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Leaf conditioning of Brazilian Cerrado species for DNA microextraction

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Proper conditioning of leaf tissues in collection expeditions can affect the quantity and quality of DNA in the extraction process. The aim of this work was to define a method of preserving foliar tissue suitable for obtaining DNA from Brazilian Cerrado trees. Young leaves of species (Mangaba and Baru) were collected and conditioned in five different treatments during a period of six days. Genomic DNA was obtained using two alternative versions of the cetyltrimethyl ammonium bromide (CTAB) protocol. For Mangaba, no statistical differences were verified between means of DNA values obtained with diversity arrays technology (DArT) (55 ng/µL) and CTAB (48 ng/µL) methods. It was found that the amounts of DNA obtained with the methods used differed according with the conditioning type and time ($F_{20,60} = 1.98$; $p = 0.022$). For Baru, the mean of DNA extracted was significantly higher ($F_{1,60} = 42.81$; $p < 0.01$) from the CTAB method (80 ng/µL). A significant difference ($p < 0.05$) was also observed between DNA means of conditioning types ($F_{4,60} = 1.1$, $p = 2 \times 10^{-4}$), without this being detected over time. Any preservation method tested is indicated for the selection of Mangaba and Baru foliar tissue conditioning for DNA extraction in a short period (up to six days).

Key words: Conservation, germplasm, native tree, DNA purification.

INTRODUCTION

The Cerrado is considered one of the world's biodiversity hotspots, housing 1/3 of the Brazilian biota and 5% of the world's flora and fauna (Eiten, 1994; Sloan et al., 2014). In the area, it is the second largest vegetation cover in Brazil and South America (Mendonça et al., 1998; Sano et al., 2010) with a high impact on food security (FAO, 2015). This large territory, according to the United States, Department of Agriculture, allowed Brazil to achieve high levels of agricultural production, making it the largest exporter of beef, chicken, sugar cane and ethanol in the world and second to soybean, making clear the functional assets that Cerrado offers us. The model of agricultural expansion used is leading to a progressive depletion of its natural resources (Machado et al., 2004; Silva et al.,

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Table 1. Description of five treatments of Mangabeira and Baruzeiro leaf tissue in the collection for DNA extraction.

Treatment	Description of foliar conditioning	Acronym
01	Leaf conditioned in falcon tube (50 ml) with 40 ml of TE (Tris-EDTA buffer) (1 M Tris HCl pH = 8.0, 0.5 M EDTA pH = 8.0), kept at ambient temperature and without light.	TEB
02	Leaf conditioned in falcon tube (50 ml) with 40 ml of TE buffer (1 M Tris HCl pH = 8.0, 0.5 M EDTA pH = 8.0), kept without light, collection in ice (I) and kept at -20°C until extraction;	TEBI
03	Leaf conditioned in Falcon tube (50 ml), kept without light, collection in ice (I) and kept at -20°C until extraction;	I
04	Leaf conditioned in Falcon tube (50 ml), with 10 ml of silica gel (Sg) and kept without light;	Sg
05	Leaf conditioned in Falcon tube (50 ml), without substance (WS) and kept without light;	WS

2006), ranking the Cerrado as one of the most endangered regions of the planet (Hoekstra et al., 2005).

About 80% of the biodiversity in this area has already undergone severe changes in its natural space, intensifying the constant threats of local extinctions and concerns about the maintenance and viability of the natural services offered (Françoso et al., 2015; WWF, 2014). Thus, it is considered important, the preservation of this heritage and the relevance of a sustained exploitation of its natural resources. For this, the use of the productive potential of its native fruit trees is recommended (Machado et al., 2004).

Until then, approximately 60 native fruit tree species are known and traditionally used by families living in the Cerrado (Franzon, 2009). According to the author, its use is still essentially extractive and often predatory, demonstrating the importance of its cultivation. In most cases, lack of knowledge of the distribution of genetic variability, propagation techniques and phenology of the species makes it difficult to grow commercially. This fact makes it necessary that basic knowledge about the biological diversity of the Cerrado be consolidated and disclosed.

The establishment of conservation plans and adequate management is essential. Studies on the genetic makeup of native populations will assist and support the design of sampling and use strategies. DNA markers have been used successfully for this purpose (Desalle and Amato, 2004). The good quality of extracted genomic DNA is a major step in obtaining a DNA marker via Polymerase Chain Reaction (PCR).

For purification of plants DNA, different DNA extraction protocols have been used (Edwards et al., 1991; Doyle, 1990; Dellaporta et al., 1983). Certain protocols are not suitable for some species, which does not lead to obtaining a sufficient amount of DNA or, when obtained, presenting a very low purity, which may lead to problems and amplification failures (Romano and Brasileiro, 1999), since different species have specific biochemical behaviors, such as the release of free and secondary radicals, which reduce the quality and quantity of DNA extracted (Cavallari et al., 2014).

The adequacy of extraction techniques is often carried out for several species of plants (Edge-Garza et al., 2014; Lade et al., 2014; Mogni et al., 2016), but few or no reference has been made to the proper way of conditioning the foliar tissues, obtained in collection expeditions. The way to preserve the tissue for DNA micro extraction is as important as the choice of the DNA extraction protocol so that, optimizations are necessary to facilitate the operation and ensure a better quality of extracted DNA (Tamari and Hinkley, 2016).

The literature is lacking in studies related to the native species of the Cerrado, so the determination of an ideal way to conserve the tissues, as well as a specific extraction protocol for such species is important, to promote the obtaining of genetic material for molecular studies. The objective of this work was to determine a suitable conditioning method for the preservation of foliar tissues samples, collected from the Cerrado for purification of genomic DNA using two alternative versions of cetyltrimethyl ammonium bromide (CTAB) protocol.

MATERIALS AND METHODS

Plant and conditioning

The Mangabeira and Baruzeiro trees were the species chosen for study. The reason for this choice was the contrast in the leaves of each plant, as to the external morphological aspects.

The leaf of the Mangabeira is thick, hairy and chartaceous, and the presence of latex, whereas the one of the Baruzeiro is thin, leathery and less hydrated. Six young and whole leaves were collected from a single plant using 50 mL falcon tubes and conditioned separately in each treatment for six days after collection (Table 1). Five treatments were defined based on the methods of preservation of the samples of leaves used for collection expeditions until the moment of extraction, such as: 1) TE buffer; 2) TE buffer with ice and maintained at -20°C (TEBI); 3) with ice alone and maintained at -20°C (I); 4) only with silica gel; 5) without substance (WS). The accessions belong to the Germplasm

Bank *in vivo* of the Goiás State University Campus Ipameri and Instituto Federal Goiano Campus Urutaí.

DNA extraction and quantification

About 1 g of tissue corresponding to the leaf limb was used, which was macerated in a porcelain mortar, through mechanical maceration with the aid of liquid nitrogen. Part of the macerate was placed in a 2.0 ml plastic tube, occupying about ¼ of its volume.

The DNA was extracted separately using two DNA purification methods, one based on the protocol of 2% CTAB (Ferreira and Grattapaglia, 1996), often referenced in the literature; and a less common one called plant DNA extraction protocol for (diversity arrays technology) DArT, an alternative version of the 2% CTAB, recommended by Diversity Arrays Technology PTY LTD. The difference between the protocols is the composition of the extraction buffer used, and the 2% CTAB protocol employs the use of polyvinylpyrrolidone (PVP) and β-mercaptoethanol, whereas the working buffer presented by DArT employs the use of sorbitol in its composition.

The obtained genomic DNA was diluted in 100 µL Milli-Q autoclaved water. Quantification was performed at 0.8% (w/v) agarose gels submitted to electrophoresis. 2.0 µl aliquots of DNA from each obtained sample were applied to the gel wells beside a series of known concentrations of λ phage DNA (50, 100 and 200 ng). Sample concentrations were estimated by visual comparison of the fluorescence intensity of the λ phage DNA bands. Gels were visualized after staining with ethidium bromide (0.5%/ml) in TBE buffer solution (1X) for 10 min.

Data analysis

The quantification data of extracted DNA were tabulated and systematized in Excel and later submitted to the analysis of variance (ANOVA) according to the three-way ANOVA model, considering the extraction method, conditioning type and time factors, as well as the interactions between them.

The first two factors were studied by applying Fisher's least significant difference (LSD) test. The time factor was studied by adjusting polynomial models of first and second-degree, using as criterion the F-test and the coefficient of determination. Normality and residual homoscedasticity were previously checked. All inferential procedures were performed at the 10% level of significance. The analyses were performed using R software, version 3.2.1.

RESULTS AND DISCUSSION

Visual aspects of foliar tissues

Comparing the Mangaba foliar tissues submitted to the five methods of preservation, through the time intervals between the extracted DNA, visual changes were verified in the conditioned samples. However, it was not possible to establish a pattern that could be related to treatment or time in this short period of test.

So, for this short period, test measurements of the free radicals and oxidants would be necessary. Some samples showed color-related changes, presenting a green-yellow color with some punctual red spots, which were more evident in the samples of the fifth day. On the other days, conditioning was similar to those when they were collected. However, tissues treated with silica gel showed gradual dehydration over the period in which the extractions were performed. Total visual drying occurred in approximately 48 h (Figure 1).

Regarding the conditioning of the Baru foliar tissues, no visual changes in coloration were observed, nor were they stained with time on the samples kept in TEBI, TEB, I and WS, indicating an adequate state of conservation. The result is different from that observed in the conditioning of the Mangabeira leaf samples. On silica gel, the tissue dehydration process was also verified, however, faster than in Mangaba tree (Figure 2). The total visual drying was observed in approximately 14 h, demonstrating that it occurred more satisfactorily in Baru than in Mangabeira. Once the foliar samples are necessary more than 12 h after collection for drying, the chance of degradation of its DNA is increased, due to the accumulation of free radicals and oxidants (Sytsma et al., 1993).

The absence of visual morphological changes observed in the foliar tissues in Baru shows resistance to senescence that must be related to its thin, thickness and with the low water content, which may hinder the rapid accumulation of free radicals and oxidants after collection. This observation indicates that, there will hardly be any problem in conditioning the samples for a short period or the equivalent of one week for any of the methods chosen. Different from what has been observed for leaf tissue of Mangabeira, is thicker and juicier, indicating that care should be taken in the choice of conditioning since we can detect signs of alteration of the color of the limbus. This yellow-green is related to the early process of foliar senescence, due to the accumulation of ethylene and cytokinin reduction (Soares, 2008), which must be triggered by leaf removal. Based on these results, it was not possible to determine for sure which method of conditioning is suitable for Mangabeira, indicating that biochemical analysis also would be necessary.

Characteristics observed during the DNA extraction procedure

In the mechanical maceration stage of the tissues, the importance of the choice of young leaves for Mangabeira to avoid resistance in the grinding process was verified, due to the high density of secondary veins. Another highlight was the rapid darkening of the sample after maceration, a fact that is related to the oxidation process. This chemical reaction is damaging to DNA, since it leads to the release of free radicals, which causes the fragmentation of nucleic acids (Rajan et al., 2014).

These aspects were not observed in Baru DNA extractions from young leaf tissue. This result indicates, mainly for Mangabeira, the necessity of using DNA extraction protocols with optimized anti-oxidant concentrations, to decrease its degradation (Silva, 2010). In this context, it is necessary to test several experimental scenarios considering variations in the composition of the

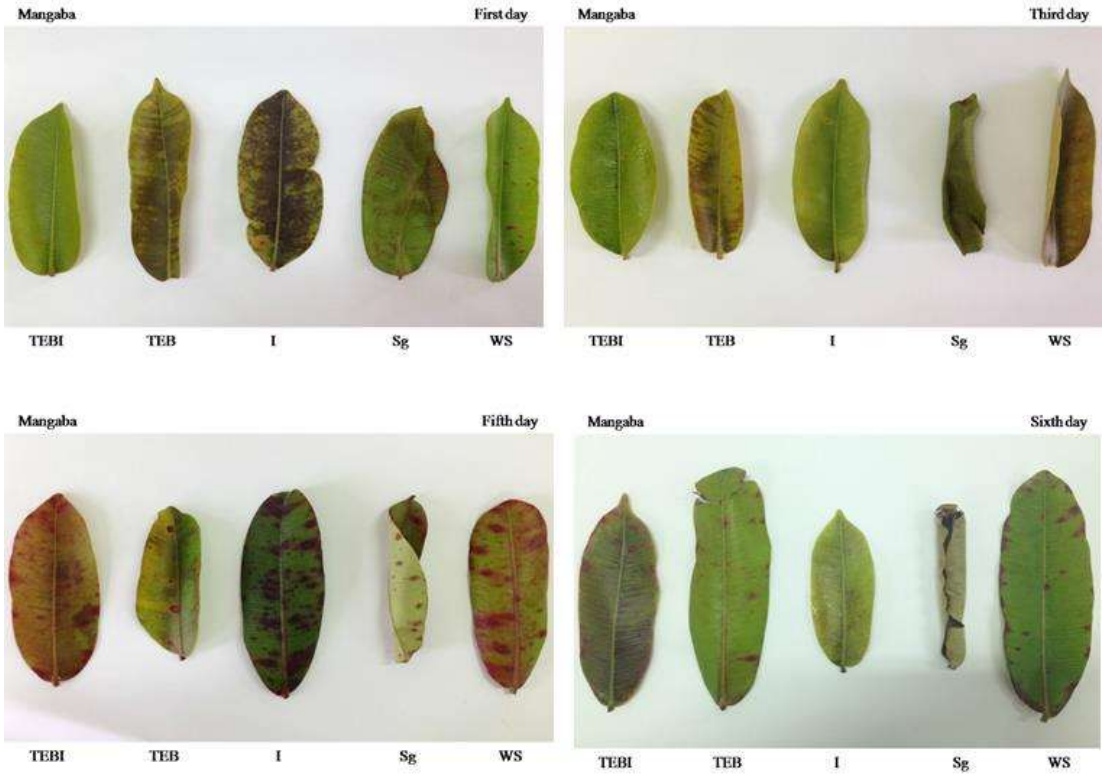

Figure 1. Visual aspects of Mangaba foliar tissues during the 1st, 3rd, 5th and 6th days of conditioning, through the five different packages used.

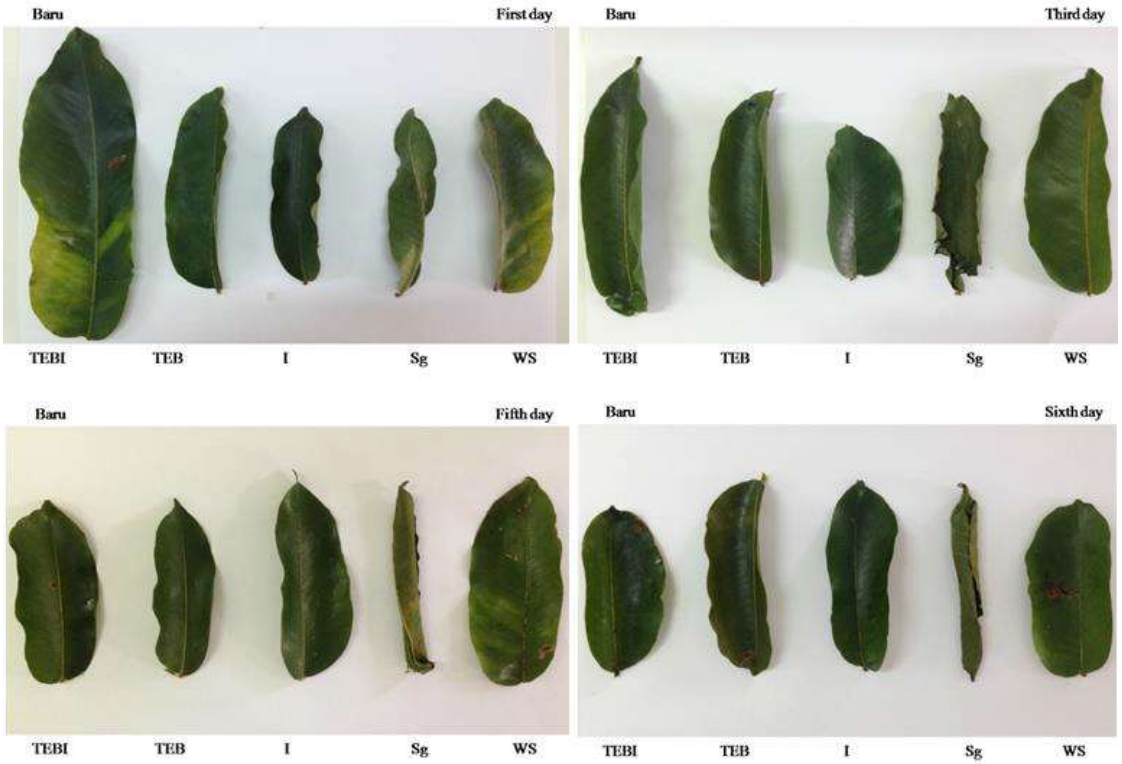

Figure 2. Visual aspects of Baru foliar tissues during the 1st, 3rd, 5th and 6th days of conditioning, through the five different packages used.

Table 2. Quantities of DNA extracted from Mangaba foliar tissue, considering the conditioning method over time according to the extraction protocol used. In the lines, upper case evidence the comparisons between the conditioning used, in the columns, lower case evidences the comparisons between the protocols used.

Time (h)	Extraction protocol	Conditioning				
		Sg	TEB	TEBI	I	WS
0	CTAB	$_{Aa}50$	$_{Aa}50$	$_{Aa}100$	$_{Aa}50$	$_{Aa}75$
	DArT	$_{Aa}15$	$_{Aa}15$	$_{Ab}40$	$_{Aa}15$	$_{Aa}50$
24	CTAB	$_{Aa}100$	$_{Aa}75$	$_{Aa}75$	$_{Aa}75$	$_{Aa}50$
	DArT	$_{ABb}50$	$_{Cb}0$	$_{Cb}0$	$_{Aa}75$	$_{BCa}15$
48	CTAB	$_{CDb}25$	$_{Db}0$	$_{Ab}100$	$_{ABa}75$	$_{BCb}50$
	DArT	$_{Ba}75$	$_{BCa}65$	$_{Aa}150$	$_{Cb}30$	$_{Aa}150$
72	CTAB	$_{Aa}75$	$_{Aa}75$	$_{Bb}30$	$_{Bb}30$	$_{Ba}0$
	DArT	$_{Ba}75$	$_{Ba}75$	$_{Ba}75$	$_{Aa}125$	$_{Ca}30$
96	CTAB	$_{Ba}0$	$_{Ab}50$	$_{Bb}0$	$_{Bb}0$	$_{Aa}50$
	DArT	$_{Ba}20$	$_{Aa}100$	$_{Ba}50$	$_{Ba}55$	$_{Ba}40$
120	CTAB	$_{Aa}100$	$_{Bb}50$	$_{Ca}0$	$_{BCa}30$	$_{Ca}0$
	DArT	$_{Aa}100$	$_{Aa}100$	$_{Bb}22.5$	$_{Ba}10$	$_{Ba}30$

extraction buffer as in the process (Lade et al., 2014).

Different from the other conditioning, the product of the extractions resulting from foliar tissues was maintained at -20°C. After the fourth day of conditioning, it showed a viscous appearance, mainly in the products from Mangaba tree foliar tissue samples. This viscous aspect is due to the presence of polysaccharides, an organic compound that hinders *in vitro* manipulations of DNA, such as PCR amplification and DNA cleavage due to inhibition of the action of DNA polymerase and restriction enzymes, respectively (Edwards et al., 1991; Fang et al., 1992; Sharma et al., 2002).

As for the pellet, the mass of extracted DNA was verified for the samples conditioned in silica gel. The smaller mass was about the other samples, both in Mangaba, as in Baru. This reduced size of the pellets certainly is associated with the lack of total maceration of the leaf tissue due to the stiffness of the dehydrated veins.

Estimation of quantities of obtained DNA

For the extractions carried out on Mangabeira leaf tissue, no statistically significant differences were observed for mean DNA values obtained using the DArT (55 ng/µL) and CTAB (48 ng/µL) methods. The amounts of DNA obtained showed wide amplitude with values 0 to 150 and 0 to 100 ng/µL of DNA, respectively. It was found that the amounts of DNA obtained from the extraction methods used differed according to the conditioning and time of preservation of the tissue ($F_{20,60}$ = 1.98, p = 0.022).

Considering this interaction frequently, larger amounts of DNA were observed among the products obtained from the DArT protocol than CTAB (Table 2). However, it was not possible to detect a significant association pattern (Figure 3). The lack of a pattern is related to the high variation of the data obtained in the micro window of pre-established observation time.

Meanwhile in Baru, the amount of DNA extracted on average was significantly higher ($F_{1, 60}$ = 42.81; p = 0), using the CTAB protocol (80 ng/µL) than DArT (46 ng/µL). There was also a significant difference in DNA means in the different conditions ($F_{4, 60}$ = 1.1; p = 2×10^{-4}). However, this difference was not detected over time. The amount of DNA obtained between the extraction methods used differed according to the conditioning and time, showing an influence of these factors on the amount of DNA obtained ($F_{20,60}$ = 1.69; p = 0.59) (Table 3). However, it was not possible to detect a clear association pattern between them in the observed observation time window (Figure 4).

Conclusion

For DNA extraction from Mangaba leaves, in short periods, the protocol DArT is the most effective. While for Baru, 2% CTAB protocol presents more efficient results,

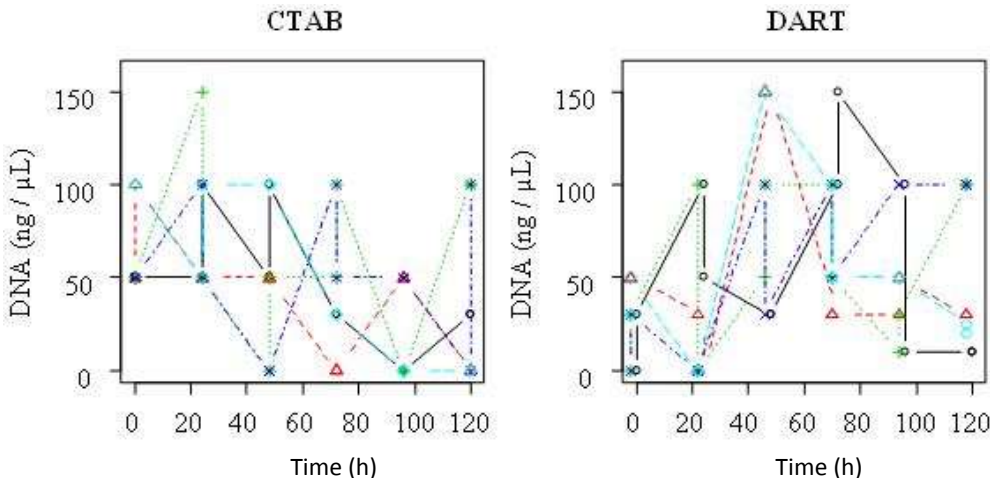

Figure 3. Graph of regression analysis of the amount of DNA separated by extraction method depending on conditioning process and preservation time for Mangaba. TEB, Dark blue line; TEBI, light blue line; I, Black line, Sg, green line; WS, red line.

Table 3. Quantities of DNA extracted from Baru foliar tissue, considering the conditioning method over time, according to the extraction protocol used. In the lines, upper case evidence the comparisons between the conditioning used, in the columns, lower case evidences the comparisons between the protocols used.

Time (h)	Extraction protocol	Conditioning				
		Sg	TEB	TEBI	I	WS
0	CTAB	$_{ABa}$75	$_{Aa}$100	$_{ABa}$75	$_{Ba}$50	$_{Cb}$0
	DArT	$_{Aa}$75	$_{Ab}$50	$_{Aa}$100	$_{Aa}$50	$_{Aa}$50
24	CTAB	$_{BCa}$40	$_{Ba}$50	$_{Aa}$100	$_{Aa}$100	$_{Cb}$0
	DArT	$_{Aa}$50	$_{Aa}$50	$_{Aa}$75	$_{Aa}$75	$_{Aa}$50
48	CTAB	$_{Aa}$90	$_{Aa}$50	$_{Aa}$100	$_{Aa}$45	$_{Aa}$100
	DArT	$_{Aa}$50	$_{Aa}$40	$_{Ab}$50	$_{Aa}$25	$_{Aa}$75
72	CTAB	$_{Aa}$100	$_{Aa}$100	$_{Aa}$100	$_{Aa}$100	$_{Aa}$100
	DArT	$_{Ab}$10	$_{Ab}$10	$_{Ab}$50	$_{Ab}$0	$_{Ab}$0
96	CTAB	$_{Aa}$100	$_{Aa}$100	$_{Aa}$150	$_{Aa}$80	$_{Aa}$100
	DArT	$_{ABb}$50	$_{Aa}$80	$_{BCb}$25	$_{Aa}$75	$_{Cb}$0
120	CTAB	$_{Aa}$100	$_{Ba}$50	$_{Aa}$125	$_{Ba}$50	$_{ABa}$80
	DArT	$_{BCb}$25	$_{Ba}$50	$_{Aa}$100	$_{Ba}$50	$_{Cb}$0

for extraction of genomic DNA.

Any preservation method tested is indicated for the selection of Mangaba and Baru foliar tissue conditioning method for DNA extraction in a short period (up to six days) of conservation. So, equally simple methods of sample preservation can be defined according to the availability of financial resources and instruments of the laboratory, where the procedures were performed.

CONFLICT OF INTERESTS

The authors declared no conflict of interest.

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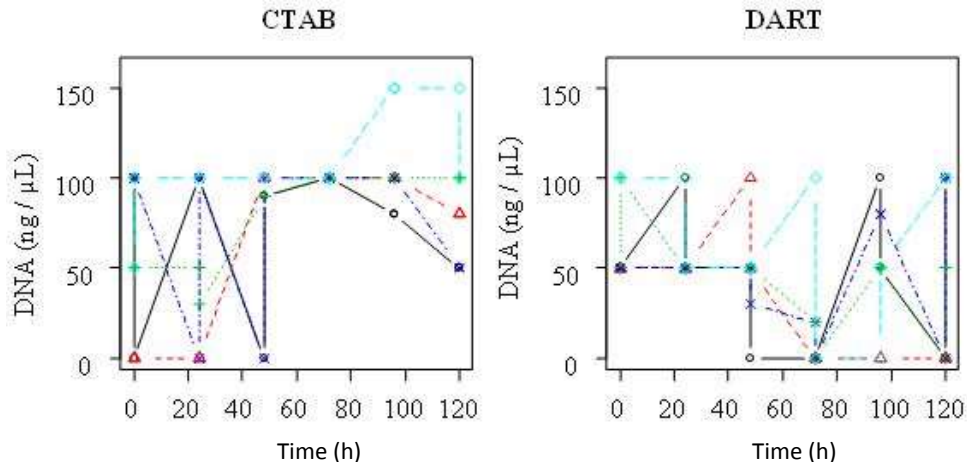

Figure 4. Graph of regression analysis of the amount of DNA separated by extraction method depending on conditioning process and preservation time for Baru. TEB, Dark blue line; TEBI, light blue line; I, Black line, Sg, green line; WS, red line.

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