“Mofumbo” seed storage

Título resumido no idioma original (Fonte Calibri 12, negrito)

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Physiological and biochemical responses on “mofumbo” seeds during storage

Autor 1[1] (Código ORCID), Autor 2[2], (Código ORCID), Autor 3[2] (Código ORCID), Autor 4[2] (Código ORCID)

Linhas numeradas. Margens de 2cm

Um máximo de seis autores

ABSTRACT: "Mofumbo" (*Combretum leprosum* Mart. (Combretaceae) is a neotropical species of wood, pharmacological, beekeeping and environmental importance. Thus, the objective was to evaluate the biochemical and physiological responses in *C. leprosum* seeds under different storage environments. The experiment was carried out in a factorial (2 x 7), with two storage conditions (natural and climate-controlled environments) and seven storage periods (0; 60; 120; 180; 240; 300 and 360 days), in four replications per treatment. At the beginning of storage and at 60-day intervals, the seeds were analyzed for water content, physiological potential (germination, germination speed index, root and shoot lengths and seedling dry mass) and biochemical (neutral lipids, lipid peroxidation, total and reducing sugars, total free amino acids, and starch). The results indicated that storing “mofumbo” seeds for 360 days reduced viability, being more pronounced after 180 days, regardless of the storage environment. “Mofumbo” seeds were stored in a natural environment for 180 days, resulting in less lipid degradation. After this period, the reduction was associated with the attack of reducing sugars on amino acids. Thus, it is concluded that “mofumbo” seeds must be stored in a natural environment for up to 180 days.

Fonte Calibri 12. Espaçamento duplo. Máximo de 200 palavras.

Máximo de cinco, que não constam no título

Index terms: *Combretum leprosum*, Combretaceae, physiological potential, seed biochemistry.

Afiliações correspondentes

[1]Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisa Agroflorestal de Roraima, Caixa Postal 133, 69301-970 - Boa Vista, RR, Brasil.
[2]Centro Universitário Ingá, departamento Agronomia, Avenida Munhoz da Rocha, Mandaguaçu, Paraná, Brasil.
* Corresponding author: xxx@hotmail.com

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RESUMO: Mofumbo (*Combretum leprosum* Mart. (Combretaceae) é uma espécie neotropical de importância madeireira, farmacológica, apícola e ambiental. Dessa maneira, objetivou-se avaliar as respostas bioquímicas e fisiológicas em sementes de *C. leprosum* sob diferentes ambientes de armazenamento. O experimento foi conduzido em fatorial (2 x 7), sendo duas condições de armazenamento (ambientes natural e climatizado) e sete períodos de armazenamento (0; 60; 120; 180; 240; 300 e 360 dias), em quatro repetições por tratamento. No início do armazenamento e em intervalos de 60 dias, as sementes foram analisadas quanto ao teor de água, potencial fisiológico (germinação, índice de velocidade de germinação, comprimentos de raiz e parte aérea e massa seca de plântulas) e bioquímico (lipídios neutros, peroxidação de lipídios, açúcares totais e redutores, aminoácidos livres totais e amido). Os resultados indicaram que armazenar sementes de mofumbo por 360 dias reduziu a viabilidade, sendo mais acentuada a partir dos 180 dias, independente do ambiente de armazenamento. O armazenamento das sementes de mofumbo em ambiente natural manteve o vigor por 180 dias, proporcionado pela menor degradação de lipídios. Após esse período, a redução esteve associada ao ataque dos açúcares redutores aos aminoácidos. Com isso, conclui-se que as sementes de mofumbo devem ser armazenadas em ambiente natural por até 180 dias.

Termos para indexação: *Combretum leprosum*, Combretaceae, potencial fisiológico, bioquímica de sementes.

Introduction

"Mofumbo" (*Combretum leprosum* Mart. (Combretaceae) is a neotropical species existent in Brazil and Paraguay (Ribeiro et al., 2020). It has wood, pharmacological, beekeeping importance and potential for restoring degraded ecosystems (Cavalcanti et al., 2019; Leal et al., 2020; Silva et al., 2020). Due to its environmental and medicinal value, studies on propagation, conservation and genetic characterization have been recommended for the survival of the species and its economic use over the next few years (Gomes-Costa and Agra, 2018). "Mofumbo" seeds are desiccation tolerant (never recalcitrant) and do not show tegumentary dormancy (Oliveira, 2012; Pacheco et al., 2014). However, there are still no studies on the time and/or conditions in which the seeds of this species can be stored to preserve maximum vigor and delay the effects of natural deterioration.

Fonte Calibri 12. Espaçamento duplo

Seed production in forest species can vary considerably from year to year (Smith and Samach, 2013). Therefore, storage under adequate conditions is essential to preserve the viability and vigor of the seeds, in addition to meeting the needs of seedling production (Oliveira, 2016). However, several factors influence seed deterioration during storage, such as temperature and high relative humidity, which, in concert, increase the intensity.

Seeds are subjected to several degenerative changes that occur after physiological maturity, whose main constituents affected are lipids, carbohydrates, proteins, and vitamins. The existence of these compounds in seeds varies according to plant species, environmental conditions, and variety (Fu et al., 2015). Seed aging affects such reserves, resulting in cell membrane degradation, changes in energy metabolism and in the structure of proteins and enzymes, reduced use of seed reserves, degradation in lipids and carbohydrates, and production of reactive oxygen species (ROS) and toxic compounds (Priestley, 1986; Fotouo-M et al., 2016). Furthermore, this process can be verified by decreased germination, increased number of abnormal seedlings and reduced vigor of

seeds and seedlings (Bilal and Abidi, 2015). In this regard, deterioration is considered an irreversible process, even under improved storage conditions (Kijowska-Oberc et al., 2021).

Therefore, the objective was to evaluate the biochemical and physiological responses in *C. leprosum* seeds under different storage environments.

Material and Methods

"Mofumbo" diaspores were collected from different matrices in the municipality of Mossoró, RN (5° 11'16.8" S, 37° 20'38.4" W and 16 m altitude) with a brown epicarp, giving a visible sign of having reached physiological maturity (Barroso et al., 2004), and placed to dry in the shade (± 30 °C and 60% relative humidity) for 72 h. Then, the seeds were manually extracted, processed, placed in single-layer Kraft paper bags, and moved to two storage environments.

Storage conditions were natural laboratory environment (26 ± 3 °C; 55 ± 12% relative humidity) and cold and dry chamber (10 ± 1 °C; 50 ± 7% relative humidity) for 360 days. Before storage (time zero) and at intervals of 60 days, the seeds were evaluated, under both conditions, for water content, germination, vigor, and biochemical determinations.

Water content in the seeds: determined by the greenhouse method at 105 ± 3 ºC for 24 h (Brasil, 2009). For this, two subsamples with 4.5 g of seeds each were used, and the results were expressed in percentage (wet basis).

Germination test in sand: carried out under laboratory conditions with four replicates of 50 seeds each. Initially, the substrate was moistened to 60% of field capacity, whose maintenance was based on daily needs. The final seedling count was performed 19 days after sowing, when germination was stabilized (Pacheco et al., 2014).

Germination speed index: evaluated in conjunction with germination, counting the emerged seedlings on a daily basis (Krzyzanowski et al., 2020).

Seedling length: at the end of the germination test, the length of regular seedlings (radicle and area part) was measured using a graduated ruler in centimeters and the results were expressed in seedling cm^{-1}.

Seedling dry mass: regular seedlings were placed in paper bags and moved to a forced ventilation oven at 65 ºC for 72 h to determine the dry mass weight (Krzyzanowski et al., 2020). The dry material was weighed using a precision analytical balance (0.001 g) and the results expressed in seedlings mg^{-1}.

Quantification of neutral lipids: performed by the gravimetric method using hexane as solvent (Soxhlet, 1879). For this purpose, 1.0 g of macerated seed was placed in a filter paper cartridge and transferred to the Soxhlet set, for a period of six hours. The extraction was performed with hexane, and after the evaporation and recovery of this solvent was complete, the samples were weighed, and the lipid contents were determined.

Lipid peroxidation: determined based on the amount of malonaldehyde (MDA) present in seeds, from the reaction of thiobarbituric acid, according to the methodology proposed by Heath and Packer (1968). For this quantification, 0.2 g of seed macerated in 2 mL of trichloroacetic acid (0.1%) was used. Soon after, the material was centrifuged at 10,000 rpm for 5 min. After centrifugation, 0.25 mL of the supernatant was collected and mixed with 1.0 mL of a solution containing 0.5% thiobarbituric acid (w/v) + 20% TCA (w/v) and kept incubated for 30 min in a water bath at 95 ºC and then placed on ice to stop the reaction.

200 mg samples of frozen seeds were macerated in 4 mL of 80% ethanol (v/v), placed in a water bath (60 °C) for 20 min and then centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatants were collected for the quantification of low molecular weight soluble metabolites (total free amino acids, total soluble sugars and reducing sugars) and the precipitates were stored to be used in starch determination.

Total and reducing soluble sugars and amino acids: determined by the anthrone method, established by Yemm and Willis (1954). For reducing sugars, the colorimetric method of dinitrosalicylic acid was used (Miller, 1959). Total free amino acids were determined by the method of Peoples et al. (1989), using the ninhydrin reagent and having glycine as the standard amino acid.

Starch: precipitates extracted from soluble compounds of low molecular weight (total free amino acids, total soluble sugars and reducing sugars) were used. For this, the precipitates were macerated in 4 ml of 30% (v/v) perchloric acid for resuspension. The extract obtained was centrifuged at 3,000 rpm for 5 min at 4 °C, and its quantification was based on the anthrone reagent method (Morris, 1948; Yemm and Willis, 1954).

Statistical analysis: the treatments were distributed in a completely randomized design, in a factorial scheme (2 x 7), with two storage conditions and seven storage periods (0; 60; 120; 180; 240; 300 and 360 days), in four repetitions. At the end of the experiment, data from each period were submitted to homogeneity and normality tests. When the significant interaction was verified, the quantitative factor (storage periods) was submitted to regression analysis. If this is not the case, the means of the qualitative factor (storage environment) were compared using the Tukey test at 5% probability. Data were analyzed using the Beta Assistat 7.7 software (Silva, 2015).

Results and Discussion

The initial water content of "mofumbo" seeds was 11.5% and during storage it ranged from 0.3% and 0.5% for the natural environment and climate chamber, respectively, for 360 days (Table 1). This small difference in the water content of the seeds is due to the permeable paper packaging that allowed the exchange of water vapor with the air in these environments.

Seed viability was maintained over 360 days, regardless of the storage environment. The decrease in vigor was evidenced by the decline in germination during storage. This fact was more

149 evident after 180 days, whose reductions were 22% and 28% for seeds from natural and climate-
150 controlled environments, respectively (Figure 1A). Unlike the seeds of *Tabebuia caraiba* Mart.,
151 germination significantly decreased during storage in a natural environment, with total loss of
152 vigor after 90 days (Guedes et al., 2012). According to these authors, the rapid loss of seed vigor
153 was due to the increase in water content during storage, a fact that was not verified for
154 "mofumbo" seeds. Similarly, Abbade and Takaki (2014) with *Tabebuia roseoalba* seeds also found
155 that the storage period significantly compromised germination, decreasing from 88% to 14% at the
156 end of 24 months.

157 The germination speed index indicated a reduction in the vigor of "mofumbo" seeds during
158 storage. Seeds that were stored in a natural environment for 180 days maintained higher vigor
159 (2.3) than those that were stored in an air-conditioned chamber (1.7) (Figure 1B). A similar fact
160 was verified by Guedes et al. (2010), whose highest germination speed indices for *Amburana*
161 *cearensis* were verified in seeds from a natural laboratory environment. On the other hand,
162 Oliveira et al. (2018) verified that the household refrigerator was efficient for the storage of seeds
163 of *Schinus terebinthifolius* Raddi. In this environment, the germination speed index and the
164 percentage of regular seedlings were higher than in the natural environment.

165 By analyzing the bimonthly mean values for seedling length, it is possible to see a decreasing
166 linear trend, with a reduction of 2.1 cm at the end of the storage period (Figure 2A). As for the dry
167 mass content of seedlings, it was found that after twelve months of storage there was also
168 variation, being initially 0.34 g and finally 0.12 g of dry mass (Figure 2B). This reduction is
169 associated with a greater consumption of seed reserves during storage, mainly for the
170 maintenance of viability for a longer period, which consequently led to a reduction in seedling
171 vigor. Results in this regard were also verified by Padua et al. (2019) on *Acacia mangium* seeds
172 stored for 15 months in household refrigerator, freezer, and climate room environments.

173 The results of seedling dry mass also showed significance for the environmental factor, with a
174 higher mean for the natural environment (0.30 g) (Table 2). In agreement with the results
175 presented, the controlled environment caused a reduction (close to zero) in the dry mass content
176 of *Amburana cearensis* seedlings at 270 storage days (Guedes et al., 2010). Thus, it was found that
177 "mofumbo" seeds stored in a natural environment maintain vigor for up to 180 days.
178 Parallel to the loss of physiological potential, there was also a loss in the biochemical quality
179 of the seeds. There was a degradation of the lipid reserve, accumulation of soluble metabolites,
180 such as sugars and amino acids, in addition to the hydrolysis of reserve polysaccharides, such as
181 starch.
182 Regarding the variable of neutral lipids, which constitutes the main component of
183 "mofumbo" seed reserve (Sousa, 2013), there was little variation over the storage period, with a
184 reduction of 1.3% of this reserve content at the end of the 360 days (Figure 3A). The lipids stored
185 in "mofumbo" seeds are degraded in a stable way regardless of the storage condition, not showing
186 that there was no direct connection with the reductions in viability (Figure 1A) and seed vigor
187 (Figure 1B), during the storage period.
188 The degradation of reserve lipids was also verified in *Tabebuia roseoalba* seeds (Abbade and
189 Takaki, 2014), with a progressive and accentuated reduction in the content of this compound only
190 after twelve months of storage. It was also found that the degradation of reserve lipids in *Moringa*
191 *oleifera* Lam. seeds occurred more intensely, both in a chamber environment and in a refrigerator,
192 after twelve months of storage (Oliveira et al., 2017).
193 In the analysis of lipid peroxidation, it showed a significant isolated effect, only for the
194 storage period. In terms of thiobarbituric acid reactive substances, there was an increase in the
195 concentration of malonaldehyde up to 240 days of storage and, after this period, the values
196 decreased (Figure 3B). Probably the decrease in the level of malonaldehyde was due to the
197 reduction in the content of unsaturated fatty acid, which is the precursor of this reaction (Bewley

et al., 2013). When lipid peroxidation occurs at the cell membrane level, it affects permeability, promoting the loss of solutes to the environment, thus causing a reduction in seed viability (Herbele et al., 2019).

Lipid peroxidation is used in several studies as one of the main indicators of seed deterioration, promoting amino acid degradation (Afzal et al., 2020; Moller et al., 2020). Thus, it is understood that there should be a correlation between these variables. However, such a relationship was not verified in this experiment because as the concentrations of malonaldehyde increased, there was an increase in the amino acid level (Figures 3B and 4D). The lack of correlation between lipid peroxidation and loss of seed quality was also observed by Borges et al. (2015) with *Melanoxylon brauna* Schott stored seeds. These results indicated that the reduction in seed viability would be associated with other biochemical processes.

Similar to the results of neutral lipids and peroxidation, the concentrations of total soluble sugars (Figure 4A) and reducing sugars (Figure 4B) also showed significant differences for the isolated factor of storage period. There was an accumulation of total soluble sugars up to 180 storage days (59.1 µmol of Gli gMF^{-1}) and, soon after this period, the behavior was marked by reduction, with an increase in the levels of reducing sugars until storage was completed. Similar results were found in *Jatropha curcas* L. seeds, whose total soluble sugar content increased up to 180 storage days, followed by a marked reduction until the end of the 265-day period (Moncaleano-Escandon et al., 2013).

During the seed deterioration process, when there is a decrease in the content of total soluble sugars, there is an increase in the levels of reducing sugars. As a result, there are losses in the capacity to use carbohydrates, affecting the mobilization of reserve tissues for the embryonic axis, with a vigor decline (Marcos-Filho, 2015). In research by Felix et al. (2020) with *Pityrocarpa moniliformis* seeds, the authors found that the quality loss of seeds stored in a growth chamber for 360 days may be related to the accumulation of reducing sugars.

223 Regarding the starch content, there was a significant effect for the isolated factor of storage
224 period. The linear decline in this polysaccharide content was verified over the storage period
225 (Figure 4C). However, diverging from this result it was found that the starch content in the seeds
226 of *Moringa oliifera* (Oliveira et al, 2017) and *Pityrocarpa moniliformis* (Felix et al., 2020) had no
227 significant changes throughout the storage periods. Considering that "mofumbo" seeds have their
228 metabolism active, it certainly provided greater amylase activity. As a result, there was cleavage of
229 carbohydrates, while the starch was broken down to maintain the stock of soluble sugars and be
230 used as a respiratory substrate (Marcos-Filho, 2015).
231 Amino acid concentrations in "mofumbo" seeds had a significant effect for the two isolated
232 factors, and seeds stored in a natural environment resulted in greater amounts (Table 3). The
233 increase in amino acid content was verified up to 180 days of storage and, from then on, there
234 was a sharp decline until the end of the 360 days (Figure 4D). Thus, it is assumed that this increase
235 in amino acid content during the storage period of the seeds is related to the increase in
236 proteolytic activity, with the likely occurrence of deterioration reactions in reserve proteins (Pádua
237 et al., 2019). On the other hand, the decrease is linked to the occurrence of the Maillard reaction,
238 considering that it is characterized by the non-enzymatic attack on amine groups by reducing
239 sugars (Veselova et al., 2015).
240 Comparing the results of reducing sugars (Figure 4B) and amino acids (Figure 4D), there is an
241 association between the data, which consequently culminated in the reduction of seed vigor. The
242 accumulation of amino acids was also verified in *Jatropha curcas* seeds, stored for 12 months, in
243 natural and refrigerated environments (Moncaleano-Escandon et al., 2013). On the other hand,
244 the amino acid content remained unchanged throughout the storage period of *Moringa oleifera*
245 seeds, regardless of the environmental condition (Oliveira et al., 2017).
246 Regardless of the environmental storage condition of "mofumbo" seeds, the physiological
247 variables of germination, germination speed index, seedling length and total dry mass had slow

248 decreases from the first 30 storage days, showing loss of vigor due to the time. Biochemical
249 evaluations of neutral lipids, lipid peroxidation, total soluble sugars and total amino acids are good
250 indicators of vigor reduction of "mofumbo" seeds, especially after 180 storage days.

251 Conclusions

252 Storing “mofumbo” seeds for 360 days reduce viability, being more pronounced after 180
253 days, regardless of the storage environment.

254 The storage of “mofumbo” seeds in a natural environment maintains the vigor for 180
255 days, resulting in less lipid degradation. After this period, the reduction is associated with the
256 attack of reducing sugars on amino acids.

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Acknowledgments

Fonte Calibri 12. Espaçamento simples

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Table 1. Water content (%) of "mofumbo" seeds stored in natural environments and climatized chamber for 360 days.

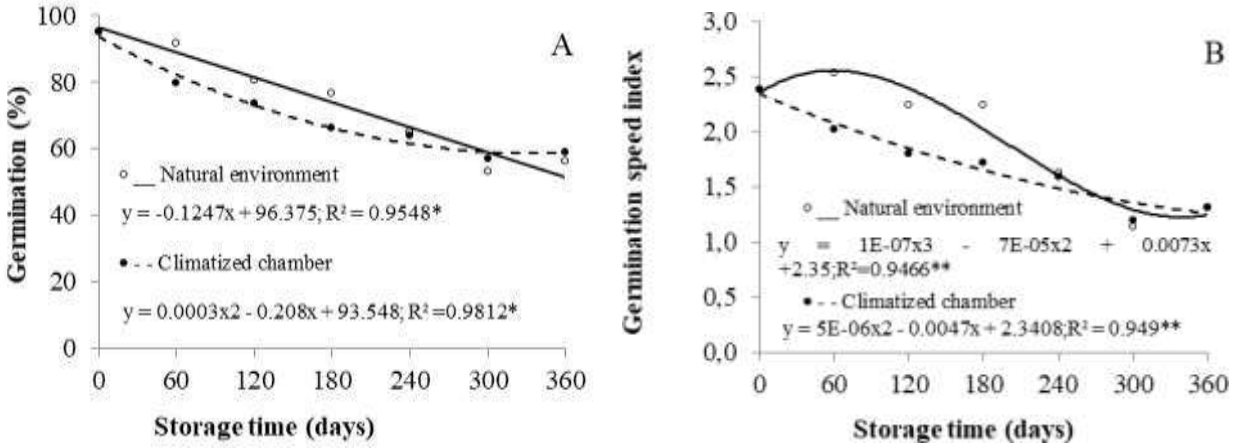
Environment	Days						
	0	60	120	180	240	300	360
Natural	11.5	11.4	11.2	11.3	11.3	11.2	11.2
Climatized chamber	11.5	11.2	11.1	11.0	11.1	11.1	11.1

Table 2. Mean values of dry mass of "mofumbo" seedlings due to the storage environment.

Environment	Seedling dry mass (g)
Natural	0.30 a
Climatized chamber	0.28 b

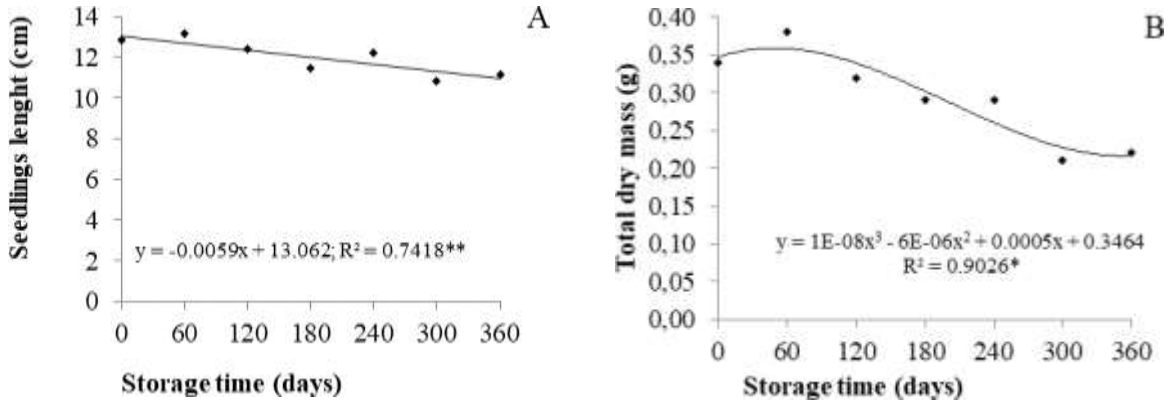
*significant at 5% probability by the F Test.

Figuras: 300 dpi, Fonte Calibri, de preferência editáveis



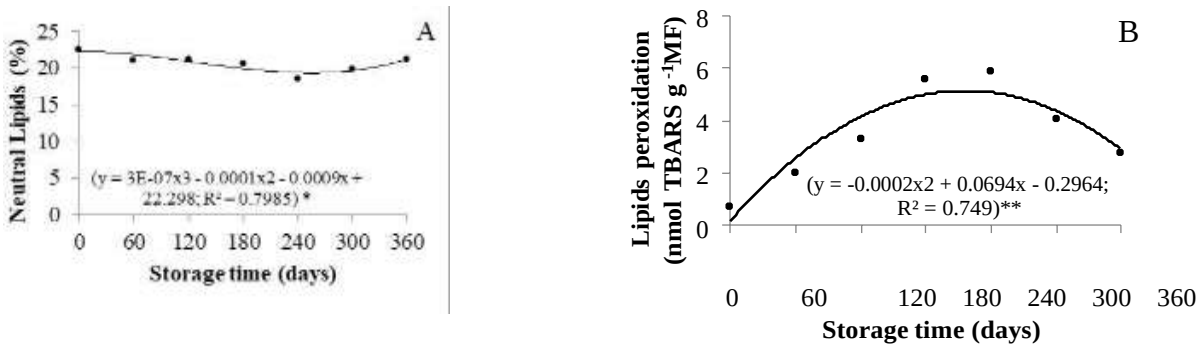
*, ** - Significant at p ≤ 0.05 and p ≤ 0.01, respectively, by the F test.

Figure 1. Germination (A) and germination speed index (B) of "mofumbo" seeds as due to the environment and storage periods.



*, ** - Significant at p ≤ 0.05 and p ≤ 0.01, respectively, by the F test.

Figure 2. Seedling length (A) and total dry mass of "mofumbo" seedlings (B) due to the storage periods.



*, ** - Significant at p ≤ 0.05 and p ≤ 0.01, respectively, by the F test.

493 Figure 3. Neutral lipids (A) and lipid peroxidation (B) of "mofumbo" seeds due to the storage
494 periods.