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Adjustment of Protocol for the Extraction of Cowpea DNA

Ajuste de Protocolo para a Extração de DNA de Feijão-Caupi

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Abstract
The use of DNA extraction protocols provides genetic material with high quality, quantity, stability and purity. Thus, the objective of this study was to adjust the protocol for DNA extraction of leaf tissue from cowpea (*Vigna unguiculata*). Adjustments were made to the DNA extraction protocol using CTAB (cetyltrimethylammonium bromide). The variables tested were CTAB (2%, 3%, 4% and 5%), β-mercaptoethanol (1% and 1.5%), Polyvinylpyrrolidone (2% and 3%), mechanical maceration of plant tissue for 30 s, 1 min and 2 min, and manual maceration (with a pistil and glass rod); liquid nitrogen and incubation in a water bath (30 min and 1 h). The results show that the 5% concentration of CTAB was more efficient, and it is thus recommended for DNA extractions of this species. The different concentrations of β-mercaptoethanol did not interfere with the result. Mechanical maceration using *TissueLyser* II at 1 min was efficient for good quality DNA extraction. The use of liquid nitrogen in the maceration was essential for the integrity of the genetic material. The reduction in water bath time resulted in time savings though did not affect DNA quality. The ISSR primer evaluated amplified regions of the genome and proved the efficiency of the extraction.

Keywords: *Vigna unguiculata*. CTAB. Liquid Nitrogen.

Resumo
A utilização de protocolos de extração de DNA deve propiciar a obtenção de material genético com alta qualidade, quantidade, estabilidade e pureza. Desse modo, o objetivo deste trabalho foi adequar o protocolo para a extração de DNA de tecido foliar de feijão-caupi (*Vigna unguiculata)*. Genótipos da Embrapa de feijão-caupi foram cultivados para a extração de tecido jovem. Ajustes foram realizados a partir do protocolo de extração de DNA utilizando CTAB (brometo de

cetiltrimetilamônio). As variáveis testadas foram CTAB (2%, 3%, 4% e 5%); β-mercaptoetanol (1% e 1,5%); Polivinilpirrolidona (2% e 3%); maceração do tecido vegetal (mecânica) nos tempos de 30 s, 1 min e 2 min, e manual (com pistilo e bastão de vidro); presença ou ausência de nitrogênio líquido e tempo de incubação em banho-maria (30 min e 1 h). Os resultados demonstraram que a concentração de CTAB de 5% foi a mais eficiente, sendo indicada para extração de DNA da espécie. As diferentes concentrações de β-mercaptoetanol não interferiram no resultado. A maceração mecânica utilizando *TissueLyser* II a 1min mostrou-se eficiente para extrair DNA com boa qualidade. A utilização de nitrogênio líquido na maceração foi imprescindível para a integridade do material genético. A redução no tempo de banho-maria resultou na economia de tempo sem afetar a qualidade do DNA. O *primer* ISSR avaliado amplificou regiões do genoma, comprovando a eficiência da extração.

Palavras-chave: *Vigna unguiculata*. CTAB. Nitrogênio Líquido.

1 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is a plant of African origin, introduced to Brazil by the Portuguese. Initially, it was cultivated in the Northeast by small- and medium-scale farmers, especially in family-based systems, being one of the staple food components for low-income populations. It is a legume of high nutritional value and an important source of employment and income, which makes it a crop of significant socioeconomic relevance, particularly in the North and Northeast regions. Its consumption is more frequent in these regions due to the crop's tolerance to irregular rainfall and high temperatures (Andrade *et al*., 2002; Frota; Soares; Arêas, 2008).

With the development of erect growth habit cultivars and more uniform maturation, cowpea cultivation has expanded across Brazil (Rocha *et al*., 2017). In the Cerrado region, it has emerged as an alternative for the second cropping season ("safrinha"), benefiting from technologies already employed in the cultivation of other crops, such as soybean (Cardoso, 2017). This strategy enhances nitrogen availability in the soil through nutrient cycling, favoring the uptake of this element by subsequent crops (Machado, 2024).

Coupled with favorable market prospects and growing demand, cowpea plays an important role in the global market. Therefore, studies in genetics and breeding are essential to provide critical information for selecting genotypes adapted to diverse environmental conditions. There is a demand for the development of improved cultivars adapted to different producing regions and with high grain yield, in order to meet both domestic and international markets (Rocha *et al*., 2013).

In this context, studies employing molecular biology tools enable the characterization of genetic diversity among individuals and the genetic mapping of plant populations (Silva *et al*., 2014), thus supporting breeders in the efficient selection of parental lines. For cowpea, a self-pollinating species, identifying parental lines with broad genetic variability is crucial (Bered *et al*., 1997) to obtain populations that allow for the selection of promising lines with desirable phenotypes. DNA markers

enable breeders to identify superior genotypes within segregating populations by distinguishing individuals based on their genetic differences (polymorphisms), which facilitates the development of genetic linkage maps and increases the efficiency of breeding crosses (Bered *et al*., 1997; Toppa; Jadoski, 2013).

Genetic diversity studies using Inter Simple Sequence Repeat (ISSR) molecular markers, which involve the use of Polymerase Chain Reaction (PCR) to amplify identical regions of DNA in opposite orientations (Dias *et al*., 2015), have proven to be effective. ISSR markers are generated using a single primer PCR reaction and have the advantage of producing a high number of bands, making them efficient for population assessment and for determining genetic diversity among individuals (Onofré, 2008).

However, the efficiency of PCR amplification relies on effective DNA extraction, with high-quality, intact DNA in sufficient quantity, presenting clear molecular bands (Edge-Garza *et al*., 2014). Various protocols can be adjusted and optimized for DNA extraction to improve DNA quality and purification according to the plant species. The cetyltrimethylammonium bromide (CTAB) method is the most widely adopted for this purpose (Ambawat *et al*., 2020). Modifications to the procedure are essential to make it simpler, faster, and more accessible, while ensuring high-quality DNA for molecular analyses. The main adjustments involve the method of grinding the leaf tissue, the use or omission of liquid nitrogen, and variations in the concentration or quantity of reagents in the extraction buffer (Sousa Lopes *et al*., 2021).

This study aimed to adjust the protocol described by Doyle and Doyle (1987) for DNA extraction in cowpea, with a view toward subsequent analyses using molecular markers.

2 Material and Methods

2.1 Genotypes and cultivars of cowpea

The study was conducted at Embrapa Agrossilvipastoril (CPAMT), located in Sinop, Mato Grosso, Brazil. Initially, seeds of cowpea materials developed by Embrapa were sown in pots and maintained in a greenhouse to allow plant development and the production of newly expanded young leaves, which were collected for DNA extraction.

2.2 DNA Extraction Protocols

Genetic analyses were carried out in the Molecular Biology Laboratory at CPAMT. All DNA extraction tests were based on the CTAB plant tissue extraction protocol described by Doyle and Doyle (1987), with the following adaptations:

1. β-mercaptoethanol concentrations of 1% and 1.5%;
2. CTAB concentrations of 2%, 3%, 4%, and 5%;

3. Mechanical maceration of plant tissue using a TissueLyser II membrane disruptor with beads, alternating maceration times of 30 seconds, 1 minute, and 2 minutes; manual maceration using a mortar and glass pestle; and maceration with liquid nitrogen;
4. Polyvinylpyrrolidone (PVP) concentrations of 2% and 3%;
5. Incubation time in a water bath: 30 minutes and 1 hour.

In Protocol 1 (P1), young cowpea leaves were collected, and 100 mg of plant tissue was weighed into a microtube. A volume of 800 µL of extraction buffer (2% CTAB) was added, supplemented with 2% polyvinylpyrrolidone (PVP) and 1% β-mercaptoethanol. Two tungsten beads were placed in each microtube, which was then subjected to a TissueLyser sample disruptor for 1 minute at a frequency of 30 Hz. Subsequently, the samples were incubated in a water bath at 65 °C for 60 minutes, with mixing every 15 minutes. After incubation, 600 µL of chloroform:isoamyl alcohol [24:1] (CIA) was added to each microtube, followed by vortexing for 1 minute and centrifugation at 12,000 rpm for 15 minutes. The supernatant was transferred to a new microtube, and 400 µL of cold isopropanol was added. The samples were kept in a freezer at −20 °C for 1 hour and then centrifuged at 12,000 rpm for 15 minutes. The DNA pellet was washed twice with 500 µL of 70% ethanol. After the final wash, the remaining ethanol was evaporated under a fume hood.The DNA was resuspended in 50 µL of TE buffer and incubated at 37 °C for 60 minutes for RNase enzyme treatment. The DNA was stored at 4 °C.

In Protocol 2 (P2), the same procedure described in Protocol 1 (P1) was followed, with the following combined variations: (1) β-mercaptoethanol concentrations of 1% and 1.5%; (2) maceration times in the TissueLyser II sample disruptor of 30 seconds, 1 minute, and 2 minutes.

In Protocol 3 (P3), the procedure from Protocol 1 (P1) was followed, with the following variables tested in all possible combinations: (1) CTAB concentrations in the extraction buffer (2%, 3%, and 4%) and (2) polyvinylpyrrolidone (PVP) concentrations (2% and 3%). For this test, β-mercaptoethanol concentration was standardized at 1.5%.

In Protocol 4 (P4), the same procedure as in P1 was adopted, but the following variables were tested: (1) CTAB concentrations in the extraction buffer (2%, 3%, and 4%); and (2) maceration times in the TissueLyser II sample disruptor for 30 seconds, 1 minute, and 2 minutes.

In Protocol 5 (P5), the procedures followed those of P1, but with 4% CTAB concentration in the extraction buffer and maceration times in the TissueLyser II of 30 seconds, 1 minute, and 2 minutes.

In Protocol 6 (P6), the following variables were tested: (1) CTAB concentration in the extraction buffer (2% and 4%); and (2) incubation times in a water bath of 30 minutes and 1 hour. The subsequent steps followed the procedure described in P1.

Protocol 7 (P7), in this protocol, plant material was macerated using liquid nitrogen.

Subsequently, 800 µL of CTAB buffer (5%) supplemented with 2% PVP (preheated to 65 °C), 14 µL of β-mercaptoethanol, and 2 µL of proteinase K were added. Samples were incubated at 65 °C for 30 minutes with agitation every 10 minutes. Then, 600 µL of chloroform:isoamyl alcohol (24:1) (CIA) was added to each microtube, gently mixed for 10 minutes, and centrifuged at 12,000 rpm for 10 minutes. The aqueous supernatant phase was transferred to new microtubes, to which 400 µL of chilled isopropanol and 60 µL of sodium acetate were added. Samples were stored at −20 °C for 1 hour. Microtubes were centrifuged for 20 minutes at 12,000 rpm at 4 °C. Subsequently, the pellet was washed twice with 1 mL of 70% ethanol and once with 1 mL of 95% ethanol. After evaporation of the alcohol in a fume hood, the DNA was resuspended in 50 µL of extraction buffer plus 2 µL of RNase and incubated at 37 °C for 30 minutes. After the procedure, samples were stored overnight at 4 °C.

Protocol 8 (P8), in P8, the evaluated variable was the maceration method: (1) mechanical using TissueLyser II with beads; (2) manual using a pestle; and (3) glass rod maceration. The following parameters were standardized: CTAB at 5%, PVP at 2%, incubation in water bath at 65 °C for 30 minutes, initial centrifugation at 12,000 rpm for 10 minutes, and final centrifugation after refrigeration at 12,000 rpm for 20 minutes at 4 °C (Thermo Scientific, Heraeus Pico 21). The pellet was additionally washed twice with 1 mL of 70% ethanol and once with 1 mL of 95% ethanol; each wash was followed by 1-minute centrifugation at 12,000 rpm, with ethanol discarded after each step. A final 1-minute centrifugation was performed to facilitate complete drying. The pellet was resuspended in extraction buffer and incubated at 37 °C for 30 minutes. Apart from the mentioned variations, all other steps followed in P8 were the same as those described for P7.

Table 1 - Genomic DNA extraction protocols applied to cowpea leaves

Protocol (P)	Macandration	Timand	CTAB (%)	β-mandrcaptoandtanol (%)	PVP (%)	Watandr bath
1	*TissuandLysandrII*	1’	2	1	-	unchangandd
2	*TissuandLysandrII*	30’’. 1’ and 2’	2	1 and 1.5	2	unchangandd
3	*TissuandLysandrII*	1’	2. 3 and 4	1.5	2 and 3	unchangandd
4	*TissuandLysandrII*	30’’. 1’ and 2’	2. 3 and 4	1.5	-	unchangandd
5	*TissandLysandr II*	30’’. 1’ and 2’	4	1.5	-	unchangandd
6	*TissuandLysandrII*	1’	2 and 4	-	-	30’ and 1h
7	*TissuandLysandrII*	1’	5	1.5	2	30’
8	*TissuandLysandrII*	1’	5	1.5	2	30’
	Glass rod	Grinding with liquid nitrogandn	5	-	-	30’
		Pandstland	5	1.5	2	30’

Source: research data.

2.3 Quantification and agarose gel electrophoresis analysis of DNA

After each tested protocol, DNA quantification was performed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). The extraction results were subjected to electrophoresis, with samples loaded onto a 1% agarose gel run at 45 V and stained with GelRed (Biotium, Hayward, CA, USA) for 2 hours. The gels were visualized under ultraviolet light using a transilluminator and photo-documented (Loccus, L-Pix EX), allowing for the assessment of DNA quality extracted from cowpea leaves in each experiment.

To confirm the quality of the extracted DNA, Polymerase Chain Reactions (PCR) were performed. The ISSR (Inter-Simple Sequence Repeats) primer UBC 809 – DIAG3'G with the nucleotide sequence AGAGAGAGAGAGAGAGG was used, with an annealing temperature of 48.2 °C. Each reaction contained 8.1 µL of MilliQ distilled water, 1.5 µL of buffer, 2.25 µL of primer (0.2 mM), 1.5 µL of dNTPs, 0.15 µL of Taq DNA Polymerase, and 1.5 µL of extracted Vigna unguiculata DNA.

The reactions were run on a Thermal Cycler, model T100, for 35 amplification cycles, with the following program: 94 °C for 4 min; 48.2 °C for 35 s; and 72 °C for 2 min. After the 35 cycles, a final extension step was carried out at 72 °C for 7 min.

The PCR amplification products were then subjected to electrophoresis in a 1.5% agarose gel, stained with GelRed, at 45 V for 2 hours and 30 minutes. The results were visualized and photographed under ultraviolet light (Loccus, L-Pix EX).

3 Results and Discussion

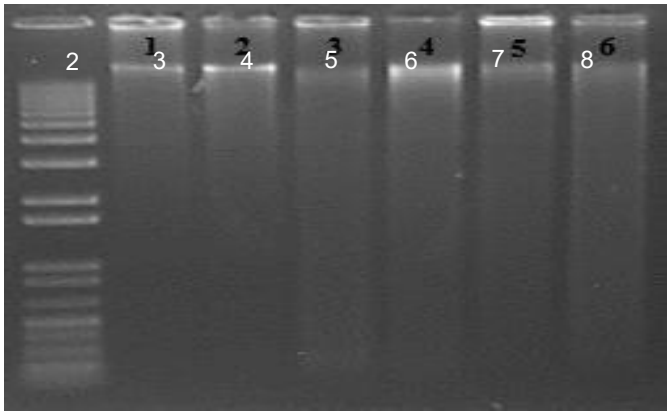
Protocols P1, P2, and P3 proved to be inefficient in terms of quality and yield in the extraction of cowpea DNA. The samples showed vertical smearing on the gel, which may be explained by contamination with DNases or mechanical shearing during the extraction process, resulting in degraded DNA. Additionally, protocols P1 and P3 showed RNA contamination, characterized by low molecular weight bands on the gel, while P2 also exhibited DNA retention in the gel wells. This result may be due to polysaccharide contamination (Romano; Brasileiro, 1999).

Due to the unsatisfactory results obtained with the previously mentioned protocols, new possibilities were tested by repeating the P1 procedure with modifications, giving rise to protocol P4. As a result, visible DNA bands were observed, confirming that increasing the CTAB concentration to 4% was effective compared to the other concentrations. Samples subjected to an additional extraction step (200 µL of 4% CTAB + 500 µL of CIA) with 2% PVP showed less vertical smearing when compared to the other repetitions.

Aiming to reduce the amount of degraded DNA in P4, the modifications made in P5 and P6 were not favorable, and did not allow for visualization of DNA bands, exposing a high amount of

degraded DNA and RNA contamination (Figure 1).

Figure 1 - Photograph of the agarose gel visualization for the P4 test, with changes in CTAB concentration and maceration times of the plant material



M. 1 bp molecular marker. 1. CTAB 2% 30 s. 2. CTAB 2% 1 min. 3. CTAB 2% 2 min. 4. CTAB 3% 30 s. 5. CTAB 3% 1 min. 6. CTAB 3% 2 min. 7. CTAB 4% 30 s. 8. CTAB 4% 1 min. 9. CTAB 4% 2 min.
Source: resarch data.

The values obtained for the A260/A280 ratio, as shown in Table 2, indicate that despite the good ratios found in the tested protocols, degraded DNA was still present. Spectrophotometry measures the amount of light absorbed by DNA in solution at a wavelength of 260 nm, and it also determines the purity of the sample using the 260/280 nm ratio (Silva *et al*., 2023).

Table 2 - Concentration and purity of genomic DNA extracted from cowpea leaf using protocol P4

P4	Concentration (ng/uL)	A260	A280	260/280
1	1368	27.369	13.864	1.97
2	1226	24.529	12.531	1.96
3	2122	42.445	21.587	1.97
4	20.4	0.408	0.296	1.38
5	1191.6	23.832	12.077	1.97
6	1133.5	22.67	11.498	1.97
7	804.5	16.09	8.181	1.97
8	333.6	6.672	3.425	1.95
9	1127.6	22.551	11.437	1.97

Source: resarch data.

With the aim of increasing DNA quality and yield, a modified protocol developed by Oliveira, Rodrigues and Hopkins (2017) based on the original protocol by Doyle and Doyle (1987) was used,

since protocols P1 through P6 were all inefficient. However, protocol P7 still did not yield satisfactory results.

In this context, the failure of DNA extraction using protocols P1 to P7 may have been due to the presence of phenolic compounds. These compounds function to induce resistance against adversities such as pathogens and physical injuries (Stangarlin, 2011), and play a fundamental role in plant metabolism, being involved in defense mechanisms and present in relatively high amounts. In this process, polyphenol oxidases have the ability to oxidize phenolic compounds into quinones, which are involved in microorganism resistance and protective actions in plant wounds (Siqueira *et al*., 2019). The challenge to the effectiveness of the extraction lies in the binding of quinones and their reduction products in the formation of superoxide radicals, which can lead to cell death. Therefore, the oxidation of phenolic compounds becomes a problem in DNA extraction (Pereira *et al*., 2009).

The use of young leaves is another extremely important factor and helps explain their relevance in DNA extraction protocols. As leaves develop, there is an increase in the concentration of polyphenols, tannins, and polysaccharides, which negatively affects DNA quality (Yamamoto *et al*., 2013). Another condition that contributed to the unsuccessful DNA extraction using the previously mentioned protocols was the procedure of storing fresh cowpea leaves in silica gel. Even when transported to the laboratory at low temperatures, this practice may contribute to the degradation of genetic material and thus hinder the adjustments tested in the extraction protocols.

As an alternative to the previous protocols and based on information from the literature, protocol P8 was tested, which, as a distinguishing factor, emphasizes the preservation of plant tissue prior to extraction. There are reports suggesting that care taken during the initial collection phase can reduce problems in DNA amplification (Pereira *et al*., 2009). Ferreira and Grattapaglia (1995) highlight the importance of using the freshest possible material, showing that samples in an active growth phase yield better results. Moreover, pre-extraction storage is a decisive phase when it comes to obtaining high-quality DNA.

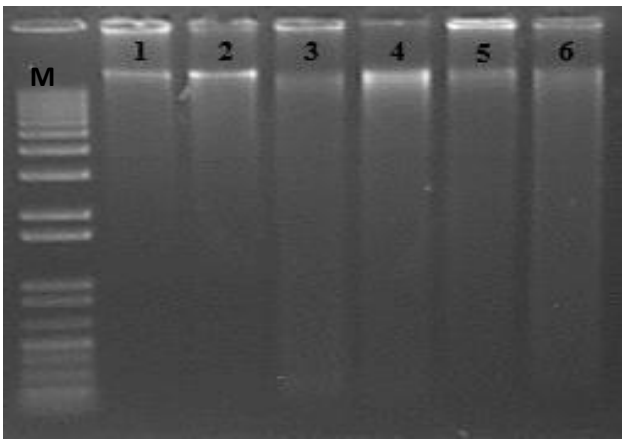
The results obtained using P8 were favorable, as it allowed the visualization of clear DNA bands, minimal vertical smearing on the gel, and no RNA contamination (Figure 1). The positive outcome with P8 may be attributed to the fact that the leaves were collected and immediately frozen in liquid nitrogen. This practice halts cellular activity and prevents the oxidation of phenolic compounds (Pereira *et al*., 2009). The use of liquid nitrogen aids in breaking cell walls, causing the extrusion of cellular contents (Romano; Brasileiro, 1999), and also prevents degradation of genetic material and reduces leaf exposure time to phenolic compounds (Dalbosco *et al*., 2015).

Among the variables tested in this protocol, the concentration of CTAB in the buffer (5%) favored higher quality and yield in DNA extraction (Figure 2), resulting in intense and well-defined

bands, thus demonstrating the efficiency of this protocol in obtaining higher quantities of DNA.

Regarding the maceration method (as a variation of P8), the mechanical method using TissueLyser II with beads was more efficient, as it produced minimal vertical smearing and well-defined bands (Figure 2). This pattern was not observed with other manual methods using a glass rod or pestle, which exhibited vertical smearing and did not result in prominent bands.

Figure 2 - Photograph of agarose gel electrophoresis for the P8 test using different maceration methods



M. 1 bp molecular marker. 1 and 2 maceration with membrane disruptor (TissueLyser II). 3 and 4 glass rod/liquid nitrogen. 5 and 6 pestle.
Source: resarch data.

The quantifications performed using the Nanodrop revealed greater efficiency of maceration with TissueLyser II and beads for one minute, as the nucleic acid concentration was higher than at the other time points of 30 seconds and 2 minutes (Table 3).

Table 3 - Concentration and purity of genomic DNA extracted from cowpea leaf using protocol P8

Protocol 8	Concentration (ng/uL)	A260	A280	260/280
1 (*TissueLyser II*)	357.8	7.156	3.737	1.91
2 (*TissueLyser II*)	485.2	5.703	3.008	1.9
3 (bastão de vidro)	46.3	14.93	7.762	1.92
4 (bastão de vidro)	513.8	10.28	5.389	1.91
5 (pistilo)	84.3	12.48	6.474	1.93
6 (pistilo)	53.2	10.66	5.594	1.9

Source: resarch data.

Some studies, such as those by Soares *et al*. (2016) and Schmitt *et al*. (2014), which aimed to extract DNA from leaf tissue of *Inga edulis*, *Inga laurina*, and *Curcuma longa*, also demonstrated

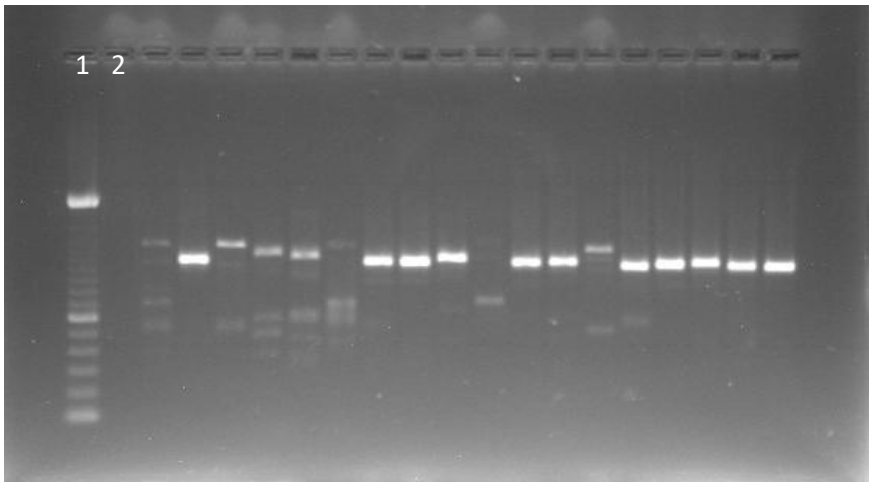
similar results using 5% CTAB in the extraction buffer, confirming that increasing the concentration allows for the extraction of intact DNA with less viscosity, both in terms of quality and quantity. Tan *et al.* (2013) tested the use of 2% CTAB in the extraction of cowpea DNA and emphasized its importance in the cell lysis process, facilitating the release of DNA into most of the solution.

Regarding protein contamination, Sousa Lopes *et al.* (2021) state that the CTAB protocol is more efficient. However, when considering contamination by carbohydrates, phenols, EDTA, or other buffer contaminants, the SDS detergent-based protocol by Dellaporta, Wood and Hicks (1983) yielded better results in fava bean (*Phaseolus lunatus L.*).

CTAB promotes the separation of nucleic acids from polysaccharides, as it causes differential solubility between them (Romano; Brasileiro, 1999). This indicates that lower CTAB concentrations were not effective, given that cowpea leaves may contain high levels of polysaccharides, thus requiring a higher CTAB concentration.

The modifications made to the original CTAB protocol were essential to identify which protocol was the most efficient for DNA extraction from cowpea. After confirming the efficiency of P8 for extracting genomic DNA from the plant tissue of the species under study, it was observed that the PCR reaction was successful (Figure 3). The DNA extracted from the lines was amplified using ISSR 809 as a test primer (Figure 3).

Figure 3 - Photograph of 1% agarose gel electrophoresis showing amplification of DNA extracted from cowpea lines using primer 809. 1. 100 bp molecular weight marker. 2. Blank solution



Source: resarch data.

By analyzing the PCR results (Figure 2) using a 100 bp marker in agarose gel, a high

concentration of DNA with a homogeneous and consistent amplification profile can be observed, as a result of the extractions performed with protocol P8. Thus, the efficiency of P8 for the extraction of cowpea DNA is associated with the use of young leaves immediately frozen in liquid nitrogen, macerated using TissueLyser II with beads for one minute, and with extraction buffer containing 5% CTAB and 2% PVP.

4 Conclusions

The extraction of cowpea DNA was efficient and satisfactory using an extraction buffer containing 5% CTAB and leaves collected at the time of extraction and immediately frozen in liquid nitrogen.

The mechanical maceration method using TissueLyser II with beads was effective and enabled the extraction of high-quality and high-yield DNA. The recommended leaf maceration time is one minute.

The recommended PVP concentration for cowpea DNA extraction can be standardized at 2%, and it is possible to reduce the water bath and centrifugation times to save time without compromising DNA quality.

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