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Selection of protein extraction method for electrophoretic analysis of seeds of the Physic nut genus

Seleção do método de extração proteica para análise eletroforética de sementes de genótipos de Pinhão-manso

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Resumo:

O objetivo do estudo foi extrair e purificar parcialmente as proteínas presentes nas sementes de *Jatropha curcas* L., selecionar o melhor método de extração para caracterizar o perfil eletroforético proteico de genótipos pertencentes ao Núcleo de Melhoramento Genético e Biotecnologia, priorizando as proteínas inativadoras de ribossomos (RIPs). A padronização foi conduzida em quatro modos, que se distinguiram na forma de extração das proteínas e nas soluções extratoras, sendo estas: I) Tampão Acetato de Sódio (0,45 M) e II), HCl (0,1%)/NaCl (0,6 M), III) NaOH (1M) e IV) Tampão fosfato-salino (PBS). O método de extração proteica com melhor resolução eletroforética foi com PBS, utilizando albúmen das sementes armazenadas. As concentrações de proteínas variaram de 2,75 a 5,15 mg/mL entre os genótipos, mas não divergiram estatisticamente entre si pelo teste de Tukey a 5% de probabilidade. Pelo perfil eletroforético foi possível identificar as RIPs, com peso molecular de 28 kDa, em todos os genótipos avaliados. Perfis de bandas diferentes foram observados nos genótipos UFRB05 e UFRB15. O genótipo UFRB05 se destacou, quando associado com a qualidade das sementes armazenadas e teor de óleo.

Palavras-chave: *Jatropha curcas* L.; proteínas inativadoras de ribossomos; SDS-PAGE.

Abstract:

The study aimed to partially extract and purify the proteins present in the seed of *Jatropha curcas* L., select the best extraction method to characterize the protein electrophoretic profile of genotypes belonging to the Center for Genetic Improvement and Biotechnology, prioritizing ribosome-inactivating proteins (RIPs). The standardization was conducted in four ways, which distinguished in the form of protein extraction and in the extraction solutions, which are: I) Sodium Acetate Buffer

(0.45 M) and II), HCl (0.1%)/NaCl (0.6 M), III) NaOH (1 M) and IV) Phosphate-saline buffer (PBS). The method of protein extraction with the best electrophoretic resolution, which was with PBS, using albumen from the stored seeds. The protein concentrations of the samples ranged from 2.75 to 5.15 mg / mL between the genotypes but did not differ statistically from each other by the Tukey test at 5% probability. It was possible to identify RIPs through the electrophoretic profile, with a molecular weight of 28 kDa, in all evaluated genotypes. Profiles from different bands were observed in the UFRB05 and UFRB15 genotypes. The UFRB05 genotype stood out when associated with the quality of stored seeds and oil content.

Keywords: *Jatropha curcas* L.; ribosome-inactivating proteins; SDS-PAGE.

1. Introduction

The physic nut (*Jatropha curcas* L.), also known as pinha de purga, purgueira, and grão-de-maluco, is an oilseed belonging to the Euphorbiaceae family (Abdelgadir& Van Staden, 2013). A shrub with small, greenish-yellow leaves, with dry fruits, smooth and dark seeds, formed by a hard and woody bark, which grows well in tropical and sub-tropical climates. It stands out as one of the main alternative renewable sources for biofuel production in Brazil since its oil has high fluidity and composition (Laviola et al., 2014; Wani et al., 2012).

In addition to biodiesel, the physic nut has other diverse applications, being used by popular medicine to treat paralysis, rheumatism, gout, skin infections, as well as by industry, in paint and soap manufacture, in agriculture, as a living fence, and recovery of vacant lots (Laviola et al. 2014; Virgens et al., 2017).

Its agronomic yield performance varies between 1,328 to 1,543 kg ha^{-1} in different regions, has greater adaptability, and can be cultivated in soils with low fertility and not irrigated, as it is considered a drought-tolerant crop (Laviola et al., 2014; Laviola et al., 2017).

In general, the genetic diversity of physic nut still needs to be adequately explored, mainly due to the growing world demand for renewable biofuel production to replace traditional petroleum-based fuels given that Brazil stands out as an example of a country that encourages use of biofuel, for example, law number 13.033/2014 determines the addition of 7% of biodiesel to diesel oil sold throughout the country, despite this stimulus very little biodiesel was obtained from physic nut(Pereira et al., 2018; Almeida et al., 2016).

So far, there are no commercial cultivars since the crop is still in the domestication process, and the breeding studies of the species are in the development stage. This situation is mainly due to the lack of uniformity in fruit maturation and harvesting. This species has also suffered losses due to attacks by several phytopathogens (Pereira et al., 2018; Laviola et al., 2014).

At the Center for Genetic Improvement and Biotechnology (NBIO) of the Universidade Federal do Recôncavo da Bahia (UFRB), Cruz das Almas - Bahia, Brazil, there is the Breeding Program of *J. curcas* L., which maintains clones from crosses between half-sibs using vegetative propagules (cuttings), conserving the species' genetic resources and selecting genotypes with favorable oil content for later indication of a new cultivar(s) for biofuel production (Pestana-Caldas et al., 2016; Almeida et al., 2016).

The genotypes obtained from NBIO result from intense work on genetic diversity through the morpho-agronomic characterization and yield performance of clonal accesses (Pestana-Caldas et al., 2016). Currently, the breeding program is developing new studies that aim to analyze genotypic characteristics since the *J. curcas* L. seeds, rich in oil, also have high protein content. However, toxic components, such as phorbol esters and curcin, a toxic ribosome-inactivating protein, prevent this crop from being used as an animal food source (Wang et al., 2020; Lin et al., 2010).

The standardization of a more straightforward method of protein extraction from seeds and studies of the electrophoretic profile of SDS-PAGE proteins (*Sodium Dodecyl Sulphate-*

Polyacrylamide Gel Electrophoresis) in different genotypes may be necessary for this species improvement because despite being old, it is the most practical, cheapest, and used method for qualitative protein analysis among proteomic techniques.

The current study aimed to extract and partially purify the proteins expressed from *J. curcas* L. seeds, select the best extraction method and maturation stage, and characterize the profile protein electrophoretic of genotypes belonging to the NBIO Breeding Program, prioritizing ribosome-inactivating proteins to increase the knowledge base about the referred species.

2. Material and methods

The study was carried out in the Center for Genetic Improvement and Biotechnology (NBIO) laboratory of the Universidade Federal do Recôncavo da Bahia (UFRB). The seeds were obtained from the seed bank stored in the cold chamber (-8 ºC, 34-36% humidity) of the NBIO and from the experimental field of the UFRB Campus, in Cruz das Almas-BA, located in the physiographic region of RecôncavoBaiano, showing the geographical coordinates of 12o40'39' S', 3906'23" W and an average altitude of 220 m. According to the Koppen classification, the climate is sub-humid, with an average annual rainfall of 1.170 mm, ranging between 900 and 1.300 m. The higher rain volumes were verified From March to August. The annual average temperature is 24.1oC, and the soil is classified as LatossoloAmareloÁlicoCoeso, with a clay texture and flat relief.

The seeds from the seed bank were collected, identified, and stored in plastic tubes in a cold chamber (-8 ºC, 34-36% humidity). The seeds from the experimental field, on the other hand, were collected and cleaned for the extraction procedure on the same day of collection.

The method selection test was conducted in four different ways, which distinguished in the protein extraction form. In the extraction solutions, the sample buffer and the gel concentration were the same for all methods.

Following the method of Stephan et al. (2010) with some changes, 6 g of defatted cake were used, obtained from oil extraction, with hexane ≥ 99%, by the Soxhlet method (1879) with adaptations, and 30 mL of the following extraction solutions: I) Acetate buffer Sodium (0.45 M), and II HCl (0.1%) / NaCl (0.6 M). The samples were macerated, homogenized in Vortex (Kylin-Sino Laboratory Instruments CO., LTD.) for 5 minutes, and kept under magnetic stirring, without refrigeration, for 55 minutes. Then they were filtered through a paper filter (Mellita) and stored at 4 °C; III) for the extraction with NaOH (1M). 1.0 g of the defatted cake was used, macerated, and kept in a water bath at 65 °C for 10 minutes. The sample was filtered through a paper filter (Mellita) centrifuged at 1,000 rpm for 10 minutes, and then the supernatant was removed to obtain the protein precipitate.

According to Lin et al. (2010), with adaptations, 10mL of the extracting solution was used for the extraction process with a phosphate-saline buffer of 2 g of seeds (integument and albumen). The sample was macerated, homogenized in Vortex (Kylin-Sino Instruments Laboratory CO., LTD.) for 5 minutes, and kept under magnetic stirring for 55 minutes. Then, they were filtered and centrifuged in a refrigerated centrifuge (Vision) at 12,000 rpm for 20 minutes. The intermediate phase was collected and maintained at 4 °C for approximately 12 hours in ammonium sulfate ($(NH4)_2SO_4$) at 30% and 60% precipitation.

Ahead, the samples were again centrifuged at 12,000 rpm for 20 minutes, the supernatant was removed, and 750 µL of phosphate-saline buffer was added. After all these steps, the samples were dialyzed using dialysis cassettes (Slide-A-Lyzer) of 10,000 MWCO with a capacity of 3-12 mL.

After choosing the best method, extractions were carried out at different stages of the seed's maturation and separate parts (integument and albumen). Green seeds, ripe (yellowish), dried directly from the tree, and seeds stored in a cold chamber. It was only possible to separate the integument and albumen from the dry seeds. In the other stages, whole seeds were used.

After selecting the extraction and electrophoresis method, proteins of different genotypes were extracted to characterize their electrophoretic profile. For this, six genotypes (UFRB03, UFRB05, UFRB09, UFRB11, UFRB13, UFRB15) from the NBIO Germplasm Bank of the Universidade Federal do Recôncavo da Bahia (UFRB) were used. The availability of stored seeds was the criterion for choosing the genotypes.

Protein quantification was performed using the LabTest Albumin kit. The absorbance reading was performed on an Sf 325nm spectrophotometer (Tecnal©) with a wavelength of 630nm, following the instructions for using the Albumin kit.

Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard protein, in increasing concentrations of 0.15 to 1.2 mg / mL, diluted in ultrapure water (Milli-Q) to obtain a calibration curve. Afterward, it was mixed with the green bromocresol reagent, and after 2 minutes, the absorbance measurements were made.

For the reading on the spectrophotometer (Tecnal©), the samples of the genotypes were diluted 1/10 in distilled water. The concentrations were in a range in which the equipment could perform the reading and within the calibration curve range. Then, they were mixed with the reagent, 1 mL of sample and 100 µL of the reagent, and then were read on the spectrophotometer. The value found was multiplied by ten to obtain the concentration of the pure sample (100%).

The calibration curve was constructed using the Excel program using the linear regression method, from measuring the absorbance of the standard protein increasing concentrations for further analysis of the correlation coefficient (R2). Tukey's statistical test was performed on the R program (R Development Core Team, 2020) at 5% probability. After that, samples from all genotypes were diluted and normalized to 2 mg / mL.

The analysis by electrophoresis was performed using sodium dodecyl sulfate (SDS), as a denaturing agent, according to the method of Laemmli (1970) with modifications, in the proportions of 5% and 10% for the stacking and separation gels, respectively. The samples were dissolved in Hydroxymethyl aminomethane-HCl 80 mM pH 6.8 containing (SDS) 2%, glycerol 10%, and bromophenol blue (0.1%). 25 µL of the sample were applied to each well and 10 µL of the protein standard. The run was carried out at 72 V until the samples passed from the stacking gel to the separation gel and 135 V until the end of the resolving gel. As molecular weight markers, Bio-Rad (10 to 250 kDa) and Novex® pre-Stained (3.5 to 260 kDa) protein markers were used. The gels were stained with Brilliant Coomassie Blue R-250 (VETEC).

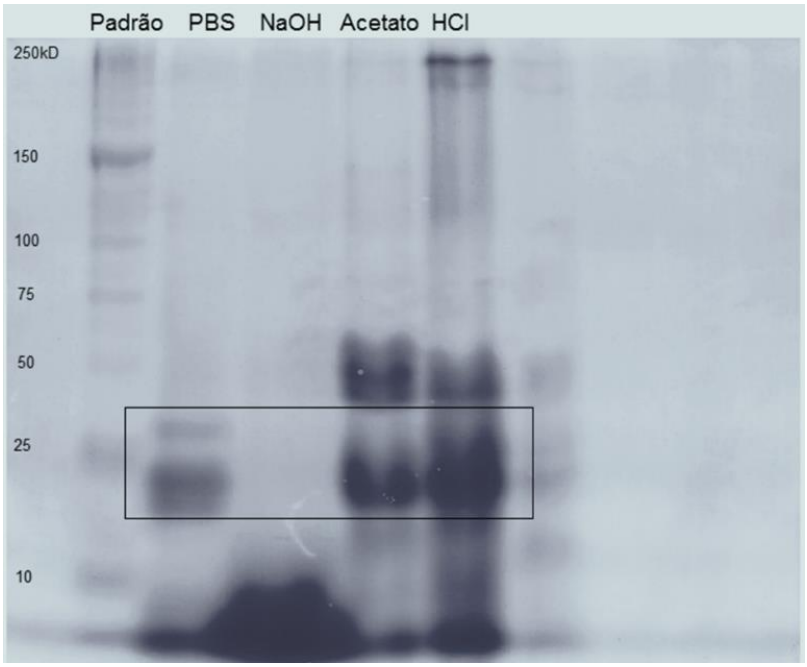
The use of data from the genome of the species J. curcas L. was registered in the National System for the Management of Genetic Heritage and Knowledge, which generated the following code: A80A30C.

3. Results and discussion

The analysis of the protein extracts of J. curcas L., in SDS-PAGE, under different extraction modes, using stored seeds, showed a different band profile, evidencing the extraction power of each method. Technically, the protein bands present in the range between 20 to 40 kDa (kiloDalton) within the rectangle in the figure suggesting the presence of RIPs (Figure 1).

Figure 1. Electrophoretic profile in one-dimensional SDS-PAGE gel of fractions from protein extracts of *J. curcas* L. seeds obtained by different extraction methods. Bio-Rad protein marker pattern (10 to 250 kDa); PBS: *Phosphate-buffered saline*. NaOH: Sodium hydroxide. HCl: Hydrochloric Acid. kDa: kiloDalton

Figura 1. Perfil eletroforético em gel SDS-PAGE unidimensional de frações de extratos proteicos de sementes de *J. curcas* L. obtidas por diferentes métodos de extração. Padrão de marcador de proteína Bio-Rad (10 a 250 kDa); PBS: solução salina tamponada com fosfato. NaOH: hidróxido de sódio. HCl: ácido clorídrico. kDa: kilodalton



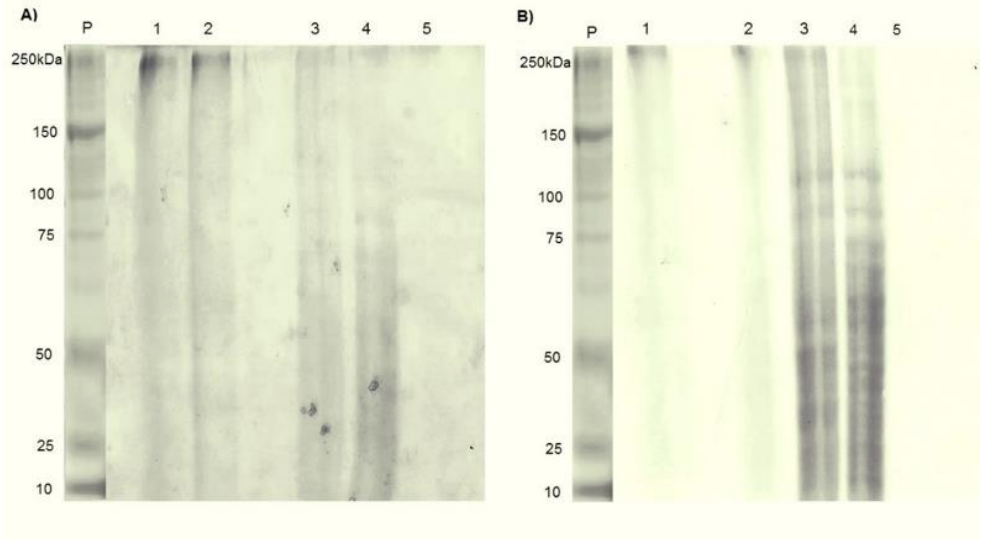
According to Figure 1, it is possible to observe that the electrophoretic profile, from extracts obtained by the extraction method using sodium hydroxide (NaOH), did not present any band. Despite the extracts obtained by Acetate Buffer and Hydrochloric Acid (HCl) presented stronger bands, the use of phosphate-saline buffer as the extracting solution was the one presenting the best band resolution. With this, we opted to use the phosphate-saline buffer as an extraction method for the next steps (Figure 1).

Besides presenting a better resolution of the bands in the electrophoretic profile, the extraction of proteins using the phosphate-saline buffer is simpler and easier since it is unnecessary to degrease the seeds, thus reducing the cost and time. Additionally, this method preserves the proteins by preventing them from being denatured when subjected to high temperatures in oil extraction using the Soxhlet extraction method and losing their functions, which would be much more interesting for tests against fungi.

The second test was carried out with different stages of seed maturation (green, yellow/ripe, and dry harvested from the plant and the ground, using whole seeds (albumen + tegument) for all types of seeds. Typically, only the albumen was tested, but only the tegument was analyzed for dry and harvested seeds in the plant and the ground. In the electrophoretic profiles, it was observed that the banding trace only appeared in the albumen extractions of the seeds harvested in the ground, with the method selected with phosphate-saline buffer. It is assumed that these proteins are most expressed when they reach full maturity and/or when they are exposed to infections caused by fungi (Figure 2), corroborating with Qin et al. (2010), who report that the curcin found in the J. curcas L. seeds reaches its peak of expression during the mature embryonic period.

Figure 2. Electrophoretic profile in one-dimensional SDS-PAGE gel of fractions from protein extracts of *J. curcas* L. seeds at different stages of maturation. Protein extracts were obtained using a phosphate-saline buffer. A) 1- Seeds of green fruits (albumen + tegument); 2- Seeds of ripe fruits (albumen + tegument); 3- Seeds harvested from the plant (albumen + tegument); 4- Seeds harvested from the plant (albumen); 5- Seeds harvested from the plant (tegument); B) 1- Seeds of green fruits (albumen + tegument); 2- Seeds of ripe fruits (albumen + tegument); 3- Seeds harvested from the ground (albumen + tegument); 4- Seeds harvested from the ground (albumen); 5- Seeds harvested from the ground (tegument); P: Molecular Weight Standard, Bio-Rad protein marker (10 to 250 kDa)

Figura 2. Perfil eletroforético em gel SDS-PAGE unidimensional de frações de extratos proteicos de sementes de *J. curcas* L. em diferentes estágios de maturação. Extratos proteicos foram obtidos usando um tampão fosfato-salino. A) 1- Sementes de frutos verdes (albumen + tegumento); 2- Sementes de frutos maduros (albumen + tegumento); 3- Sementes colhidas da planta (albúmen + tegumento); 4- Sementes colhidas da planta (albumen); 5- Sementes colhidas da planta (tegumento); B) 1- Sementes de frutos verdes (albumen + tegumento); 2- Sementes de frutos maduros (albumen + tegumento); 3- Sementes colhidas do solo (albumen + tegumento); 4- Sementes colhidas do solo (albumen); 5- Sementes colhidas do solo (tegumento); P: Padrão de Peso Molecular, marcador de proteína Bio-Rad (10 a 250 kDa)



The sample's protein concentrations did not differ statistically from each other by the Tukey test at 5% probability (R Development Core Team, 2020). The highest concentration was given to the UFRB15 genotype, and the lowest was to the UFRB11 genotype. Although these do not diverge, in the future, this character may be correlated with the others in the search for superior genotypes (Table 1).

Table 1. Average Protein Concentrations present in the samples of physic nut genotypes

Tabela 1. Concentrações Médias de Proteínas presentes nas amostras de genótipos de pinhão-manso

Genotypes	Absorbances	Average concentration in 10% (mg/ml)	Total concentration average (mg/ml)
UFRB03	0.304	0.428	4.283[a]
UFRB05	0.282	0.367	3.672[a]
UFRB09	0.294	0.400	3.996[a]
UFRB11	0.249	0.275	2.754[a]
UFRB13	0.325	0.488	4.877[a]
UFRB15	0.335	0.515	5.155[a]

Means followed by the same letter, in the same column, do not differ from each other using the Tukey 5% significance test. **Source:** Research data.

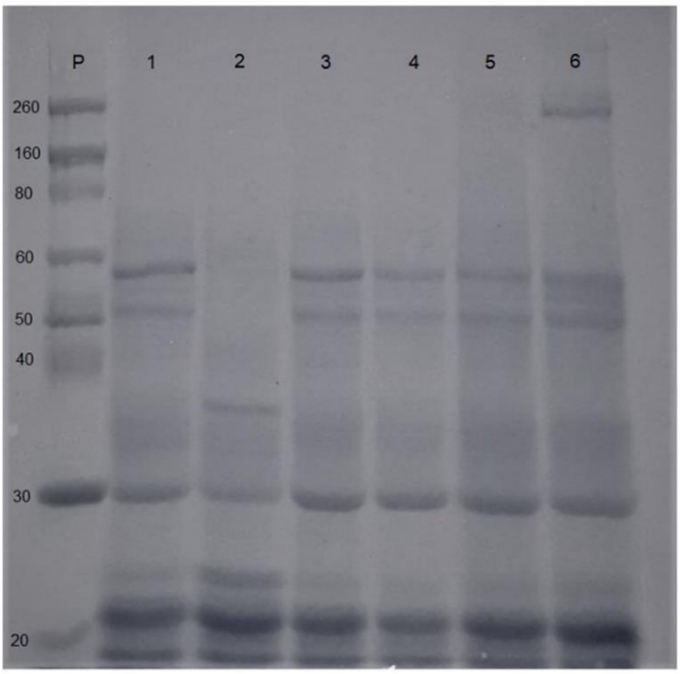
For the electrophoretic profile analysis of proteins, the samples were diluted to a concentration of 2 mg/mL to ensure no influence on the band identification between genotypes with different protein concentrations.

In the genotype electrophoretic profile characterization, it was possible to observe some differences in the band pattern (Figure 3). The same was verified in protein profile studies between

species of the genus Capsicum obtaining indications of genetic diversity, showing the seed and leave electrophoresis effectiveness for characterizing, identifying, and differentiating plants (Olatunji &Morakinyo, 2015).

Figure 3. Electrophoretic profile in one-dimensional SDS-PAGE gel of fractions from seed protein extracts of *J. curcas* L. genotypes. Concentration of 2 mg / mL. P: Novex Pre-Stained Protein Molecular Weight Standard (3.5 to 260 kDa) Genotypes: 1 - UFRB3; 2 - UFRB5; 3 - UFRB9; 4 - UFRB11; 5 - UFRB13; 6 - UFRB15

Figura 3. Perfil eletroforético em gel SDS-PAGE unidimensional de frações de extratos proteicos de sementes de genótipos de *J. curcas* L.. Concentração de 2 mg/mL. P: Padrão de peso molecular de proteína pré-corada com Novex (3,5 a 260 kDa) Genótipos: 1 - UFRB3; 2 - UFRB5; 3 - UFRB9; 4 - UFRB11; 5 - UFRB13; 6 - UFRB15



All genotypes presented bands with a molecular weight of ~ 20 and 30 kDa in their electrophoretic profile (Figure 3). Lin et al. (2010), when extracting and purifying proteins from physic nut seeds, obtained extracts with a molecular mass similar to that reported in this work, of 28.2 kDa, identifying it as curcin, ribosome-inactivating protein. Based on this, it is suggested that the extracted protein was the one expected to obtain.

The class of proteins in question is considered toxic. They act by preventing the elongation of protein synthesis through the cleavage of an adenine located in the loop of the ribosomal RNA, which can cause cell death (Liu, 2017). Also, they have different roles in the plant defense system, acting against biotic and abiotic stresses. However, these proteins' action mechanism in defense has not yet been fully established. Nevertheless, several studies show that RIPs have antifungal, antibacterial, antiviral roles and insecticide activity.In vitro studies proving these antifungal and antiviral functions have already been carried out with several species. The transgenic plants receiving RIP genes showed greater tolerance to fungi and viruses (Zhu et al., 2018; Zhu et al., 2020).

It is suggested that when plants undergo some stress, RIPs are released from their compartments and inactivate the protein synthesis machinery, leading to programmed cell death, preventing the pathogen's proliferation (Rust et al., 2017).

The UFRB05 genotype showed a weaker band profile in the region between 50 kDa and 60 kDa, also showing a stronger band between 30 kDa and 40 kDa, and a band between 20 and 30 kDa that did not appear in any other genotype. It can be suggested that this genotype presented, in addition to the RIP type 1, 30 kDa, a RIP type 2, with two polypeptide chains (Figure 3).

The seeds used in the research were collected and stored in a cold chamber, altrought under controlled conditions, some genotypes showed much fungal contamination (Figure 4).

Figure 4. Photograph of the seeds of the genotypes used in the research
Figura 4. Fotografia das sementes dos genótipos utilizados na pesquisa

However, the UFRB05 genotype presented seeds in better conditions of use, with few fungi contaminations (Figure 4). Thus, it is assumed that these proteins found in this genotype may be associated with seed resistance, ensuring better quality during storage. In the study by Farias (2018), working with identical genotypes, it was found that the UFRB05 genotype was among the superior ones in terms of oil content.

Jatropha has a Type II RIP in its genome, a ricin-like protein, more cytotoxic than type I RIPs, with a molecular weight of ~ 60 kDa. Such proteins are composed of 2 polypeptide chains; the A chain is a RIP domain and a lectin-like B chain. When going through the denaturation process, disulfide bridges break due to the action of sodium dodecyl sulfate (SDS), appearing separated with 32 and 34 kDa molecular weight bands (Souza et al., 2018).

It is assumed that the bands appearing between 50 and 60 kDa in the gels are Globulins, more specifically Globulin 11S, a reserve protein already reported in several studies with seed protein electrophoretic, also present in the Jatropha genome, with a molecular mass of ~ 53.63 kDa (Cavalcanti & Bora, 2010).The UFRB15 genotype also showed significant differences. In this case, it exhibited a 260 kDa band, not yet described in the literature.

It was not possible fully purifying and eliminating the other storage proteins in the seeds so that only curcin remain. It would be necessary for the samples to go through a few more steps for this to happen. Here, a partial purification was carried out, proceeding to the dialysis stage, removing excess salts (ions) from the sample buffer.

Such a study will be essential to guide the other studies being developed by the Nucleus. Some tests have already been carried out with phytopathogenic fungi of interest to the crop, such as Lasiodiploidia theobromae and Colletotrichum gloeosporioides, but they are still in progress. In the following stages of the study, we intend to carry out new tests with new methods, for later use of these RIPs, in a purified form, as biopesticides as already described for other species as well (Suharti&Djam'an, 2019). Besides, this study is another step, among many more in a genetic improvement program, to obtain new cultivar(s).

4. Conclusions

The best extraction method is with the addition of phosphate-saline buffer, easy to perform, low-cost, and expressing better band resolution.

The electrophoretic band profile varies between genotypes. When associated with the quality of stored seeds and oil content, the UFRB05 genotype stands out.

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