AN EFFECTIVE DNA EXTRACTION METHOD FROM *Cajanus cajan* SEEDS SUITABLE FOR PCR ANALYSIS

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ABSTRACT

The presence of co-extracted impurities in seeds can make DNA extraction challenging, as these contaminants can interfere with PCR amplification, both of which, extraction and PCR, are important steps for ongoing breeding and selection programs. A new protocol is evaluated in this study as an alternative for extracting DNA from embryonic radicle tips of pigeonpea seeds that yield DNA readily amenable to PCR. The proposed protocol showed great promise as an alternative method, as it is low-cost, straightforward, and effective for PCR amplification. It also has the advantage of not requiring hazardous or expensive reagents and can be easily scaled up, demonstrating its potential as a valuable resource for scientists studying pigeonpea genetics and breeding.

Additional keywords: DNA yield, guandu, HotShot method, molecular markers, PCR amplification

RESUMEN

Un método efectivo de extracción de ADN en semillas de guandú (*Cajanus cajan*) adecuado para análisis por PCR

La presencia de impurezas extraídas conjuntamente en las semillas puede dificultar la extracción de ADN, ya que estos contaminantes pueden interferir con la amplificación por PCR; ambos procesos son pasos importantes en los programas de reproducción y selección. En este estudio se evaluó un nuevo protocolo como alternativa para extraer ADN de los ápices de la radícula embrionaria de semillas de guandú, las cuales producen ADN fácilmente adecuado para la PCR. El protocolo propuesto mostró un gran potencial como método alternativo, ya que es económico, sencillo y efectivo para la amplificación por PCR. También tiene la ventaja de que no requiere reactivos costosos o peligrosos y puede ampliarse fácilmente, lo que demuestra su potencial como un recurso valioso para los científicos que estudian la genética y el mejoramiento del guandú.

Palabras clave adicionales: Amplificación por PCR, guandú, marcadores moleculares, método HotShot, rendimiento de ADN

INTRODUCTION

The adoption of biotechnological techniques has played a crucial role in improving crop yields and adapting them to different climatic conditions. Forage plants have benefited from biotechnological advancements in plant-breeding programs, and further progress are likely to be made to increase animal production (Kumar, 2011; Kulkarni et al., 2018).

Molecular-based analyses aimed at the genetic improvement of plants typically involve DNA extraction, which is crucial for advances in breeding programs. However, high concentrations of co-extracted DNA contaminants such as polysaccharides, polyphenols, and secondary compounds of plants pose a challenge for DNA extraction (Sahu et al., 2012; Teoh, 2016). Many of these contaminants often co-precipitate with DNA and can reduce or even inhibit downstream enzymatic reactions such as in PCR assays. To ensure efficient and cost-effective genomic DNA extraction, it is necessary to develop faster, simpler, and more effective protocols that can yield PCR-quality DNA for marker-assisted selection (Aydin et al., 2018).

Although DNA can be extracted from various tissues such as leaves, stems, and endosperm, isolating DNA directly from seeds before sowing can be advantageous for marker-assisted plant-breeding programs. DNA extracted from such source material can reduce workload and costs, saving time and effort on many molecular analyses, such as PCRs, in breeding programs where maintaining numerous genotypes is expensive (Chen et al., 2009; Junior et al., 2016).

However, isolating DNA from seeds can be challenging, as seeds contain an important amount of mucilage polysaccharides and oils that might interfere with DNA quality to be extracted and used for further analyses (Sudan et al., 2017; García-Abolafio et al., 2023). Hence, there is a pressing need for effective and reliable DNA extraction protocols that not only facilitate rapid and cost-effective DNA extraction but also ensure PCR-quality DNA by reducing or even eliminating any potential interference caused by the presence of secondary compounds during the extraction process.

Pigeonpea (*Cajanus cajan* L. Millsp.) is a versatile leguminous crop that grows perennially in tropical and subtropical regions of several countries. This crop has multiple uses, such as grain and livestock feed (Gowda et al., 2012). To improve its grain yield, forage quality, and overall productivity, various breeding programs have been launched for pigeonpea. However, optimizing every step of the breeding process, starting from sample DNA extraction, is necessary to accelerate the pigeonpea breeding program in tropical and subtropical regions.

In this study, we present a modified method for rapid and cost-effective DNA extraction from embryonic radicle tissue of pigeonpea seeds. We also compared the efficacy of this developed procedure with other protocols for amplification of targeted-DNA, using both nuclear and organellar molecular markers.

MATERIAL AND METHODS

Plant Material. For the analyses, 25 samples from 5 accessions of *Cajanus cajan* were chosen from the Embrapa's Pigeonpea Core Collection, located at coordinates 3°45'03" S, 40°20'37" W in Sobral, Ceará, Brazil. Each accession (3PL1, 63PL1, 29PL1, 49PL3, 12PL2) consisted of five samples. Pigeonpea seeds were collected at random and stored with silica gel crystals prior to extraction. Before DNA extraction, individual dried seeds were washed in a 1.5 mL microcentrifuge tube with 500 µL of nuclease-free ddH$_2$O, and then dried on filter paper.

DNA Isolation. Three distinct methods were employed for extracting pigeonpea DNA, two of which were chosen to allow comparison of the extraction process itself, and PCR amplification of DNA regions of interest, with the new proposed protocol. These were as follows: (1) a commercially available, low-cost, yet efficient DNA extraction kit, (2) *TNES* buffer-based DNA extraction (Tris, NaCl, EDTA, SDS) as described by Ssekamatte et al. (2018), (3) a new protocol based on the HotShot method described by Truett et al. (2000).

(1) Commercial extraction Kit

The organic material, consisting of whole seeds, was macerated in a mortar and pestle until a fine powder was achieved. Subsequently, 100 mg of this material was loaded into a kit column. The Blood-Animal-Plant DNA Preparation Kit from Cellco Biotech Ltda., following the manufacturer's guidelines, was then employed to extract DNA from the powdered pigeonpea seeds.

(2) TNES buffer-based extraction

To extract DNA from individual pigeonpea seeds, a volume of 700 µL of lysis buffer (0.2 M Tris-HCl, 0.8 M NaCl, 20 mM EDTA and 1% SDS) and 12 µL of proteinase K (20 mg/mL) were added to a 1.5 mL microcentrifuge tube containing 100 mg of powdered seeds. Tubes were incubated at 65 °C for an hour and then centrifuged at 13,200 *g* for 10 min. The resulting supernatant was transferred to a new 1.5 mL tube, to which 150 µL of 5 M NaCl and 900 µL of absolute alcohol were added. After incubation for 2 h, at room temperature, tubes were centrifuged at 13,200 *g* for 10 min, and the supernatant was discarded. The pellet was washed twice with 70% ethanol at room temperature, and then allowed to dry at room temperature. Finally, 70 µL of 1 × TE buffer (10 mM Tris-HCL pH 8.0 and 1 mM EDTA) was added to the pellet to rehydrate and suspend the extracted DNA.

(3) Modified HotShot (modHotShot)

Seeds were individually placed in 2.0-mL microcentrifuge tubes and incubated in nuclease-free ddH$_2$O at room temperature until the tip of the embryonic radicle began to sprout off the seed coat (Figure 1). To accelerate the process and facilitate the absorption of water, scarification of the seeds was carried out. The radicle tips (0.1-0.2 cm in length) were carefully collected via a longitudinal incision. Genomic DNA was then extracted using 70 µL of Alkaline Lysis Buffer (25 mM NaOH, 0.2 mM Na$_2$EDTA, pH 12.0) in a 0.2 mL microtube. The tissue was thoroughly macerated with a mortar and pestle, and

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subsequently, 100 mg of powdered material was incubated in the Bio-Rad T100 thermal cycler (Bio-Rad) at 100°C for 20 minutes.

Following incubation, microtubes were cooled on ice, and 70 µL of Neutralization Buffer (40 mM Tris-Base, pH 5.0) was added to each sample to change the pH to approximately 8.0. These samples were centrifuged at 10,000 × g for 1 min to pellet the debris, and the supernatant (DNA solution) was transferred to a new 2.0-mL microcentrifuge tube. The DNA samples were stored at -20°C until further analysis.
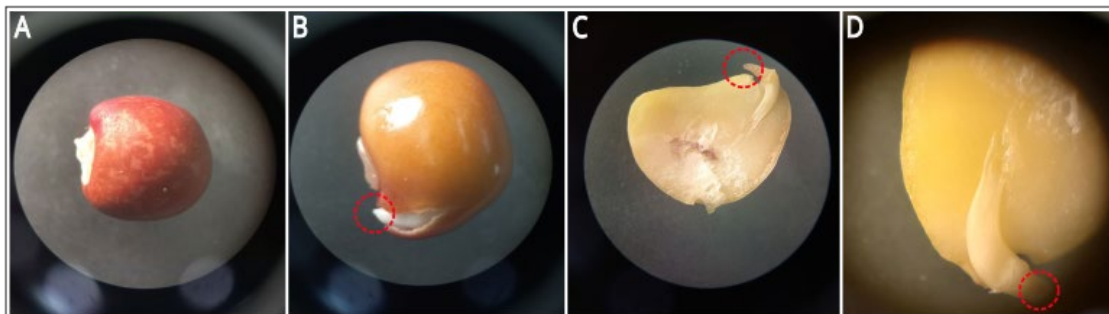
Figure 1. Different parts of pigeonpea seeds from which genomic DNA was isolated using a modHotShot protocol. (A) Dried seeds; (B) the emergence of the embryonic radicle excised for DNA extraction; (C) terminal part of radicle; (D) cotyledon and radicle tip

Spectrophotometric analyses of DNA. To assess the quality and quantity of extracted DNA, the samples were analyzed using the BioDrop-DUO UV/Vis spectrophotometer (BioDrop, Cambridge, UK) with 1 µL of each sample. The absorbance ratios at 260/280 nm, as well as the A260/A230 mm, were measured to determine DNA purity.

Agarose gel electrophoresis. Genomic DNA and amplicons were analyzed by agarose gel electrophoresis using 0.8-1.0 % agarose gel. Electrophoresis was performed using 0.5× Tris–Borate EDTA (TBE) buffer at a constant voltage of 110 V for 80 min. The gel was stained in an ethidium bromide bath, containing 0.5 TBE buffer and 1 µg/mL of ethidium bromide (EtBr) for 40 min. The integrity and concentration of DNA was confirmed by electrophoresing the samples on agarose gel, observing under UV light

PCR amplification. The internal transcribed spacers of nuclear ribosomal DNA (ITS1, 5.8S, and ITS2; amplified by P1a/P4 primers) and the chloroplastidic gene encoding the RuBisCO protein (*rbc*L1/*rbc*L3ambigR primers), both largely used DNA markers, were initially selected for comparing extraction protocol performances. Subsequently, the modHotShot protocol's efficacy in amplifying other DNA regions (ITS 1, primers P1a/P2B or P1a/P2K; ITS 2, primers P3K/P4; sucrose synthase 1 gene, primers SuSy1-F/SuSy1-R; *rpl*20-*rps*12 intergenic spacer region, primers *rpl*20-*rps*12) was further examined using the DNA extract. We have also evaluated the amplification profile of the multi-locus Inter simple sequence repeat (ISSR) marker (UBC-840).

Each DNA sample was diluted to a working concentration of 20 ng·µL. Both DNA regions, ITS and *rpl*20-*rps*12 spacer, were amplified in T100 Thermal Cycler (Bio-Rad Laboratories Inc., Germany) using the primers described on Table 1.

PCR amplification was carried out in a final volume of 10 µL containing 2.0 µL of DNA-template, buffer concentration of 1.25, 2.5 mM $MgCl_2$, 0.8 mM dNTP, 0.5 µM of each primer, and 0.5 U of *Taq* Pol-I HotStart (CellCO Biotech Ltda). The PCR thermal cycling conditions for the targeted DNA regions, as previously mentioned, consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at the specified temperature (Ta) for 1 minute, extension at 72°C for 1 minute, and a final extension step at 72°C for 6 min. The amplification conditions of ISSR markers were initial denaturation step at 94°C for 5 min, followed by 45 cycles consisting of denaturation at 94°C for 30 s, annealing at the specified temperature (Ta) for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 6 min. DNA extraction was considered successful when PCR band(s) were clearly visible on the agarose gel.

Statistical analysis. To determine significant differences in yields (ng/µL) and quality (260/280 nm and 260/230 nm) of extracted DNA by different extraction protocols, with three repetitions, the Kruskal-Wallis test followed by a post hoc Dunn test, from the R Package Stats (Bates et al., 2023) was used. Dunn P-values were calculated for each pairwise comparison and a value≤0.05 was considered as statistically significant between each two results.

Time and cost involvement. The cost analysis included an assessment of three factors: i) reagent cost per sample, ii) consumables cost, and iii) labor cost. Reagent cost per sample covered the components involved in cell lysis, while consumables cost included the main plastic-based materials used in these assays, such as microtubes and tips. Labor costs were calculated based on the values established by the National Council for Scientific and Technological Development and the Coordination for the Improvement of Higher Education Personnel (CAPES) from Brazil for undergraduate students (R$ 100 or USD 19.2 per week) and postgraduate candidates (R$ 550 or USD 105 per week).

The cost of DNA extraction for the commercial kit was estimated by dividing the total price of the kit by the number of preparations. The time taken to complete the extraction process was also considered as a cost factor.

RESULTS AND DISCUSSION

Refining the protocol for nucleic acid extraction in pigeonpea seeds is a crucial step towards enabling future molecular genetic investigations. A robust protocol must be developed to yield genetic material that meets the quality and quantity criteria necessary for PCR essays, with minimal or no secondary metabolite contamination.

DNA extraction revealed varying yields among the three protocols, ranging from 4.58 ± 1.76 (Commercial kit) to 114.00 ± 38.10 ('TNES buffer') (Table 2). The varying yields and purities of the DNA extracts indicate that the outcome of extraction analyses is influenced by the protocol used.

The extraction gel image from the commercial kit revealed faint bands and smears, indicating possible DNA degradation during the extraction process (Figure 2). The protocol yielded limited amounts of DNA, resulting in the lowest performance among the three tested protocols, with an average DNA concentration of 4.58 ng/µL and a range of 1.64 to 8.57 ng·µL^{-1}. The A260/A280 ratio was the most significant indicator, with values ranging from 1.31 to 2.13. Notably, 71 % of the amplified samples fell within the acceptable range of 1.6 to 2.2. However, the mean A260/A230 ratio was relatively low, varying from 0.25 to 1.17, indicating poor quality of the DNA samples extracted using this protocol. It was the second lowest among the tested protocols, as shown in Table 2.

The 'TNES buffer' protocol, adapted for beans by Ssekamatte et al. (2018), yielded intense bands, although some samples showed significant DNA degradation and retention in the wells. This method performed the best in terms of DNA yield, ranging from 43.2 to 184.0 ng/µL, with an average yield of 114 ng/µL. The A260/A280 and A260/A230 ratios ranged from 1.14 to 1.88 and 0.773 to 1.66, respectively (Table 2). It should be noted that half of the samples had A260/A280 ratio values within an acceptable range, and the A260/A230 ratio values were also within the recommended range of 1.5 to 1.8 (Aboul and Oraby, 2019).

The commercial kit and the 'TNES buffer'-based extraction also produced extracts of low A260/A230 ratios, which corroborate findings reported in the literature that polysaccharides can co-precipitate during DNA purification (Chen et al., 2009). The presence of these contaminants can lead to DNA degradation, lower yield, and poor spectrophotometric quantification, as well as interfere with the PCR amplification by the DNA polymerase enzyme (Júnior et al., 2016). One possible solution to eliminate these compounds is to increase the concentration of NaCl or KCl in the extraction buffer, or to use surfactant solutions such as Cetyltrimethylammonium bromide-CTAB (Junior et al., 2016). However, even the use of high concentrations of NaCl (5M) in combination with the surfactant detergent Sodium Dodecyl Sulfate (SDS) did not completely remove these contaminants using the 'TNES buffer' method. Additionally, the buffer that binds DNA to the silica membrane in the Commercial kit may have contributed to the co-elution of polysaccharides along with DNA.

Table 1. List of primers used to amplify specific nuclear and chloroplast DNA regions using DNA extracted from pigeonpea (*Cajanus cajan*) seeds as a template in PCR amplification

Locus	Primer name	Primer sequence (5'-3')	Ta (°C)	PCR product size (bp)	Reference
Internal transcribed spacer1 (ITS1)	P1a	GGA AGT AAA ACT CGG TAA ACA AGG	50	400	Downie and Katz (1996)
	P2K / P2B	CTC GAT GGT TCA CGG GAT TCT GC / CTC GAT GGA ACA CGG GAT TCT GC			Kim and Jansen (1994)
Internal transcribed spacer2 (ITS2)	P3K	GCA TCG ATG AAG AAC GTA GC	55	400	Kim and Jansen (1994)
	P4	TCC TCC GCT CAT TGA TAT GC			White et al. (1990)
Sucrose synthase 1 (SuSy1)	SuSy1-F	GCA CTT GAG AAG ACC AAG TAT CCT G	55	691–948	Choi et al. (2006)
	SuSy1-R	TTC CAA GTC CTT TGA CTC CTT CCT CC			
rbcL	*rbc*L*1*	ATG TCA CCA CAA ACA GAR ACT AAA GC	50	400	Olmstead et al. (1992)
	*rbc*L*3*ambigR	GGC GGA CCT TGG AAR TAT AAG			
Ribosomal protein L20	*rpl*20	TTT GTT CTA CGT CTC CGA GC	53	884-900	Hamilton (1999)
Ribosomal protein S12	*rps*12	GTC GAG GAA CAT GTA CTA GG			
ISSR marker	UBC-840	GAG AGA GAG AGA GAG AYT	35	200-1,350	University British Columbia

Notes: 1) PCR amplification of ITS1, 5.8S, and ITS2 were accomplished using primers P1a and P4; 2) Primer pairs for ITS1 were either P1a and P2B or P1a and P2K; 3) Primers for ITS2 amplification were P3K and P4

Table 2. Mean and median DNA yields (ng/μL) and quality (A260/280 and A260/230) of extraction protocols tested on pigeonpea seeds*

| Protocol	Yield (ng/μL)		A260/A280		A260/A230	
	Mean ± SD	Median (interquartile)	Mean ± SD	Median (interquartile)	Mean ± SD	Median (interquartile)
Commercial kit	4.58 ± 1.76	4.58 (2.02)c	1.69 ± 0.22	1.72 (0.28)a	0.78 ± 0.25	0.81 (0.33)b
'TNES buffer'	114.00 ± 38.10	116.00 (48.30)a	1.56 ± 0.21	1.59 (0.29)a	1.16 ± 0.23	1.80 (0.29)a
modHotShot	21.90 ± 9.79	20.50 (5.87)b	1.13 ± 0.04	1.14 (0.05)b	0.44 ± 0.07	0.43 (0.08)c

* The mean and median values were derived from DNA extractions of the following pigeon pea accessions (3PL1, 63PL1, 29PL1, 49PL3, 12PL2) belonging to Embrapa's Pigeonpea Core Collection.

When evaluating the results of DNA extraction through agarose gel electrophoresis, we observed that both the modHotShot protocol and the commercial kit did not yield significant amounts of high molecular weight DNA, as shown in Figure 2, but it did yield intermediate volumes of nucleic acids (Table 2). Nucleic acid concentrations based on spectrophotometer measurements yielded 9.23 to 59.2 ng/µL with a mean of 21.9 ± 9.79 ng/µL (modHotShot). While presented the lowest values in the A260/A280 and A260/A230 ratios, being from 1.05 to 1.24 and 0.272 to 0.615, with means of 1.13 and 0.44, respectively (Table 2).

The method exhibited a lower A260/A280 ratio (1.13), compared with the other methods, which is probably due to the protein distribution in the embryo (Abebe, 2022), as the protocol does not include a purification step. This method is characterized by its speed, low cost and suitability for routine use in the processing of hundreds of samples simultaneously, such as population analyzes for breeding selection. The elimination of the purification step compensates for the use of small biological samples, resulting in less waste and allowing successful PCR amplification reactions to run smoothly (Warner et al., 2001; Quintana et al., 2022).

As anticipated, the A260/A230 ratio was found to be low in all tested protocols, particularly in modHotShot, indicating a significant presence of carbohydrates (such as starch), which are commonly found in seeds and can make up over 50% of their composition (Abebe, 2022). Despite the inclusion of a purification step in the Commercial kit and 'TNES buffer', these contaminants could not be eliminated.
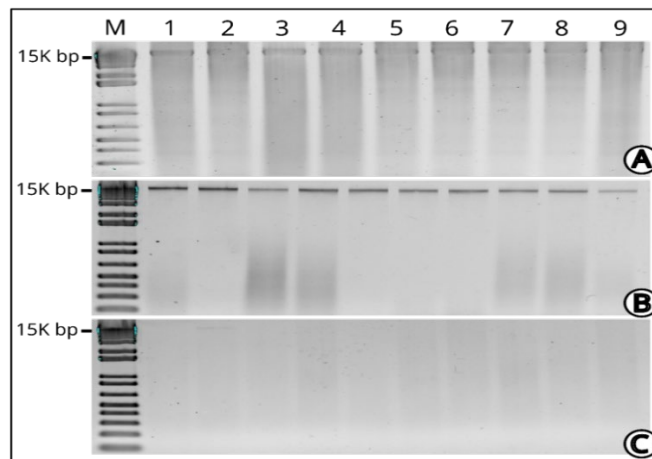

Figure 2. Inverted agarose gel images displaying DNA extracted from pigeonpea seeds (Lanes 1–9) using the following protocols: (A) Commercial kit, (B) 'TNES buffer', and (C) modHotShot. Ladder indicating the larger amplicon size (15,000 bp; Invitrogen E-Gel 1 Kb Plus DNA Ladder).

The Kruskal-Wallis test indicated a significant difference (P-value < 0.000) among the protocols for all variables evaluated. Dunn's post-hoc analysis revealed that the modHotShot protocol (median: 20.5, interquartile range: 5.87), 'TNES buffer' protocol (median: 116, interquartile range: 48.3), and "Commercial kit" protocol (median: 4.58, interquartile range: 2.02) exhibited statistically significant differences in DNA yield (chi-square (degree of freedom 2) = 62.905). Similarly, the A260/A230 ratio (chi-square = 49.232) was significantly different among the protocols, with the modHotShot protocol exhibiting values ranging between 0.425 and 0.08, "TNES" protocol exhibiting values between 1.8 and 0.297, and "Commercial kit" protocol exhibiting values between 0.808 and 0.328 (Figure 3).

Interestingly, the "TNES" (1.59 to 0.297) and "Commercial kit" (1.69 to 0.217) protocols showed similar DNA purification efficiency (chi-square = 47.398) for the A260/A280 ratio (Table 2). This finding suggests that both protocols have similar performance in terms of DNA purity. The results of this study demonstrate that the choice of DNA extraction protocol can significantly influence both DNA yield and purity.

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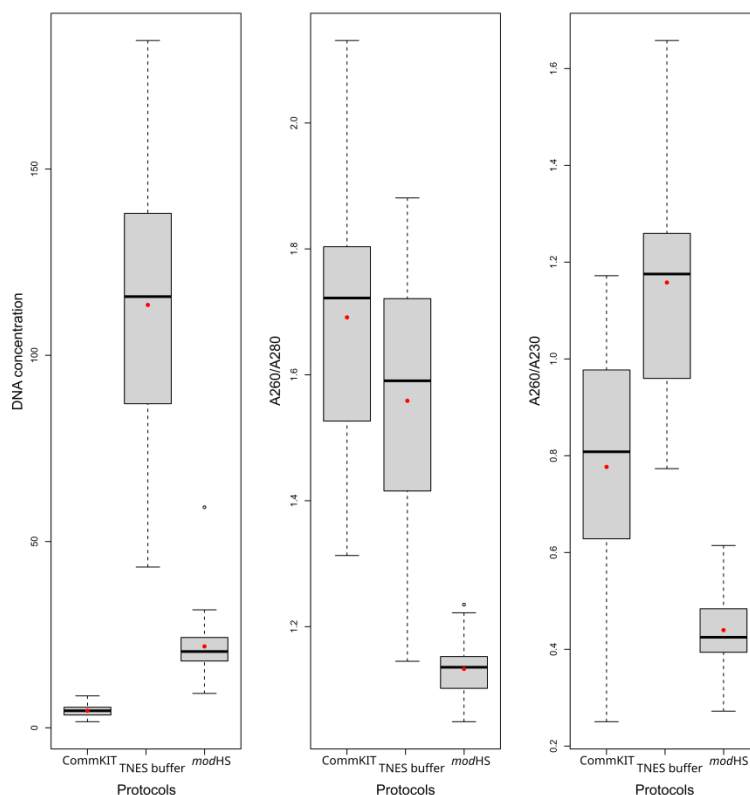
Figure 3. Boxplots of the DNA yield, A260/A280 and A260/A230 of the modHotShot, Commercial kit and TNES buffer methods. The red point corresponds to the mean, the dash inside the box corresponds to the median, and the inferior and superior limits of the box are equal to the first and third quartiles, respectively. The box length equals the degree of sample dispersion. The inferior limit of the dotted line equals the minimum value and the superior limit of the dotted line equals the maximum value of the sample.

The PCR amplification tests conducted on the DNA extracted by the protocols assessed in this study exhibited varied outcomes. Notably, all tested extraction methods were able to successfully amplify DNA of relatively long genomic regions, including the ITS 1, 5.8S, and ITS 2 region (Figure 4). Due to issues with liquid handling, lane 1 showed a very faint band, almost imperceptible.

While the 'TNES buffer' protocol was successful in amplifying ITS fragments, it failed to amplify a shorter fragment, namely the partial RuBisCO protein with primers rbcL1/rbcL3ambigR (Figure 5). Additionally, the ISSR marker UBC-840 showed a smear-like pattern in the gel electrophoresis, indicating the presence of PCR inhibiting substances probably interfering with primer annealing or even removing or chelating Mg ions essential for the

DNA polymerase to work (Sissi and Palumbo, 2009) (Figure 6).

The high content of polysaccharides in the seed endosperm, which was used as the DNA source material in this protocol, might have contributed to this outcome, as polysaccharides are known to inhibit PCR amplification (Rådström et al., 2004).

On the other hand, all markers were successfully amplified using the modHotShot DNA extraction method, demonstrating that the residues present in the samples did not inhibit PCR amplification, most likely because the very small amounts of secondary metabolites (Figure 4-6). This DNA extraction method provides consistent and reliable PCR amplification in pigeonpea seeds.

Despite having lower yield, modHotShot extracts were advantageous for PCR performance,

likely due to the reduced amount of co-extracted contaminants when compared with 'TNES-buffer' extracts. Not only that, but also the NaOH ability

to denature DNA into a single-stranded form and dissolve all organic structures of plant cell walls (Tsugama et al., 2011).
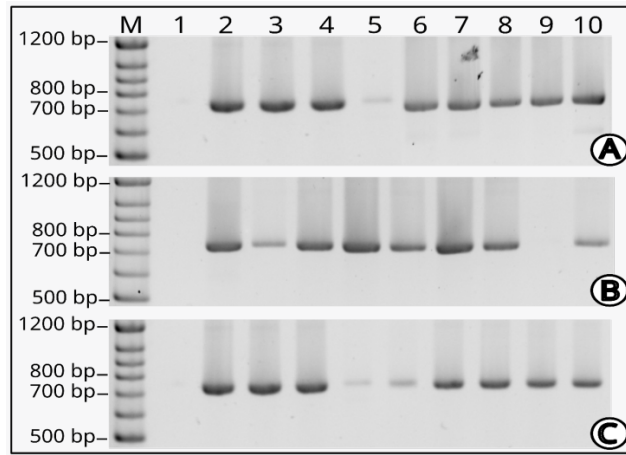
Figure 4. Inverted ethidium bromide-stained agarose gels showing results of PCR amplification of ITS1, 5.8S, and ITS2 with primers P1a/P4. Lanes 1-10 contain DNA extracted from *Cajanus cajan* seeds using the following protocols: (A) Commercial kit, (B) 'TNES buffer', and (C) modHotShot. Lane M: 100bp Ladder Plus, Ready-To-Use (Sinapse Biotecnologia, Brasil)
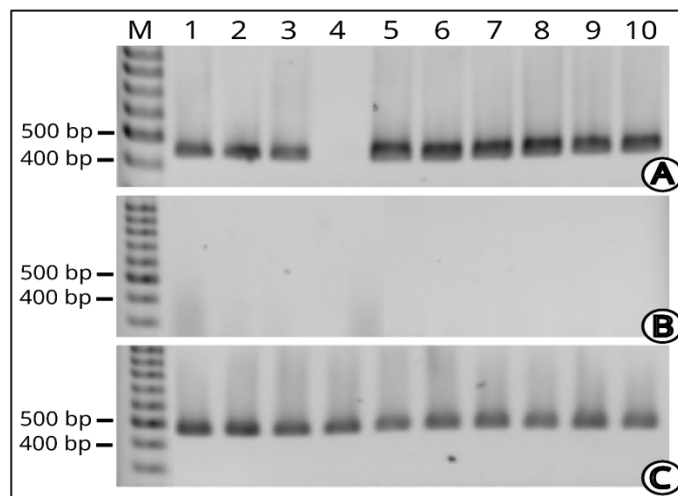
Figure 5. Inverted ethidium bromide-stained agarose gels showing results of PCR amplification of the RuBisCO protein with primers *rbc*L1/*rbc*L3ambigR. Lanes 1-10 contain DNA extracted from *Cajanus cajan* seeds using the following protocols: (A) Commercial kit, (B) 'TNES buffer', and (C) modHotShot. Lane M: 100bp Ladder Plus, Ready-To-Use (Sinapse Biotecnologia, Brasil).

Among the samples tested, only one sample that had its DNA extracted using the commercial kit was successfully amplified for the ISSR marker, while the others failed (Figure 6).

This outcome may have been due to the high amount of polysaccharides present in the sample, which potentially influenced the amplification, despite the successful amplification of the ITS and the chloroplast marker (Figure 4). Furthermore,

commercial kits, while providing better-quality genomic DNA (Figure 2), can be expensive and easily replaced by simpler and less expensive methods (Jafar et al., 2023). These extraction kits are often the preferred choice for laboratory tests due to its short extraction times compared with other more traditional methods. However, we were unsuccessful when using it for cotyledons from seed samples. Using a commercial DNA extraction kit provides numerous benefits, such as practicality, simplicity, the elimination of corrosive reagents (e.g., phenol and chloroform), and consistent reproducibility in DNA production. Despite these advantages, a few studies in the literature have indicated that DNA extraction with

commercial kits resulted in insufficient DNA yields for feasible molecular analyses, and the higher cost is also acknowledged as a drawback (Bitencourt et al., 2007).

In contrast, the modHotShot method utilizes the physical property of high temperatures to denature proteins and enzymes, in combination with the alkaline buffer, enabling successful DNA extraction in just 21 min using a temperature of 100°C. Similar NaOH-based methods have also been successfully applied in a recent study (García et al., 2023), and have also proven to be a promising option for DNA extraction in seeds due to its low cost, simplicity and high efficiency.
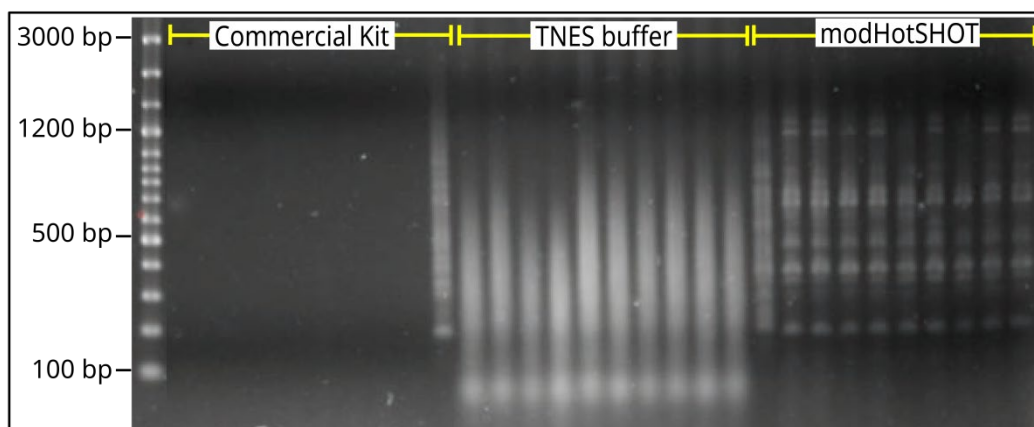
Figure 6. Ethidium bromide-stained 2% agarose gels showing results of ISSR-PCR amplification of *Cajanus cajan* DNA with primer UBC-840 using the following protocols: (A) Commercial kit, (B) 'TNES buffer', and (C) modHotShot. Lane M: 100bp Ladder Plus, Ready-To-Use (Sinapse Biotecnologia, Brasil)

Following the successful PCR amplifications using the modHotShot method, we conducted further tests to amplify other DNA fragments of interest, including the internal transcribed spacer 1 and 2 (separately), sucrose synthase 1, and the ribosomal protein L20-ribosomal protein S12 intergenic spacer (Figure 7). The positive results obtained from these amplifications serve as an additional evidence of the new protocol's effectiveness in amplifying DNA from pigeonpea seeds, even when the extraction did not yield significant quantities of high molecular weight DNA. Despite the lower concentration of DNA in the extracts, there is often a corresponding reduction in the concentration of inhibitors. This, in turn, increases the likelihood of successful amplification of the desired DNA fragment.

Our findings emphasize the significance of selecting an appropriate DNA extraction protocol capable of efficiently amplifying DNA regions of interest from pigeonpea seeds. In this context, the utilization of embryonic radicle tips rather than cotyledon tissue as source material for DNA extraction in the modHotShot method exhibits high potential as a favorable alternative for DNA extraction. One benefit of using radicle tips is that it helps to maintain the seed's integrity, which allows the individual to be included in a selection program.

The modHotShot extraction method offers considerable savings in both time and reagents compared to other methods. This newly modified method, specifically designed for pigeonpea seeds, was found to be the most cost-effective

DNA extraction method, with total costs ranging from R$ 3.47 (USD 0.67) to R$ 6.53 (USD 1.25), primarily due to its low reagent consumption (Table 3). On the other hand, the 'TNES buffer' method had the highest total cost R$ 24.74 (USD 4.76) to R$ 58.28 (USD 11.20) compared to the other methods, primarily due to the longer labor time (3h 50 min) required for this method.
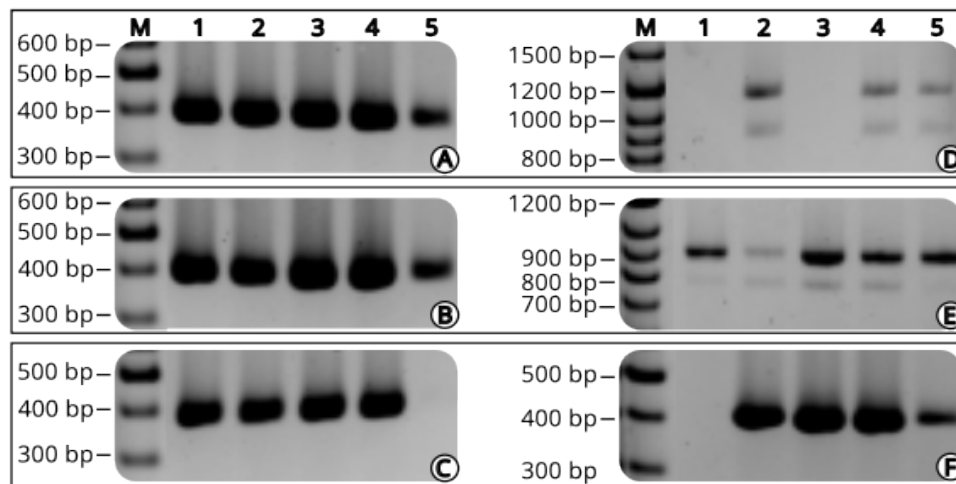
Figure 7. Inverted ethidium bromide-stained agarose gels showing PCR amplification results from DNA extracted by the *mod*HotShot method. The amplified regions were as follows: (A) Internal transcribed spacer1 - ITS1, P1a/P2K, (B) ITS1, P1a/P2B, (C) Internal transcribed spacer2 - ITS2, P3K/P4rev, (D) Sucrose synthase 1, SuSy1-F/SuSy1-R, (E), Ribosomal protein L20-ribosomal protein S12 intergenic spacer, *rpL*20/*rpS*12, and (F) RuBisCO protein, *rbc*L*1*/*rbc*L*3*ambigR. Lanes 1-5: Genomic *DNA* was prepared from seeds. Lane M: 100bp Ladder Plus, Ready-To-Use (Sinapse Biotecnologia, Brasil)

Table 3. Assessment of the estimated time and cost of the protocols tested in function of the Brazilian currency (R$) or US dollar (USD)

Protocols	Reagent cost (R$/USD)	Consumable cost (R$/USD)	Estimated Time	Labor cost (R$/USD)	Total cost (R$/USD)
Commercial kit*	-	-	42 min	3.50-9.63/ 0.67-1.85	15.42-21.55/ 2.97-4.14
TNES buffer	2.58/0.50	2.99/0.58	3 h 50 min	19.17-52.71 3.69-10.14	24.74-58.28 4.76-11.21
modHotShot	0.013/0.0025	1.71/0.33	21 min	1.75-4.81 0.34-0.93	3.47-6.53 0.67-1.26

*: The calculation done for the commercial kit was carried out to in the function of the total price of the kit by preparation. This calculation does not include any preparation time (as for pipetting).

CONCLUSIONS

While all the methods assessed in this study share the advantage of not relying on toxic reagents typically used in DNA purification and not requiring specialized laboratory equipment, such as an exhaust cabinet, the newly designed modHotShot method demonstrated superior efficiency in PCR amplification of various regions of interest, encompassing both nuclear and organellar DNA. The DNA extracted through this method efficiently amplified all tested regions (ITS1, ITS2, Sucrose synthase 1, *rpL*20/*rpS*12 intergenic spacer, and RuBisCO protein), although the yield and quality of template DNA were intermediate, ranging from low to high molecular

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weight. Therefore, the modHotShot method is not suitable for extracting large quantities of high-quality, unfragmented DNA, although this does not affect PCR results. The modified HotShot method's cost-effectiveness and simplicity make it a promising option for DNA extraction in pigeonpea seed studies. In contrast, the commercial kit and 'TNES buffer' methods were found to be inefficient in amplifying the targeted DNA regions and failed to amplify microsatellite-anchored markers.

Additionally, the modHotShot method, utilizing an alkaline buffer for cell lysis, was the sole method in this study that did not need a purification step, owing to the use of a small amount of biological material. The method also enables efficient DNA extraction within 21 minutes, utilizing only a single 0.2 mL microtube per sample. With minimal handling requirements, this allows for easy scaling up of reactions from a few extractions to hundreds per day, therefore suitable for low to high throughput.

Alkaline lysis extractions (modHotShot) allowed DNA isolation at a fraction of the cost of other extraction methods tested in this research, an important low-cost solution for projects with a small budget but a large number of samples.

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