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SHORT COMMUNICATION

Comparison of three genomic DNA extraction methods from soursop leaves (*Annona muricata* L.)

Comparación de tres métodos de extracción de ADN genómico de hojas de guanábana (*Annona muricata* L.)

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ABSTRACT

The new molecular biology techniques require high-quality DNA. Most of the DNA extraction methods rely on commercially available kits and/or the cetyltrimethylammonium bromide (CTAB) method. Up to date, there is a lack of molecular studies in soursop (*Annona muricata* L.) and no DNA extraction protocol has been reported. Therefore, the development of a method to extract high-quality soursop DNA is necessary. In this study, we compared three methods to isolate genomic DNA from lyophilized soursop leaves. The DNA was extracted using the

MO BIO Laboratories Inc. PowerPlant^{R} Pro DNA Isolation Kit and two CTAB-methods with some modifications. The parameters evaluated were concentration, purity, and integrity. Besides, the maturase K (*matK)* gene was amplified by polymerase chain reaction (PCR) to test the effectiveness of DNA. Our results showed higher DNA concentration and purity using the Sagahi-Maroof method in comparison with the Doyle & Doyle and the molecular kit. Therefore, the combination of 3% polyvinylpyrrolidone (PVP) and 3% CTAB in the lysis buffer improved the quality and concentration of the DNA extracted from soursop leaves. Further, the *matK* gene with a size of 796 bp was successfully amplified by PCR from the DNA isolated with the Sagahi-Maroof method in all samples tested. In conclusion, the Sagahi-Maroof CTAB-method with modifications was the most efficient method to extract high-quality DNA, which will serve for future molecular studies.

Keywords: amplification, CTAB, molecular, isolation, PCR, purification.

RESUMEN

Las nuevas técnicas de biología molecular requieren un ADN de alta calidad. La mayoría de los métodos de extracción de ADN se basan en kits disponibles en el mercado y/o en el método del bromuro de cetiltrimetilamonio (CTAB). Hasta la fecha, en guanábana (*Annona muricata* L.) se carece de estudios moleculares y no se ha reportado un protocolo de extracción de ADN. Por lo tanto, se necesita el desarrollo de un método de extracción de ADN de guanábana de alta calidad. En este estudio se compararon tres métodos para aislar ADN genómico a partir de hojas liofilizadas de guanábana. El ADN fue extraído usando el kit MO BIO Laboratories Inc. PowerPlant^{R} Pro DNA Isolation Kit y dos métodos basados en el protocolo de CTAB con algunas modificaciones. Los parámetros evaluados fueron pureza, concentración e integridad. Además, se amplificó el gen maturase K (*matK*) por reacción en cadena de la polimerasa (PCR) para comprobar la utilidad del ADN. Los resultados mostraron una mayor concentración y pureza de ADN usando el método de Sagahi-Maroof en comparación con el método de Doyle & Doyle y el kit molecular. Por lo tanto, la combinación de 3% polivinilpirrolidona (PVP) y 3% CTAB en el buffer de lisis mejoró la calidad y concentración del ADN extraído de hojas de guanábana. Además, el gen *matK* con un tamaño de 796 pb fue amplificado exitosamente por PCR a partir del ADN aislado con el método de Sagahi-Maroof en todas las muestras analizadas. En conclusión, la metodología de CTAB de Sagahi-Maroof con modificaciones fue el protocolo más eficiente para la extracción de ADN de buena calidad, lo cual servirá para futuros estudios moleculares.

Palabras clave: aislamiento, amplificación, CTAB, molecular, PCR, purificación.

1. INTRODUCTION

The soursop (*Annona muricata* L.) is a climacteric fruit which has gained importance due to the various organoleptic and medicinal properties that have been attributed to it, such as: anti-cancer, anticonvulsant, anti-parasitizing, among others (Moghadamtousi *et al*., 2015; Gaviria *et al*., 2018). The investigation carried out on soursop has been mainly focused to prolong the postharvest shelf life through the application of waxes, emulsions, 1-methylcyclopropene, refrigeration and the combination of these. Moreover, little information at the molecular level related to the genetic diversity of soursop can be found, the main reasons that no commercial variety exists and its genome is not fully sequenced (Berumen-Varela *et al.,* 2019).

Current protocols in molecular biology such as genome sequencing, diversity studies, and genetic variability, among others, require the use of high-quality DNA. The quality of DNA is mainly related to the physiological and morphological condition of the plant material used (Moreira & Oliveira, 2011) since extraction in some cases becomes a laborious and complicated technique, due to a large amount of phenolic compounds and polysaccharides present in plant tissues (Deochand *et al.,* 2014). It has been reported that soursop leaves have a high content of phenols, flavonoids, alkaloids, and lipids (Agu & Okolie, 2017). The presence of these compounds in the DNA causes the degradation of their quality, decreasing their yield and inhibiting the activity of some enzymes in molecular biology experiments (Sánchez-Coello *et al.,* 2012; Olvera *et al.,* 2018). Most DNA extraction methods are modifications of the CTAB protocol which vary depending on the plant tissue used for extraction, differing in time and cost. CTAB has been widely used in the extraction of DNA due to its characteristic as a cationic detergent since it solubilizes polysaccharides (Abubakkar *et al*., 2012). Another alternative to extracting DNA is the use of commercially column-based extraction kits, which have been useful to extract pure DNA from plant species. However, there is a significant loss of DNA depending on the column used. Nowadays, no specific and accurate protocol for the extraction of soursop DNA can be found. Therefore, we need to compare protocols to obtain genomic DNA with good concentration, purity, and integrity. Additionally, DNA requires to be free of substances that inhibit PCR and thus, it should be verified. In this regard, the utility of DNA can be confirmed thought the amplification of the chloroplast gene maturase K (*matK*), which has been proposed as a locus for DNA barcoding of plants species by the Consortium for the Barcode of Life (CBOL) Plant Working Group (Hollingsworth *et al.,* 2009).

The objective of the present study was to evaluate three methods of DNA extraction in soursop leaves to establish the most adequate, simple and reliable protocol for its isolation.

2. MATERIALS AND METHODS

2.1 Plant material

Juvenile leaves of soursop were collected from 20 ungrafted trees in an orchard located in the Ejido of Venustiano Carranza, Tepic, Nayarit (21°30'26 LN and

104°53'37 LW) and immediately transported to the laboratory. Leaves without physical, pest and pathogenic damage were selected, washed with distilled water and then stored at -80ºC for 48 h in a Thermo Scientific freezer, model ULT1.3-86-3-A41, LCC (USA). Five leaves per tree were lyophilized in a LABCONCO Free Zone 2.5 (Houston, Texas, USA) at -46ºC/420 mBar for 48 h and then crushed in a mill with Krups steel blades model GX4100 (Germany) to obtain a fine powder. Subsequently, 50 mg of the powder were transferred to a 1.5 mL microcentrifuge tube to isolate DNA for each of the extraction methods. Twenty DNA samples were individually subjected to the three DNA extraction protocols.

2.2 DNA extraction

2.2.1 Molecular kit MO BIO Laboratories Inc. PowerPlant^{R} Pro DNA Isolation Kit (13400-50)

In this DNA extraction method, the manufacturer's instructions were followed without modifications.

2.2.2 Doyle & Doyle (1987) with modifications

The extraction buffer was adjusted from the original procedure by increasing the concentration of CTAB. The extraction buffer used in this investigation contained 3% CTAB, 1M Tris-HCl at pH 8.0, 5M NaCl, 5M EDTA at pH 8.0. In order to extract genomic DNA, 1 mL of the extraction buffer was added to the powder, vortexed and immediately incubated in a water bath at 60 °C for 45 min in constant agitation. Subsequently, 500 µL of chloroform-isoamyl alcohol (24:1) was added, mixed by inversion for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant was removed, placed in a sterile tube and then 700 µL of cold isopropanol was added for the precipitation of DNA. The pellet was washed with 1 mL of 76% ethanol, mixed by inversion and then centrifuged at 10,000 rpm for 10 min. A second wash was performed with 1 mL of 90% ethanol and then allowed to dry at room temperature for 60 min. Finally, DNA was re-suspended with 200 µL of TE solution (10 mM Tris-HCl at pH 8.0, 1 mM EDTA at pH 8.0) and stored at 4 °C until further evaluation and quantification.

2.2.3 Saghai-Maroof *et al.* (1984) with modifications

A second protocol to extract genomic DNA based on the CTAB method was used. The extraction buffer was modified from the original protocol by increasing the concentration of CTAB and the addition of PVP. The extraction buffer consisted of 3% CTAB, 1 M Tris-HCl at pH 7.5, 0.7 M NaCl, 0.05 M EDTA at pH 8.0, 0.32 M 2-Mercaptoethanol and 3% Polyvinylpyrrolidone (PVP). The DNA extraction procedure was performed by adding 1 mL of extraction buffer (preheated at 65ºC) to the powder. Then, the samples were incubated for 60 min at 65 °C under constant agitation. Subsequently, 700 µL of chloroform-octanol (24:1) was added, it was mixed by inversion for 15 min and then centrifuged at 14,000 rpm for 15 min. The supernatant was decanted in a new tube and the previous step was repeated

for greater elimination of plant tissue. Next, the supernatant was transferred to a sterile tube, 10 µL of RNase (10 mg/mL) was added and then incubated for 30 min at 37 °C. DNA was precipitated with 1 mL of absolute ethanol and then centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and the DNA pellet was washed with 1 mL of 75% ethanol, centrifuged at 10,000 rpm for 10 min and a second wash with 90% ethanol was carried out. After, the DNA pellet was allowed to dry at room temperature and dissolved in 200 µL of TE solution. The DNA was stored at 4 °C until further use.

2.3 Quantification and Determination of DNA integrity

The concentration and purity of the DNA were determined using 1 µL of each sample in a Nanodrop 2000® nano-spectrophotometer (Thermo Scientific, Waltham, MA, USA) with the absorbance ratios A260/A280 nm, A260/A230 nm and reported in ng/uL. The integrity of the DNA was evaluated visually by electrophoresis in 1.0% agarose gels. The electrophoresis conditions consisted of a constant voltage of 120 V for 2 h in 1X TBE buffer (500 mM Tris-HC1, 60 mM boric acid and 83 mM EDTA) containing 1X GelRed as the staining agent. The gel was visualized in the transilluminator Bio-Image System 312 nm, Neve Yamin, Israel, and the images were acquired using the Carestream Molecular Imaging Software, Version 5.0.

2.4 Amplification of the *matK* gene

The utility of the DNA was verified by amplifying the *matK* gene by PCR, which expected size is 796 bp and has been used as a bar code for the taxonomic identification of plants (Hollingsworth *et al.*, 2009; Bieniek *et al*., 2015). The PCR reaction was carried out using the 2X REDTaqR ReadyMix ™ PCR Reaction Mix kit following the manufacturer's instructions using 12.5 ng/µL of DNA per reaction. The amplification conditions by PCR were: 94 °C for 10 min of denaturation, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min. The reactions were carried out in the Prime Thermal Cycle thermocycler (5PRIME/02, Cole-Parmer, Stone, Staffordshire, OSA, UK). The primer sequences for the amplification of the *matK* gene were those reported by Larranaga & Hormaza (2015) as universal markers with a fragment size of 796 bp. The DNA was analyzed by electrophoresis in agarose gels using the previously mentioned conditions.

2.5 Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with a statistical significance of 5%. Tukey's multiple comparison tests were used to determine significant differences among methods in concentration and absorbance ratios ($P \leq 0.05$). The statistical analysis was carried out using SAS 9.0 (USA, 2002).

3. RESULTS

Figure 1 shows the box-plot diagrams of the quantitative parameters of the DNA from the three extraction methods used. According to Figure 1a, average values of 737.95 ng/μL, 147.08 ng/μL and 1101.145 ng/μL were obtained by the molecular kit, Doyle & Doyle (1987) and Saghai-Maroof *et al*. (1984), respectively. Further, it can be clearly appreciated that the DNA concentrations obtained by the Doyle & Doyle (1987) and Saghai-Maroof *et al*. (1984) methods were more consistent (similar DNA concentration), showing a lower DNA concentration variability in comparison with the molecular kit (Fig. 1a). Moreover, Saghai-Maroof *et al*. (1984) showed greater DNA concentration compared to the other two methods ($P \leq 0.001$).

On the other hand, the purity of the DNA samples by the three methods was reported from the ratio of the absorbance's A260/A280 and A260/A230 (Figs. 1b – 1c). The A260/A280 ratio obtained in the molecular kit and the method based on Doyle & Doyle (1987) with modifications presented values in a range of 1.6-2.0 and the A260/230 ratio between 0.8 to 2.0. The samples extracted with the Saghai-Maroof *et al*. (1984) showed the greatest purity, with average values of 2.0 in both relations (A260/A280, A260/230). Additionally, it can be seen that the Saghai-Maroof method showed more uniform absorbance ratio values (little variation in A260/A280 and A260/A230 absorbance) compared to the other two methods (Figs. 1b - 1c).

Also, we visualize the integrity of the DNA and the amplification of the *matK* gene as shown in Figure 2. DNA integrity was evaluated by electrophoresis in agarose gels, observing that all genomic DNA samples of the different extraction methods presented a single defined band of high molecular weight, without scavenging, free of inhibitory compounds and without RNA contamination (Fig. 2a). According to the values obtained in the absorbance ratio A260/A280 and A260/A230 together with the results observed by gel electrophoresis, the method of Saghai-Maroof *et al*. (1984) with modifications was the best method to isolate pure high-quality DNA. To evaluate the utility of the DNA, we analyze the *matK* gene by PCR. Figure 2b shows the results of the *matK* gene amplification from the isolated DNA by the three extraction methods. The DNA samples isolated from the molecular kit and the method based on Doyle & Doyle (1987) showed no amplification of the *matK* gene. Only the sample of lane 1 showed positive amplification of the *matK* gene using the method of Doyle & Doyle (1987). In contrast, all samples tested from the CTAB method of Saghai-Maroof *et al*. (1984) with modifications showed successful amplification of a single band of a size of 796 bp, which coincides with the expected fragment size (Fig. 2b).

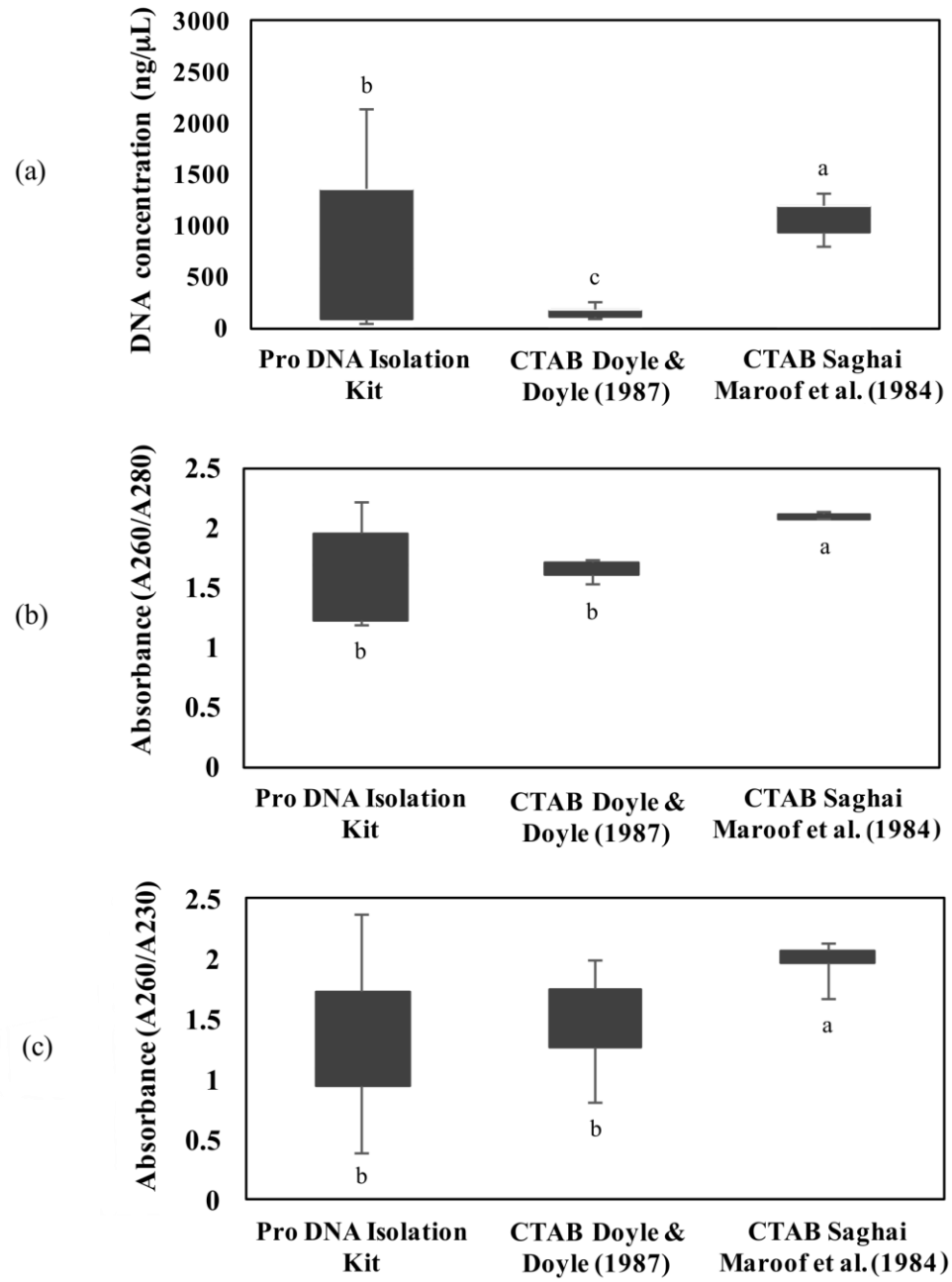

Fig. 1. Box-plot diagram of (a) DNA concentration, (b) A260/A280 and (c) A260/A230 obtained for each extraction method. Each box indicates the values of the quartiles, the limits of the distribution represent the maximum and minimum values. Different letters indicate significant differences ($P \leq 0.005$) among the evaluated methods according to the Tukey's test.

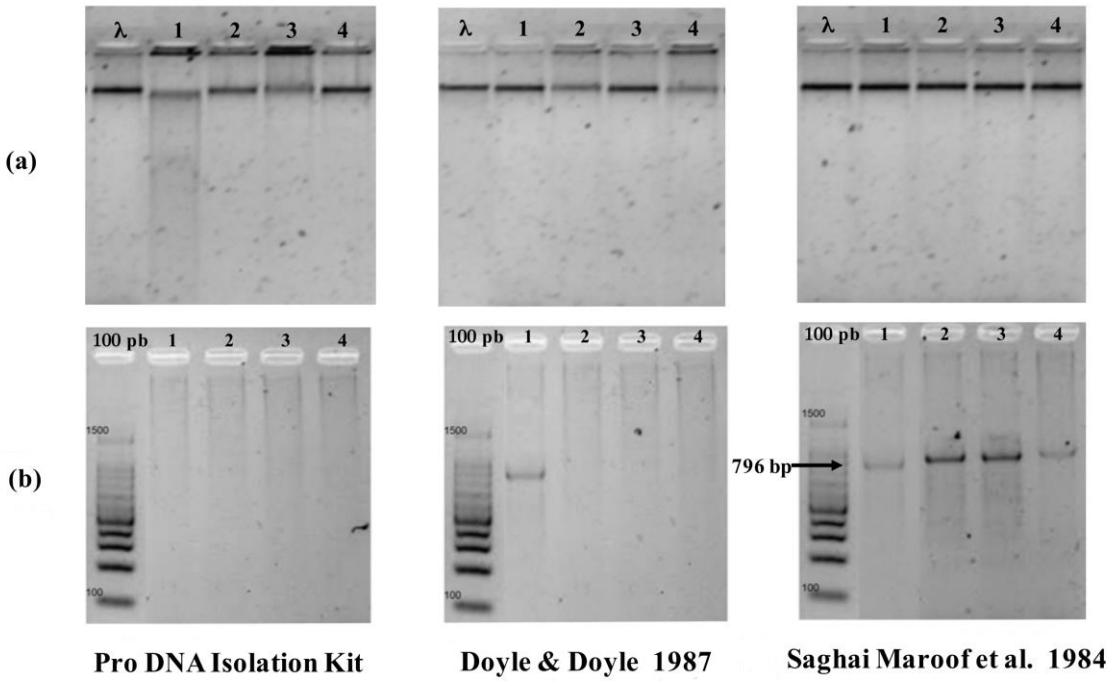

Fig. 2. Agarose gel electrophoresis. (a) genomic DNA isolated from soursop leaves by three methods. λ is the Lambda DNA marker (48,502 bp), numbers 1 to 4 represent the isolated DNA. (b) Amplification of the *matK* gene from genomic DNA isolated by three methods. 100 bp is the molecular marker, numbers 1 to 4 are the amplified DNA.

4. DISCUSSION

Several methods for the DNA extraction have been published, however, a specific protocol for the DNA extraction of soursop has not been reported in the literature. Even when some authors have extracted DNA from soursop leaves based on the CTAB methods, no data regarding the methodology, DNA concentration, and absorbance ratios can be found due to the objective of that investigations were to analyze the genetic diversity using molecular markers (Brown *et al.*, 2003, Suratman *et al*., 2015; Hasan *et al.,* 2017; Anuragi *et al.,* 2018).

Tissue sample collection and right storage conditions before the DNA extraction is an important step to avoid DNA degradation (Till *et al.,* 2015). Thus, most of the studies to isolate DNA from plant species freeze the tissues in liquid nitrogen followed by grinding. However, in this study, we lyophilized the tissue to extract DNA. Lyophilization is a cheap and practical alternative to remove water from tissues and allows the storage of samples for many months prior to DNA extraction (Till *et al.,* 2015). In addition, one of the advantages of lyophilized plant material compared to freeze the tissues in liquid nitrogen is that is easy to transport and is stable at room temperature. Lyophilization produced high-quality genomic DNA for high throughput targeting induced local lesion in genomes assays (Till *et al.,* 2004). Further, Betancourt-Olvera *et al.* (2018) isolated DNA from Mexican hawthorn

(*Crataegus mexicana* Moc. & Sessé) using six extraction methods. These authors reported that the lyophilized plant material showed the highest DNA yield and quality compared to fresh shoots and dry leaves in all the extraction methods used.
Alfonso *et al.* (2016) reported the DNA extraction of young leaves from different Annona species such as *A. reticulata* L., *A. glabra* L., *A. muricata* L. and *A. squamosa* L. using the kits NucleonPHYTOpure Amersham (without columns) and the DNeasy® QIAGEN Kit (with columns), obtaining concentrations of 30 ng/μL by grinding 0.1 g of tissue with liquid nitrogen. Our results showed that DNA concentration obtained by any of the methods established in this investigation are clearly superior to those previously mentioned. These results can be attributed that we lyophilized the tissue instead of frozen, suggesting that lyophilization concentrated the DNA. Further, RNase was added after DNA extraction in the molecular kit and Saghai-Maroof *et al.* (1984) methods to eliminate RNA, which helped to increase the concentration of DNA.
The method of Saghai-Maroof *et al*. (1984) with modifications showed the highest DNA concentration among all the methods tested (Fig. 1a). The difference in the reagents used in this method is that the buffer contained PVP, 2-Mercaptoethanol and a higher concentration of CTAB. PVP has been used in CTAB based methods of plant species to remove phenolics compounds by forming hydrogen bonds with them (Maliyakal, 1992; Sahu *et al*., 2012*).* The addition of PVP and the increase in the concentration of CTAB in the lysis solution has been shown to allow the solubilization of polysaccharides and the elimination of phenolic compounds that can inhibit the enzymatic action (Abubakkar *et al.,* 2012), allowing the high removal of secondary metabolites. Furthermore, the addition of 1-2% PVP has been used in CTAB methods of various plant species to avoid the oxidation of polyphenols (Maliyakal, 1992; Sahu *et al*., 2012*).* Likewise, 2-Mercaptoethanol helps inactivate nucleases, remove tannins, proteins and other polyphenols present in plants (Varma *et al.,* 2007).
Besides, two centrifugation steps after the addition of chloroform-octanol (24:1) were performed in the method of Sagahi-Maroof *et al.* (1984) with modifications to eliminate plant tissue, suggesting the removal of phenolics compounds by precipitation (Abdel-Latif & Osman, 2017; Olvera *et al.,* 2018). Therefore, this extra centrifugation step in combination with the PVP, 2-Mercaptoethanol and the increase of CTAB concentration aided to eliminate phenolics compounds, which probably explains the high concentrations of DNA obtained by the method modified of Saghai-Maroof *et al*. (1984) compared with the Doyle & Doyle (1987) and the molecular kit.
In the case of the purity values (ratio of the absorbance's A260/A280 and A260/A230) we recorded differences between the methods evaluated. Lower values than 1.7 suggest the presence of proteins and higher than 1.9 indicate the presence of RNA in the samples (Latif, and Osman, 2017). According to Betancourt-Olvera *et al*. (2018) a high purity DNA (A260/A280) and without the presence of proteins or phenols shows values between 1.7-2.0, having an optimal value of 1.8 and values of 2.2 for the A260/230 ratio.
The results obtained by the molecular kit were lower compared with the CTAB based methods (Figs. 1b – 1c), probably due to the presence of phenolics compounds that can interfere in the quantification by UV absorption (Olvera *et al.,*

2018). On the other hand, the two CTAB based methods presented average to optimal absorbance values. NaCl in the extraction buffer present in both methods might remove the proteins and carbohydrates that bonds to the DNA and increases the solubility of polysaccharides (Abdel-Latif & Osman, 2017; Olvera *et al.,* 2018). Ukwubile (2014), reported modifications based on the CTAB method for DNA extraction in *A. senegalensis* Pers. using sodium acetate and isoamyl alcohol, obtaining quality values between 1.8 and 2.0 (A260/A280).

In this regard, the method of Saghai-Maroof *et al*. (1984) with modifications showed the greatest purity values (average of 2.0) as shown in Figures 1b – 1c. These results can be ascribed by the use of chloroform-octanol instead of chloroform-isoamyl alcohol as in the Doyle & Doyle 1987 and molecular kit because it has been reported that octanol can enhance the isolation of nucleic acids (Harisha, 2005). Further, the additional wash step with octanol can reduce the co-precipitation of polysaccharides with the DNA.

Also, we evaluated the integrity and utility of DNA by electrophoresis and PCR, respectively (Figs. 2a – 2b). Although all DNA extraction methods were equally able to isolate genomic DNA with good integrity (Fig. 2a), no amplification of the *matK* gene was detected when the molecular kit was used (Fig. 2b). This result can be attributed to the presence of inhibitors in PCR such as the phenolic compounds or proteins (presents in the DNA). On the other hand, only one sample of the method based on Doyle & Doyle (1987) showed amplification of the *matK* gene.

This is probably because that sample contained good quality DNA because as mentioned above, a high variability of absorbance ratios was observed. Further, the DNA extracted by both methods previously mentioned had absorbance's below 1.8, which indicates the presence of phenolic compounds in the DNA that interfere and/or inhibits the PCR amplification.

Nonetheless, we successfully amplified the *matK* gene using the Sagahai-Maroof *et al*. (1984) method with modifications, producing a single and clean product of 796 bp in length (Fig. 2b). This confirmed that the DNA extracted by this method is free of inhibitory compounds and is suitable for further molecular applications.

Based on the previously mentioned, the method of Saghai-Maroof *et al*. (1984) with modifications indicated that the isolated DNA is free of contamination and with the quality necessary for manipulation in molecular analysis. Therefore, the combination of 3% PVP and 3% CTAB in the lysis buffer improved the quality and amount of DNA extracted.

In conclusion, we demonstrated that DNA extracted with the CTAB method proposed by Saghai-Maroof *et al*. (1984) with modifications was the most efficient, useful, and accurate protocol to obtain high-quality DNA from soursop leaves, indicating that the isolated DNA is free of contamination and can be used for future molecular studies.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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