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Improved methods for storing and extracting DNA from *Ilex paraguariensis* (Aquifoliaceae) tissue samples

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ABSTRACT. Mate (*Ilex paraguariensis*) leaves are popular for consumption as an infusion, which provides various health benefits due to its nutraceutical properties. Leaf samples oxidize after harvesting, requiring special handling to avoid DNA damage or degradation by enzymatic or oxidative activities. The objectives of this work were to evaluate several methods for sample storage and DNA extraction to identify practical and efficient protocols to guarantee the DNA quantity and integrity for molecular studies of mate. Total DNA was extracted from fresh leaves and compared with DNA extracted from leaves stored in silica gel at room temperature for 14 days or in CTAB (cetyltrimethylammonium bromide) buffer. The leaves were cleaned with absorbent paper and stored in 50 mL Falcon tubes containing approximately 25 g of silica gel or in 2 mL Eppendorf tubes containing approximately 1 mL of CTAB buffer. Samples treated with silica gel were stored at room temperature for 14 days, and the ones with CTAB buffer were stored either at 4°C for 14 and 90 days or at room temperature for 90 days. The DNA was quantified using a Nanodrop spectrophotometer and agarose gel electrophoresis. DNA purity (with regard to the presence of enzyme inhibitors) was tested by PCR amplification of fragments of the plastid gene, *trnL-trnF*. Samples of

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mate leaves can be stored on silica gel or in CTAB buffer for up to 90 days at room temperature without reduction in DNA quality. Samples stored in CTAB buffer can be refrigerated at 4ºC to minimize oxidation of phenolic compounds. The improved methods for sample storage and DNA extraction with CTAB maintain quantity and integrity for conducting molecular studies of mate.

Key words: Mate; CTAB; Silica gel; DNA; Molecular studies

INTRODUCTION

Ilex paraguariensis (Aquifoliaceae), popularly known as mate, is an arboreal species native to southern Brazil, Argentina and Paraguay (Heck and Mejia, 2007). In addition to various industrial applications, mate is especially appreciated in the form of tea, chimarrão and tererê. The leaves and stems present high levels of phenolic compounds that once infused, present diuretic, digestive, and stimulant properties (Sturion and Resende, 2010). Mate is therefore particularly important among infusions and teas produced by countries in South America, with more than 600,000 tons of leaves produced in Brazil alone annually (FAO, 2013).

Owing to advancements in DNA technology, considerable efforts have been made to characterize species like mate through studies in population genetics, phylogenetics, phylogeography, ecology, and conservation genetics (Holsinger, 2010; Carrió and Rosselló, 2014). Studies developed by Debat et al. (2014) analyzed the transcriptome and presented an estimation of 32,355 genes and 12,551 genetic variants or isoforms present in the genome of *I. paraguariensis*, an important basic information for the discovery of biologically and agronomically important characteristics.

Molecular studies provide critical information to formulate conservation strategies, improve selection for the species, genetic improvement, and management of the plants. However, a prerequisite for conducting molecular studies is obtaining DNA in adequate quantity and quality, since these are restrictive factors for successful polymerase chain reaction (PCR) (Agbagwa et al., 2012). The quality of DNA is directly dependent on the condition of tissues and methods of collection, extraction, and purification.

An ideal procedure for genomic DNA extraction from plants should be simple, fast, and inexpensive, and should be able to obtain intact DNA from small amounts of tissue with adequate quantity and purity (Agbagwa et al., 2012). Extracted DNA from fresh tissue is ideal for molecular studies (Ferreira and Grattapaglia, 1998). However, fresh samples are not always readily available; therefore, optimized methods to avoid damage or degradation of nucleic acids by enzymatic or oxidative activity are necessary (Prendini et al., 2002). Studies focused on preservation and storage methods of plant tissue samples are currently scarce for native species(Nagy, 2010). However, many methods have been tested and optimized by several research institutions and laboratories, but have not been published (Nagy, 2010).

Preservation of plant tissue samples by irreversible denaturation of enzymes can be achieved by physical and chemical treatments (Nagy, 2010). A commonly used physical method for sample storage is rapid dehydration with silica gel to inhibit enzymatic activity (Funk et al., 2017; Gostel et al., 2016). Unfortunately, preservation of a completely dried sample is not always possible. Alternatively, chaotropic agents and extraction buffers, such as CTAB (cetyltrimethylammonium bromide) buffer, can be used to stabilize enzymatic activity (Nagy, 2010). Storage of leaves in CTAB buffer saturated in NaCl has proven to be effective (Rogstad, 1992), and has also been tested for conservation of fungal spores (Huang et al., 2016a).

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DNA from mate leaves has successfully been extracted using kits (Cascales et al., 2014; Gottlieb and Poggio, 2014) and protocols using CTAB buffer (Vidor et al., 2002a; Vidor et al., 2002b; Wendt et al., 2007; Friedrich et al., 2017). In both techniques, fresh samples (Friedrich et al., 2017), samples dried with silica gel (Cascales et al., 2014; Gottlieb and Poggio, 2014) or frozen samples kept at -80ºC can used (Wendt et al., 2007; Kubiak et al., 2010). However, the lack of detail in these protocols impedes optimization and reproducibility for molecular DNA analysis. Therefore, we evaluated several methods for sample storage and DNA extraction to identify practical and efficient protocols to guarantee DNA quantity and integrity for molecular studies of mate.

MATERIAL AND METHODS

The protocol for total DNA extraction was adapted from Doyle and Doyle (1987): 2% hexadecyltrimethylammonium bromide; 100 mM of Tris-HCl pH 8.0; 1.4 M NaCl; 20 M ethylene-diamine-tetra-acetic acid (EDTA). The lysis buffer was modified with the addition of 1g of polyvinyl pyrrolidone (PVP) and an increase of the concentration of β-mercaptoethanol from 0.2 to 2%. These changes were made because the increase of antioxidant substances favors the DNA extraction of mate leaves (Wendt et al., 2007).

Collection of samples and methods for storage

Young branch leaves were collected in September 2017 from 51 adult mate plants. The leaf samples were of different sizes depending on the vegetative growth of the sampled plant. The different sizes of the collected leaves, representative of all analyzed samples, are presented in Figure 1. The leaves were cleaned with absorbent paper and stored in 50-mL Falcon tubes containing approximately 25 g of silica gel or in 2-mL Eppendorf tubes containing approximately 1 mL of CTAB buffer. Samples were stored in silica gel at room temperature for 14 days, in CTAB buffer at 4°C for 14 or 90 days, or at room temperature for 90 days. DNA was also immediately extracted from fresh mate leaves.

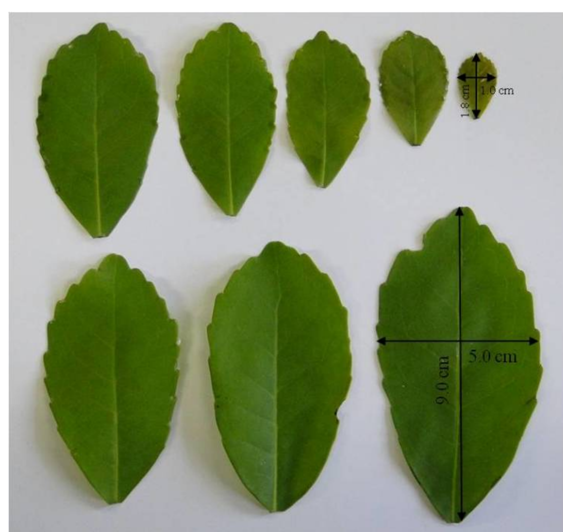

Figure 1. Different sizes of sampled mate leaves highlighting the variation in length and width of the leaves used.

DNA extraction and analysis protocol

Initially, the mate leaves were weighed and macerated in liquid nitrogen and transferred to 2-mL Eppendorf tubes containing 650 µL of modified CTAB buffer and 13 µL of β-mercaptoethanol (2%) that was previously heated to 65°C. The tubes were incubated in a water bath at 65°C for 60 min. The tubes were cooled to room temperature and 650 µL of chloroform-isoamylalcohol (24:1) was added, homogenized for 1 min on a shaker (Tornado Agitator, Arsec TS 200), and centrifuged (Centrifuge 5410 Eppendorf) at 12,000 rpm for 5 min.

Refrigerated isopropanol was added to the supernatant in a 1:1 ratio and the tubes were kept for 24 h at approximately -18°C. After this period, the tubes were centrifuged for 5 min and the supernatant was discarded to dry the pellet at room temperature. After drying, the pellet containing the nucleic acids was washed for 20 min in 500 µL of washing solution composed of 23.8% water, 76% absolute ethanol and 0.2% 5M ammonium acetate. After discarding washing solution, DNA was resuspended in 100 µL Tris-EDTA buffer (TE), and precipitated in 50 µL 5 M ammonium acetate and 370 µL absolute ethanol. Again, the sample was centrifuged and the supernatant was discarded and completely dried. The pellet was finally resuspended in 100 µL deionized water and kept in a refrigerator for 24 h. After this period, the samples were incubated for 60 min at 37°C with 10 $\mu g.mL^{-1}$ RNase and subsequently stored at -4°C.

The concentration of DNA in each of the samples was quantified by a spectrophotometer (Nanodrop 2000, Thermo Scientific) and the quality of the samples was evaluated by PCR and agarose gel electrophoresis. To analyze DNA quality, five samples of mate leaves were taken at random from each of the storage. To test quality of the total DNA, 1 µL of each sample was run on a 1% agarose gel stained with GelRed®. DNA quality was also tested with PCR with a universal primer pair for a non-coding region of the plastid genome, *trnL-trnF* (primer C - CGAAATCGGTAGACGCTACG and primer F - ATI'TGAACTGGTGACACGAG) (Taberlet et al., 1991). The DNA samples were diluted to 25 $ng.\mu L^{-1}$ and the procedures for PCR were adapted from Williams et al. (1990). PCR mix contained 25 ng DNA, 1X PCR Reaction Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.6 mM $MgCl_2$, 0.12 mM dNTP mix, 0.16 µM of each primer, 1 U Taq DNA polymerase (Platinum, Invitrogen™) with sterile ultrapure water to a final volume of 25 µL. Amplification conditions consisted of initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C, 55ºC for 1 min and 72°C for 2 min, and then a final extension at 72°C for 3 min.

PCR amplifications were loaded onto 2% agarose gels prepared in 1X Tris-borate-EDTA buffer (TBE) pH 8.0, stained with GelRed®, and electrophoresis was performed for approximately 2 h at 60 V. The gels were visualized with an ultraviolet transilluminator and recorded with a photodocumentation system (EDAS 290 - Kodak).

RESULTS AND DISCUSSION

Regardless of the extraction methodology and plant species used, high-quality DNA samples are necessary for molecular studies. Many species produce natural substances that can interfere with DNA isolation and purification. These substances include polyphenols, polysaccharides, and other secondary metabolites, which exhibit intraspecific variations

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(Silva, 2010; Agbagwa et al., 2012; Corrêa et al., 2013). Among these substances, known as contaminants, the polysaccharides were observed in 6% of the evaluated mate leaves samples (in three plants), giving the final product a viscous and unusual appearance in PCR. Even though they are present in only a small portion of the samples, the level of polysaccharides in foliar tissue of some mate plants was enough to impede DNA isolation, and optimization was necessary to maximize extraction efficiency. The presence of polysaccharides can interfere with the yield and quality of the DNA extracted from the samples (Deshmukh et al., 2007), as well as inhibit the activity of various enzymes used to manipulate DNA, such as Taq DNA polymerase (Sharma et al., 2002) and the restriction enzymes HindIII and EcoRI/MseI (Schlink and Reski, 2002). It is possible that higher concentrations of CTAB buffer (above 2%) could improve extraction, by eliminating polysaccharides from samples, as observed for *Curcuma longa* (Schmitt et al., 2014) and *Anacardium giganteum* (Silva et al., 2014). In these studies, DNA isolation was optimal in 5% buffer. The results of the studies are consistent with those obtained with Cerrado native plants, when the DNA was also extracted in CTAB buffer (Silva, 2010).

Another frequent contaminant that affects sample storage and DNA are the phenolic compounds. Here, we show that mate leaf samples subjected to different storage treatments (Figure 2). Samples stored in CTAB at 4ºC for 14 days or up to 90 days showed no oxidation of polyphenols (Figure 2C and 2D), despite the high concentrations of polyphenols usually present in the leaves of this species. However, when the samples were stored in CTAB at room temperature for 90 days (Figure 2E), both the leaf tissues and the liquid part of the sample had brown coloration, characteristic of polyphenol oxidation.

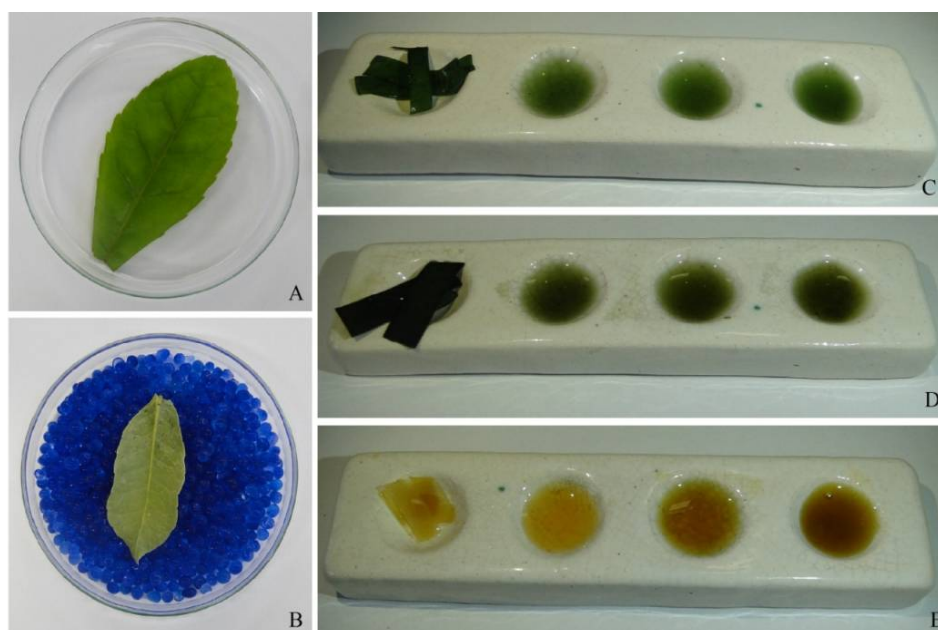

Figure 2. Mate leaves samples subjected to different storage treatments: (A) Fresh sample, (B) stored on silica gel for 14 days at room temperature, (C) stored in CTAB buffer at 4ºC for 14 days, (D) at 4ºC for 90 days, (E) or at room temperature for 90 days.

During homogenization of foliar tissues to initiate DNA extraction and isolation, even in freshly collected samples, phenolic compounds are irreversibly oxidized, binding to the protein and nucleic acids (Soares et al., 2016) and making DNA unsuitable for molecular studies. Phenolic compounds are the main substrate for polyphenol oxidase enzymes that are activated during oxidation. These react with intra-and extracellular phenolic substrates, resulting in darkening of plant tissues (Silva et al., 2009). β-mercaptoethanol is a commonly used anti-oxidant (Romano and Brasileiro, 1999), which when used in conjunction with CTAB extraction buffer, improves quality and quantity of DNA (Silva, 2010). In this study, β-mercaptoethanolwas used at 10 times higher concentration (2% instead of 0.2%) than the original protocol (Doyle and Doyle, 1987), and in conjunction with CTAB extraction buffer, was sufficient to reduce oxidation of the phenolic compounds present in the samples of mate leaves.

DNA extracted from fresh samples of mate leaves, stored with silica gel, or in CTAB buffer, was isolated in variable quantities with adequate concentrations that were quantified by spectrophotometry (Table 1). It should be noted that the extractions were carried out with different amounts of the starting sample as a result of using dried out and fresh leaves. Therefore, it is not possible to make conclusions about the efficiency of the DNA extraction process. Although fresh leaves are preferred, some samples must be stored under certain circumstances, such as when collecting leaves of adult plants in natural populations, *ex situ* cultivation of species, or for transport to research centers. Thus, proper sample storage is necessary to avoid enzymatic degradation and oxidation of the phenolic compounds, as well as maintaining DNA integrity. Since enzymes are active in the presence of water, the elimination of water from tissues, such as storage with silica gel, is a critical procedure in the vast majority of storage techniques (Nagy, 2010).

Table 1. Concentrations (ng μL^{-1}) of genomic DNA extracted from samples of mate leaves submitted to different storage treatments.

Storage of samples			Initial sample weight (mg)[1]	Average DNA concentration ($ng.\mu L^{-1}$)[2]	Range of DNA isolated ($ng.\mu L^{-1}$)[3]
Form	**Days**	**Temperature**			
Fresh	0	Room temperature	100	520.4	267.2 – 972.0
Silica gel	14	Room temperature	50	815.5	187.3 – 1675.9
CTAB[4]	14	4ºC	180	593.7	175.3 – 1548.5
CTAB[4]	90	Room temperature	180	103.8	41.0 – 205.6
CTAB[4]	90	4ºC	180	180.8	53.6 – 300.5

[1]Average initial weight amount of sample used for the extraction of genomic DNA. In the case of storage in CTAB buffer, the entire sample was used for DNA extraction. [2]Quantified by spectrophotometer (Nanodrop 2000, Thermo Scientific). [3]Lowest and highest concentration of genomic DNA obtained from the samples. [4]CTAB (cetyltrimethyl ammonium bromide) buffer.

Silica gel was an efficient method for storage and preservation of DNA (Figure 2B and Figura 3A and 3B), indicating that it can be used for the collection of samples of field-grown adult mate plants. These results with silica gel are consistent with those presented by Cascales et al. (2014) and Gottlieb and Poggio (2014), for storing young mate leaves. In contrast, dehydration of *Kielmeyera lathrophyton* samples with silica gel was not adequate (Feres et al., 2005). Silica gel is commonly used to preserve plant samples as it is simple and conserves DNA integrity, even though moisture levels must be maintained after an

extended period of time (Gostel et al., 2016; Funk et al., 2017). Dehydrated tissue is advantageous as it can be comminuted with greater ease and DNA can be extracted easier since removing the water in samples reduces enzyme activity (Murray and Thompson, 1980).

The ratio of silica gel to the sample should exceed 10:1, and ideally the sample should be broken down into small pieces to facilitate desiccation (Nagy, 2010). However, in this study, a ratio of approximately 30:1 was used (25 g of silica gel for 0.8 g of sample) to adequately dry out of the samples (Figure 2B). This indicates that, although efficient for storage, a lot of silica gel is required and may be a limitation since acquiring silica gel in remote areas is difficult and is not ideal for long-term storage in countries with limited research budgets (Carrió and Rosselló, 2014). In addition, the space required to store just a few samples in silica gel exceeds that of those stored with other methods, such as in CTAB buffer.

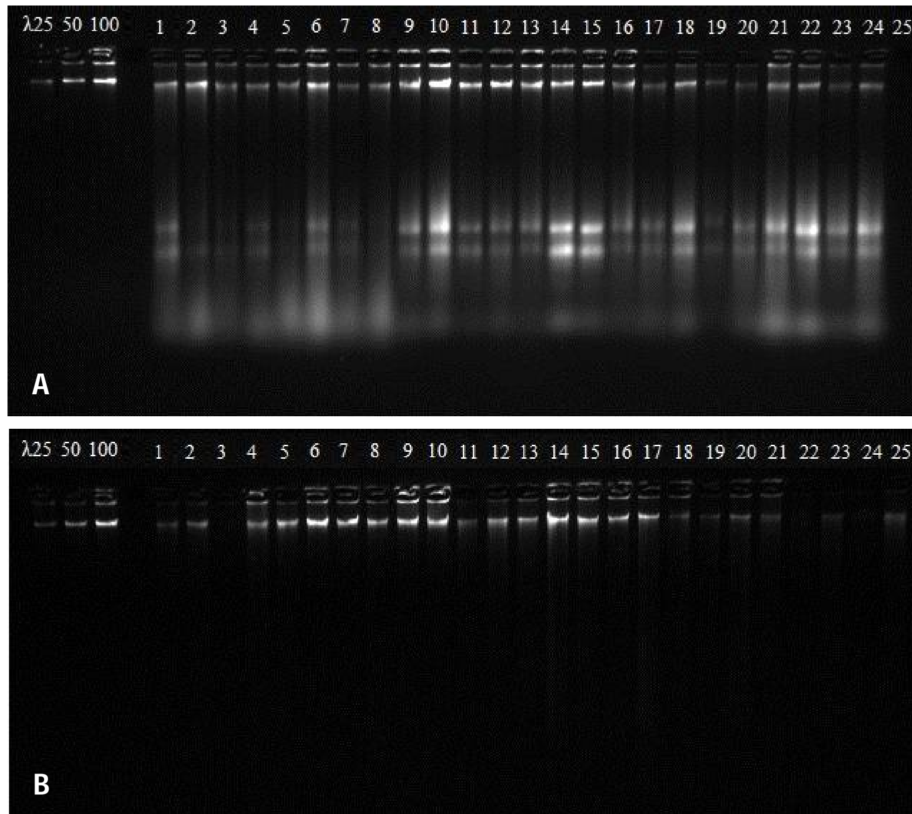

Figure 3. Electrophoretic analysis of DNA extracted from leaf samples of adult mate plants on 1% agarose gel without (A) and with (B) RNase treatment, respectively. The different concentrations of the molecular weight standard (phage λ) correspond to 25, 50 and 100 ng of DNA. Numbers 1-5 correspond to leaf samples stored with silica gel at room temperature for 14 days. Numbers 6-10 correspond to samples of fresh leaves. Numbers 11-15 correspond to samples stored in CTAB at room temperature for 14 days. Numbers 16-20 correspond to samples stored in CTAB at 4ºC for 90 days. Numbers 21-25 correspond to samples stored in CTAB at room temperature for 90 days.

Large amounts of genomic DNA were successfully isolated from leaves stored in CTAB buffer at 4ºC for 14 days and up to 90 days. Samples can therefore be collected and maintained for up to 90 days at low temperatures or even at room temperature. Although rusty in appearance (Figure 2E), mate leaves stored in CTAB buffer at room temperature for 90 days also allowed for isolation of similar amounts of DNA when compared to refrigerated samples (Table 1). We observed that DNA from mate leaves ran slowly on the agarose gel, characteristic of RNA (Figure 2A), so we treated the samples with RNase (Figure 2B). In addition, DNA from fresh leaves showed the highest intensity of the bands, as well as from samples stored with silica gel, with bands of intensity similar to the marker band with a known weight of 100 $ng.\mu L^{-1}$ (Figure 3A and 3B). These results may be associated with the different amounts of sample used (Table 1) and/or the degradation process that begins shortly after the removal of leaves from the plant, with fresh tissue being the best source of high molecular weight DNA (Almakarem et al., 2012).

Considering the results of this work, the samples of mate leaves can be collected directly in CTAB buffer and maintained even at room temperature until the beginning of the DNA extraction and isolation process. This greatly facilitates the collection, transport or storage processes for research, especially in remote areas. In addition, CTAB may be a viable alternative for the collection of desiccation-sensitive samples that are difficult to preserve using other methodologies. These results obtained with the CTAB buffer should be associated with the presence of chemicals that help prevent DNA degradation, such as Tris-HCl, which provides good buffering capacity, and EDTA, which chelates the cations Mg^{2+} and Ca^{2+} and inhibits DNAse activity (Huang et al., 2016b). In addition, we used PVP, which prevents oxidation of phenolic compounds due to its antioxidant action.

Although there are several methodologies for extracting and isolating genomic DNA from plants, the conventional methods of extraction are not necessarily reproducible for all species due to the large variability in the composition of the plant tissue used (Chiari et al., 2009), requiring several adaptations and modifications (Aras et al., 2003). As observed in previous studies, the CTAB extraction method described by Doyle and Doyle (1987) have been adapted for a large number of plant species and forest species, such as *Apuleia leiocarpa* (Lencina et al., 2016) and *Plinia cauliflora* (Danner et al., 2011). However, the method does not allow for DNA extraction for *Kielmeyera lathrophyton* and *K. petiolaris* (Feres et al., 2005). This methodology is characterized by the use of the cationic detergent CTAB in the extraction buffer, which is necessary for separating nucleic acids from polysaccharides, since polysaccharides have different solubilities in the presence of this detergent (Romano and Brasileiro, 1999).

CTAB buffer is advantageous for use in DNA extraction for small tissue samples, and can also be used in several types of plant tissue, such as leaves, roots, seeds, endosperm, embryos, pollen, cells in suspension (Corrêa et al., 2013) and wood (Danner et al., 2011). This is primarily because this method does not rely on RNA or protein degradation enzymes, resulting in greater process agility, lower cost and high extraction efficiency (Oliveira et al., 2007). The method of preserving plant tissue with CTAB buffer was successfully originated in a study with high quality DNA extracted successfully from leaf samples that were preserved for 30 days (Rogstad, 1992), and samples from the stems of *Plinia cauliflora*, in which it avoided the oxidation of phenolic compounds (Danner et al., 2011). This method was also used for the conservation of fungal spores, maintained at room temperature or at 4ºC for two weeks (Huang et al., 2016b). Contrary to these results,

DNA extraction in CTAB buffer was not successful from leaves of *Rheum palmatum*, as a result of its degradation (Huang et al., 2016b).

From the analysis of the amplification products of the plastid fragment *trnL-trnF*, we observed that with all of the storage methods, or with fresh leaves, we obtained DNA without the presence of residues that impede the action of Taq DNA polymerase (Figure 4). Inappropriate storage methods may result in DNA degradation, as well as co-precipitation of PCR inhibitors (Huang et al., 2016b), which apparently did not occur in the samples, since there was amplification of the plastid fragments. The same was not observed for *R. palmatum* and *K. lathrophyton*, for which conservation methods influenced DNA quality and PCR reactions. Samples of *R. palmatum* preserved in NaCl-CTAB were not amplified, while silica gel-desiccated samples yielded high quality fragments (Huang et al., 2016b). In contrast, in *K. lathrophyton*, storage with silica gel made it impossible to amplify the fragments, where as freezing at -20 ºC favored the preservation of the samples (Feres et al., 2005). In the present study, it should be noted that oxidation of phenolic compounds present in the leaf samples of mate stored in CTAB, at room temperature for 90 days, did not impede DNA amplification. This is an important result, because even if a lower concentration of DNA was obtained, based on the observations made on the agarose gel (Figure 3), its quality was sufficient to allow for amplification (Figure 4).

Using fresh leaves is preferred, since higher concentrations of amplifiable DNA can be obtained from the same amount of sample. However, it is not always possible to have fresh samples; therefore, it is necessary to optimize methods of storing samples to guarantee the integrity and quality of the DNA for future studies. Mate leaf samples can be stored in silica gel or in CTAB buffer for up to 90 days at room temperature, resulting in DNA qualitatively and quantitatively suitable for molecular studies. In addition, the results of this work indicate a greater flexibility of material collection and planning for the subsequent stages of DNA extraction, without significant losses in quality and/or quantity, and without compromising the molecular techniques for amplification of DNA fragments. Finally, even though an efficient method for storing samples that allows the obtaining of high-quality DNA for molecular studies does not necessarily indicate applicability to other forest species, the results of the present work suggest new possibilities for collection and storage of samples that are difficult to acquire, especially native species.

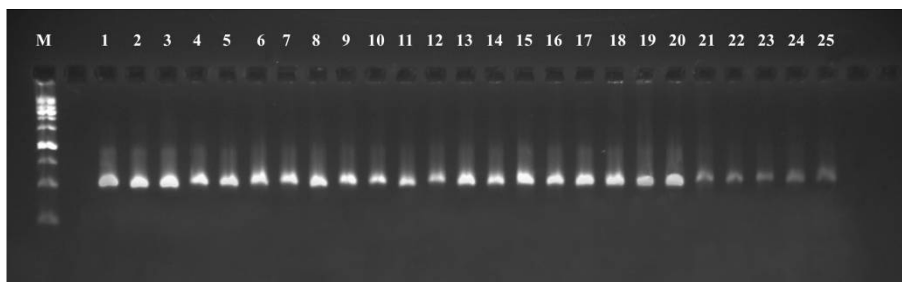

Figure 4. Electrophoretic profile of the PCR amplification products of the fragment *trnL-trnF* of cpDNA in leaf samples of mate on 2% agarose gel. M = 100 bp ladder. Numbers 1-5 correspond to leaf samples stored on silica gel at room temperature for 14 days, numbers 6-10 correspond to samples of fresh leaves, numbers 11-15 correspond to samples stored in CTAB at room temperature for 14 days, numbers 16-20 correspond to samples stored in CTAB at a temperature of 4ºC for 90 days and numbers 21-25 correspond with samples stored in CTAB at room temperature for 90 days.

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CONCLUSIONS

Samples of mate leaves can be stored on silica gel or in CTAB buffer for up to 90 days at room temperature. Samples stored in CTAB buffer can be refrigerated at 4ºC to minimize oxidation of phenolic compounds. The improved methods for sample storage and DNA extraction with CTAB maintain quantity and integrity for conducting molecular studies of mate.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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