands, possibly because the leaf material was not completely submerged by the extraction buffer, resulting in a low amount of extracted DNA, with an average concentration of 0.25 ng/ µL, not allowing the visualization of bands on the gel. Regarding the amount of β-mercaptoethanol used, there were no significant changes in the presence or intensity of the DNA bands, suggesting the use of the lowest tested amount of β-mercaptoethanol (6 µl).

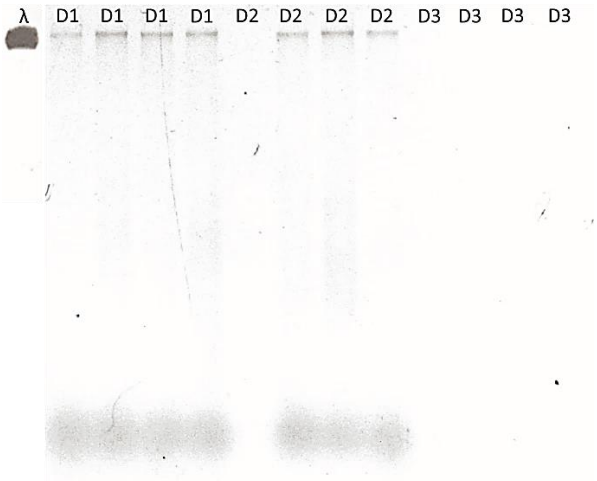

Figure 2. Electrophoretic profile of DNA marker (λ uncut DNA) and DNA extracted from young leaves of *M. urundeuva* in 0.8% agarose gel using the modified Doyle and Doyle (1987) protocol, with altered amounts of leaf tissue and β-mercaptoethanol. D1= 100mg of leaf tissue and 6µl of β-mercaptoethanol; D2= 100mg of leaf tissue and 8µl of β-mercaptoethanol; D3= 170mg of leaf tissue and 6µl of β-mercaptoethanol.

The amplification reactions were conducted in order to prove the efficiency of the extraction protocols regarding the quality and quantity of the genomic material. The bands from samples D1 and D2, extracted according to the modified Doyle and Doyle (1987) protocol, were visible and amplified with the ISSR UBC 812 primer after PCR, indicating the presence of intact and good-quality DNA (Figure 3).

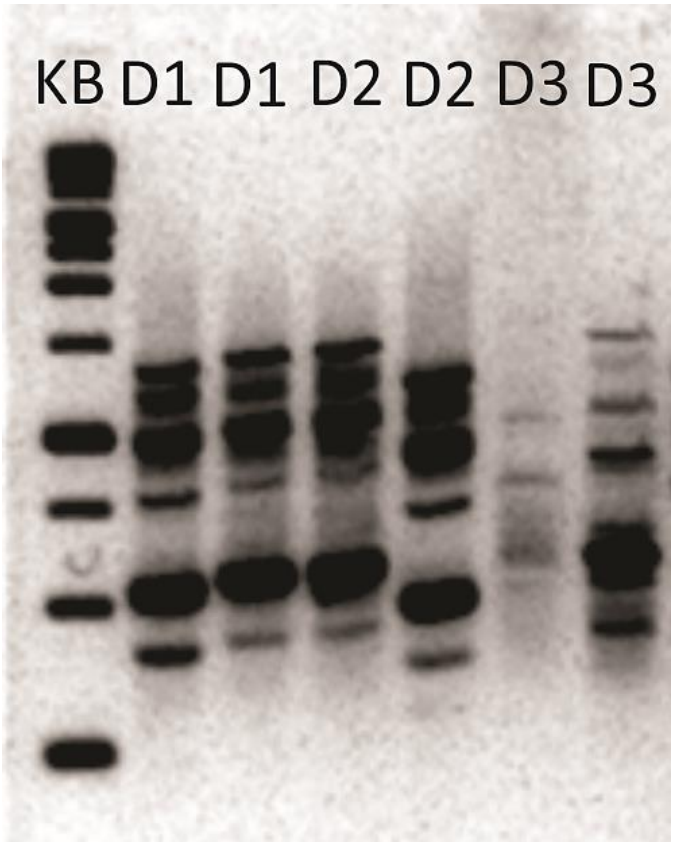

Figure 3. Electrophoretic profile of PCR products using the ISSR *primer* UBC 812 in samples of *M. urundeuva, using* the modified Doyle and Doyle (1987) protocol, with altered amounts of leaf tissue and β-mercaptoethanol, respectively: sample D1 (100mg of leaf tissue and 6µl of β-mercaptoethanol); sample D2 (100mg of leaf tissue and 8µl of β-mercaptoethanol); sample D3 (170mg of leaf tissue and 6µl of β-mercaptoethanol).

4. Discussion

Different molecular techniques are important in the assessment of genetic diversity and help in genetic conservation studies (Palmieri et al. 2010; Viana et al. 2016; Gomes et al. 2020; Bhandari et al. 2021; Eghlima et al. 2021; Gomes et al. 2021). There is a wide utilization of commercial DNA extraction kits, which could not work well in many species of plants. This is due to the great heterogeneity existing in the chemical composition of the vegetable cells (Ferreira and Grattapaglia 1995; Raimundo et al. 2018). For these species, it is fundamental the modification and optimization of the protocols of DNA extraction with adjustments of the concentrations of reagents, lysis buffers, and precipitation buffers, for obtaining DNA with minimal contamination.

The variation observed in the efficiency of the five DNA extraction protocols tested, as well as in the use of different amounts of leaf tissue and concentrations of β-mercaptoethanol, is probably related to the variability present in the biochemical composition of *M. urundeuva* (Sousa et al. 2022). In addition, the evaluated methods have specific concentrations for each reagent, but the detergents used in plant DNA extraction protocols in the literature are generally cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS). Among the protocols evaluated in this study, only the method proposed by Dellaporta et al. (1983) does not use CTAB in the DNA extraction buffer. Therefore, we suggest using CTAB based methods are more suitable for DNA extraction from young *M. urundeuva* leaves. DNA precipitation is facilitated because CTAB is positively charged, and DNA is negatively charged (Stefanova et al. 2014; Xia et al. 2019).

In the present work, the modified Doyle and Doyle (1987) protocol, using 100 mg of leaf tissue and 6 µL of β-mercaptoethanol, presented a better cost-benefit ratio, that is, a fast and inexpensive protocol that provides DNA of good quality and in sufficient quantity to carry out amplification reactions using PCR. According to Castro et al. (2020), aroeira leaves extract presents high concentrations of tannins (polyphenols). Based on this, the efficiency of the Doyle and Doyle (1987) method for DNA isolation in *M.*

urundeuva was expected, since this method is often used for DNA extraction from plant cells with high levels of polyphenols (Silva et al. 2010; Leza et al. 2017).

5. Conclusions

The use of the modified protocol described by Doyle and Doyle (1987) is suggested, with the use of 100 mg of leaf tissue and 6µl of β-mercaptoethanol, for the extraction of DNA from *M. urundeuva* leaves, as they generate visible bands after DNA amplification reactions through PCR.

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