PHYSIOLOGICAL AND ENZYMATIC CHANGES DURING SEED MATURATION AND GERMINATION OF *Luehea divaricata*

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Recebido para publicação: 04/12/2016 – Aceito para publicação: 22/11/2016

Abstract

Luehea divaricata Mart. & Zucc is tree is of great ecological and economic importance. This study investigated the effect of fruit maturation on the germination and vigor of *L. divaricata*. Fruits were harvested at three maturity stages (unripe or green, brown and open) defined by digital colorimetry and seeds extracted from each stage divided into lots of earliest harvested and stored. Germination test was conducted with four replicates of 25 seeds from each fruit maturity stage in plastic boxes under temperature of 30 ± 2° C. The study was conducted in a 2 x 3 (two lots and three maturity stages) factorial arrangement in a completely randomized design with four replications. Data was submitted to analysis of variance and the means were compared by Tukey at 5%. Germination, germination speed index, average root length and moisture content showed higher values for recently harvested seeds extracted from fruits of all maturity stages compared to stored seeds. LEA protein showed higher activity in seeds from fruits with brown epicarp color of both lots while α-amylase protein activity was higher in the stored seed lot. This shows that better seeds are those that are newly collected fruits and browns.
Keywords: Açoita-cavalo; seed physiological maturity; seed proteins; seed vigor.

Resumo

Alterações fisiológicas e enzimáticas durante a maturação de sementes de Luehea divaricata Mart. & Zucc. Este trabalho objetivou quantificar a influência da maturação dos frutos na germinação e no vigor de sementes de açoita-cavalo. Os frutos foram coletados nos estádios de maturação verdes, marrons e abertos definidos por colorimetria digital cujas sementes foram divididas em dois lotes de sementes recém colhidas e armazenadas. O teste de germinação foi realizado com quatro repetições de 25 sementes para cada estádio de maturação, em caixas gerbox colocados em câmara de germinação a 30±2 °C. O experimento foi conduzido em esquema fatorial 2 x 3 (dois lotes e três estádios de maturação dos frutos), em delineamento inteiramente casualizado com quatro repetições. Os resultados foram submetidos a analise de variância e as médias comparadas pelo teste de Tukey a 5%. A porcentagem de germinação, índice de velocidade de germinação, comprimento da raiz e teor de água apresentaram as maiores médias no lote de sementes recém colhidas, em todos os estádios de maturação fisiológicados frutos comparadas com sementes armazenadas. A proteína LEA apresentou maior atividade em sementes extraídas de frutos com epicarpo marrom de ambos os lotes enquanto a α-amilase apresentou maior atividade no lote de sementes armazenadas.
Palavras-chave: Açoita-cavalo; maturidade fisiológica; proteínas; vigor.

INTRODUCTION

Luehea divaricata Mart. frequently known as açoita-cavalo, açoita-cavalo-miúdo, ibatingui, ivatingui, pau-de-canga, and caiboti (LORENZI, 2008) produces good timber for furniture (CARVALHO, 2008). Chiamolera et al. (2011) recommended the species for forestry programs because of its high annual increment in stem diameter.

In order to obtain high quality seeds it is necessary to pinpoint physiological maturation of both fruits and seeds which is not yet well documented in the production of reproductive structure of *L.divaricata*.

Physiological maturity of fruits is important for determination of the ideal period for harvest, processing, drying, storage and quality control of seeds (AGUIAR *et al*., 2007). Seed physiological maturity is when seeds reach maximum physiological quality, vigor, germination, size and dry matter weight. At that stage, degenerative changes begin compromising germination and vigor (CARVALHO; NAKAGAWA, 2012). Seed physiological maturity is importance for definition of the ideal time to collect seeds with high physiological quality.

For determination of the physiological maturity in seeds, several fruit characteristics are used such as changes of fruit epicarp color (VIDIGAL, 2008; DIAS *et al*., 2006), fruit size and seed weight (DIAS *et al*., 2006; COSTA *et al*., 2006), and seed moisture content (ARAÚJO *et al*., 2006). In light of the above arguments, this essay aimed to determine changes during fruit maturation on germination and vigor of *L.divaricata* seeds.

MATERIALS AND METHODS

We collected fruits of *L. divaricata* from seven mature trees located in the western region Parana state, Brazil, at three stages based on fruit epicarp color: green, brown, and ripe and open fruits. Seeds were manually extracted after collection.

Seeds from each fruit stage were divided into two lots: one formed by fresh collected seeds, while the other lot was formed with seeds stored for 30 days in an uncontrolled environment room.

Fruit mean diameter and length were measured with a digital caliper. Seed moisture content (wet basis) was determined in the laboratory by oven method at 105±3° C for 24 hours (BRASIL, 2009) with four replicates of 100 seeds from each maturity stage and seed lot.

Germination test used four replicates of 25 seeds from each maturity stage. Seeds were sown using Germitest paper, moistened with 2.5 times its weight with distilled water (BRASIL, 2009) inside plastic boxes placed in germination chamber at 30±2° C, with a photoperiod of 12 hours of light.

Measurement of germination were made daily, with normal germinated seedlings being considered as those that presented a developed root system. Data were expressed as percentage of normal germinated seedlings. At the same time, the germination speed index (GSI) was calculated according to Maguire (1962).

Average root length (ARL) was determined with a ruler. For seedling dry matter (SLDM), ten seedlings were taken at random from the substrate, placed in an oven at 65º C for 48 hours for weighing (±0.0001g).

Evaluation of epicarp color was performed by digital colorimetry (ACR-1023 by Instrutherm®) on 25 *L. divaricata* fruits from each maturity stage and reproduced with a software (Photoshop CS6) producing the colors represented in figure 1.

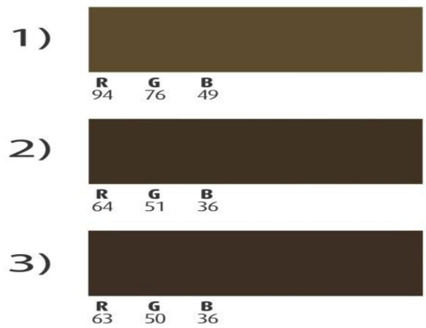

Figure 1. Mean values of the epidermis colors of fruits using a digital colorimeter, as a function of maturity. 1= green stage, 2= brown stage, 3= ripe/open stage. R=red, G= green, B=blue.

Figura 1. Valores médios da coloração de frutos de *L. divaricata* usando o colorimetro digital em diferentes estádios de maturação. 1= verde, 2= marron, 3= aberto. R=vermelho, G= verde, B=azul.

Samples of 1.5 grams of seeds from fruits of each maturity stage were homogenized in 4 mL of 0.01 M sodium phosphate buffer at pH 6.0 (extraction buffer) in a previously cooled porcelain mortar with the addition of 0.04 g of polyvinylpyrrolidone (PVP) during maceration. The homogenized product was centrifuged at 20.000 g for 20 minutes. The supernatant obtained, considered, as the fraction containing soluble proteins was stored at 4^{o} C for later biochemical analyses (LUSSO and PASCHOLATI, 1999).

Activity of α-amylase enzyme and LEA protein were determined using methodology described by Ching (1973). For determination of the α-amylase, 1 mL of starch solution was added to the extract, and the sub-samples placed in an incubator at 30° C. After 5 minutes, 1 mL of I_2+KI and 9 mL of distilled water were added.

Spectrophotometer readings at 620 nm for used 1 mL of buffer solution plus 9 mL of distilled water as blank and the substrate was composed by 1 mL of starch solution plus 1 mL of I_2+KI plus 9 mL of distilled water were made. Total activity of α-amylase enzyme was calculated by {[(substrate) x 620 - (sample) x 620]/5X} x 20 extraction/(0.1aliquot x 1.5 grams of seeds), where X=1µg, while starch = [(substrate) x 620]/1000 with the results being expressed as µg of hydrolyzed starch min^{-1} per seed.

ISSN eletrônico 1982-4688
DOI: 10.5380/rf.v47i1.44203

For determination of the LEA protein, 1 mL of T-med solution (bovine albumin, copper sulfate and sodium hydroxide) was added to the extract, and the sub-samples placed in an incubator at 30° C. After 5 minutes, 1 mL of I_2+KI and 9 mL of distilled water were added.

Spectrophotometer readings at 525 nm for LEA protein used 1 mL of buffer solution plus 9 mL of distilled water as blank and the substrate was composed by 1 mL of starch solution plus 1 mL of I_2+KI plus 9 mL of distilled water were made. The activity of the LEA protein was given by Ct=FC x At, where Ct = test concentration, FC = calibration factor of the LEA protein, At = test absorbance with data expressed in g dL^{-1} x 10.

The essay was conducted as factorial (2 x 3) composed by two seed lots and three fruit maturity stages with four replications. Normal distribution of the data was evaluated by the Lillifords test, which presented normal distribution. The mean values were compared by the Tukey test at 5% probability. Pearson simple correlation analysis was also performed between the results of the enzyme analysis tests and the germination tests using Genes software (CRUZ, 2006).

RESULTS AND DISCUSSION

Length of *L. divaricata* fruits (Table 1) did not result in differences (P>0.05) as a function of maturity stages. This result occurs after the fruit reaches maximum size. Reduction of fruit size depends on the species and on the degree of dehydration found by the end of fruit maturation (MARCOS FILHO, 2005). Different results were reported by Marini *et al*. (2011) with *Luehea grandiflora* Mart. & Zucc where fruits from the open stage exhibited the smallest lengths.

Fruits of *L. divaricata* classified as at the open/ripe stage exhibited greater diameter values (9.70 mm) compared to those classified as green and brown (Table 1). Similar trend was observed by Alves *et al*. (2005) in fruits from *Mimosa caesalpiniifolia* Benth. which showed a gradual increase in length and width throughout the maturation process. However, those authors reported that fruit visual index was not an effective predictor of physiological seed maturity.

Table 1. Average length and diameter of *L. divaricata* fruits in three stages of maturation, Marechal Cândido Rondon, 2012.

Tabela 1. Comprimento e diâmetro de frutos de *L. divaricata* em três estádios de maturidade fisiológica, Marechal Candido Rondon, 2012.

Stages of maturation	**Length**	**Mean diameter**
	-------------- mm --------------	
Green	17.37 a	8.70 b
Brown	16.78 a	8.81 b
Open/ripe	17.17 a	9.70 a
DMS	0.71	0.34
CV (%)	12.6	11.7

Mean values followed by the same letter in the column do not differ among themselves by the Tukey test at 5% probability. CV: Coefficient of variation. DMS: Minimum significant difference.

There was no interaction (P>0.05) between seed lots and seed maturity stages for germination percentage, GSI and SDM. In contrast, there was significant interaction (P<0.05) among ARL, SDM and seed moisture contents (Tables 2 and 3).

The greatest values for seed moisture content were obtained from the fresh collected seed lot of fruits classified as green and brown (Table 2). Even so, those values were smaller than those measured by Marini *et al.* (2012) with *L.grandiflora* seeds that exhibited 13% moisture in the black maturity stage.

Seeds from *L. divaricata* fruits classified as green and open/ripe exhibited greater germination values than from brown fruits. No differences (P>0.05) were detected between the fresh and the stored seed lots. In an analogous way, vigor evaluated by the GSI with seeds from unripe fruits exhibited the greatest values, which differed from those of brown fruits, without difference between the two seed lots (Table 2).

The highest ARL was measured in seedlings obtained from seeds of green and recently harvested fruits compared to those from seedlings produced from seeds of either brown or open/ripe fruit stages as well as stored seed lot (Table 3). ARL was synchronized with GSI and germination percentage, indicating that seeds from green fruits showed greater vigor and germination potential. Therefore, ARL and fruit epicarp color are good indicators of physiological maturity of *L. divaricata* seeds.

In addition, Oro *et al*. (2012) studying maturity stages of cerejeira-do-mato (*Eugenia involucrata* DC.) fruits observed that high germination speed were recorded with seeds from clear red fruits. Those authors stated that seeds from that fruit development stage showed greater vigor similarly with the results in the present essay.

Table 2. Germination percentage (%G), germination speed index (GSI) and moisture content in seed lots coming from fruit harvested at three maturity stages, 2012.

Tabela 2. Porcentagem de germinação (%), índice de velocidade de germinação e grau de umidade em sementes de *L. divaricata* recem colhidas e armazenadas em diferentes estádios de maturação do fruto, 2012.

Seed Lots	Fruit maturity stage			Mean values
	Green	**Brown**	**Open/ripe**	
		Germination (%)		
Newly harvested	62.0	29.2	64.38	51.8 a
Stored	59.0	31.0	54.0	48.0 a
Mean Values	60.5 A	30.1 B	59.1 A	
CV (%)	20.8			
DMS	18.8			
		GSI (days)		
Newly harvested	0.751	0.318	0.596	0.55 a
Stored	0.652	0.2341	0.3802	0.42 a
Mean Values	0.702 A	0.276 B	0.488 AB	
CV (%)	26.08			
DMS	0.23			
		Seed moisture content (%)		
Newly harvested	10.54 Aa	9.68 Aa	8.544 Ba	9.58
Stored	8.348 Ab	7.918 Ab	8.247 Aa	8.17
Mean values	9.44	7.29	8.39	
CV (%)	6.8			
DMS	1.09			

Mean values followed by the same small letter in the column and capital letter in the line do not differ among themselves by the Tukey test at 5% probability. CV= Coefficient of variation. DMS: Minimum significant difference.

Seed lot influenced ($P<0.05$) seedling dry matter. Highest dry matter values were recorded in seedlings from the recently collected seed lot. No differences ($P>0.05$) resulted as result of fruit maturity stages.

On the other hand, seed dry matter (SDM) was higher from seeds of green fruits from both lots. However, seeds from the recently collected seed lot resulted in a greater accumulation of dry matter when compared to stored seeds from unripe fruits (Table 3).

Table 3. Average root length (ARL), seedling dry matter (SLDM) and seed dry matter (SDM) in *L. divaricata* seed lots from fruits harvested as a function of maturity stages, 2012.

Tabela 3. Comprimento médio de raiz, massa seca de plântulas, e matéria seca de sementes de *L. divaricata* recem colhidas e armazenadas em diferentes estádios de maturação do fruto, 2012.

Seed Lots	Maturity stages			Mean values
	Green	**Brown**	**Open/ripe**	
		ARL (cm)		
Recently harvested	3.116 Aa	2.143 Ba	1.68 Ba	2.31
Stored	1.634 Ab	1.387 Ab	1.327 Aa	1.44
Mean values	2.37	1.765	1.503	
CV (%)	14.3			
DMS	0.48			
		SLDM (g Kg^{-1})		
Recently harvested	0.019	0.011	0.016	0.015 a
Stored	0.0071	0.004	0.008	0.006 b
Mean values	0.013 A	0.008 A	0.012 A	
CV (%)	29.4			
DMS	0.006			
		SDM (g Kg^{-1})		
Recently harvested	0.378 Aa	0.309 Ba	0.287 Ba	0.327
Stored	0.332 Ab	0.296 Ba	0.286 Ba	0.304
Mean values	0.355	0.302	0.286	
CV (%)	4.25			
DMS	0.02			

Mean values followed by the same small letter in the column and capital letter in the line do not differ among themselves by the Tukey test at 5% probability. CV= Coefficient of variation. DMS: Minimum significant difference.

L. divaricata seed dry matter trend was similar to that published by Pessoa *et al*. (2010) on the physiological maturity of *Piptadenia viridiflora* (Kunth.) Benth seeds. In that study, the period of greater accumulation of dry matter coincided with greater germination potential.

Seed dry matter is considered by various authors as one of the safest indicator of seed maturity because seed physiological maturity parallel with maximum seed dry matter content (COSTA *et al*., 2006). Additionally, Carvalho and Nakagawa (2012) established a maturity relationship with physical and physiological characteristics such as size, moisture content, dry matter content, germination and vigor.

LEA protein (Figure 2) expressed its greatest values in seeds from fruit at the brown maturity stage of both seed lots. For the other fruit stages, there was a difference between seed lots with greatest activity from material of the stored seed lot. Seed LEA protein not only results in tolerance to desiccation but also promotes stabilization and structuring of membrane system (BLACKMAN *et al*., 1991). LEA proteins protect cellular structures against water loss during maturation (BRAY, 1993).

Additionally, seed desiccation during development is characterized by accumulation of a particular group of mRNAs and LEA proteins. The mRNAs of LEA proteins appear in embryo tissues at the beginning of desiccation and become the most prevalent in the dry state, declining progressively various hours after seed soaking (JOSÉ *et al*. 2005). Such trend explains the high accumulation of the protein in seeds from brown and open/ripe fruit maturity stages in which there were lower values of seed moisture content (Table 2), as well as lower germination potentials.

Seeds from the stored lot showed greater activity of α-amylase enzyme when compared to the recently collected seed lot extracted from green and open/ride fruits. The greatest activity of α-amylase was quantified in samples from the green fruit stage, followed by the brown and open from the stored lot, while in the fresh collected seed lot the highest activity was observed in the brown fruuit stage, followed by the open/ride and green fruits (Figure 2).

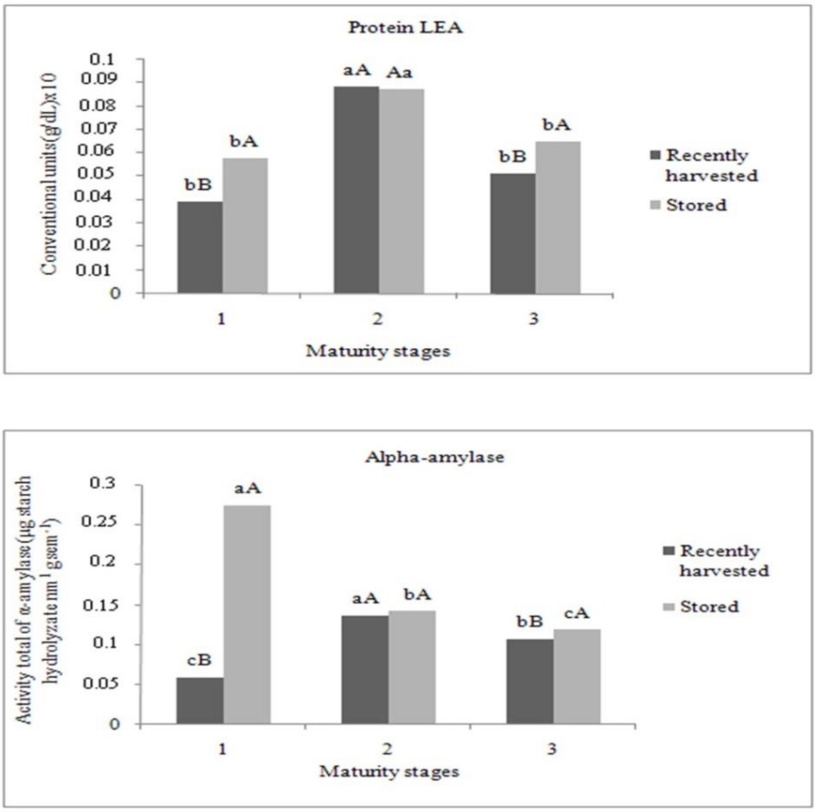

Figure 2. Activity of α-amylase and enzymes LEA protein from two *L. divaricata* seed lots as a function of fruit maturity stages - 1: Green; 2: Brown; 3: Open/ripe. Columns followed by the same lowercase letter for the different stages of maturation and upper case for the different seed lots not differ among themselves by the Tukey test at 5% probability.

Figura 2. Atividade da α-amilase e protein LEA em dois lotes de sementes de *L. divaricata* em função do estádio de maturação. 1: verde; 2: marron; 3: aberto.

During seed germination, α-amylase plays an important role in starch hydrolysis in metabolizable sugars (LIMA *et al*., 2008). Activity of α-amylase is of fundamental importance in seed germination because of the effects from high temperatures during seed drying (ROSA *et al*., 2005). The lack of synchronism between expressiveness of α-amylase and seed vigor is due to the inactivation of the germination process in the seeds used for the test resulting in unreliable data for isolated use of the enzymatic activity test of α-amylase as a vigor index in studies of *L. divaricata* seeds.

The Pearson correlation between percentage and speed rate of germination was significant and positive. On the contrary, correlation between germination percentage and GSI showed high negative values with LEA protein indicating that the greater the values of germination percentage and GSI the lower the values of LEA protein in the seeds. The other variables were independent (Table 4).

Table 4. Pearson correlation coefficient between germination percentage (%G), germination speed rate (GSI), average root length (ARL), LEA protein (LEA), α-amylase, seedling dry matter (SLDM) of *L. divaricata* seeds, 2012.

Tabela 4. Coeficiente de correlação de Pearson entre porcentagem de germinação, índice de velocidade de germinação, comprimento de raíz, proteína LEA, α-amilase e massa seca de plantulas de *L. divaricata*, 2012.

	% G	**GSI**	**ARL**	**SLDM**	**α-amylase**	**LEA**
% G	1	0.86 *	0.21^{ns}	0.52^{ns}	-0.04^{ns}	-0.96^{**}
GSI		1	0.58^{ns}	0.66^{ns}	-0.002^{ns}	-0.88^{*}
ARL			1	0.79^{ns}	-0.47^{ns}	-0.41^{ns}
SLDM				1	-0.64^{ns}	-0.62^{ns}
α-amylase					1	0.22^{ns}
LEA						1

* Significant at 5% and ** at 1% probability of error by the T test.
^{ns} Not significant. ^{b}

Meneses *et al*. (2006) reported that LEA proteins were described on genes abundantly expressed during final stage of desiccation in seed development, thus showing the negative correlation with germination percentage and GSI, which were greater in *L. divaricata* seeds with higher moisture content (Table 2).

CONCLUSION

- *L. divaricata* seeds should be harvested from fruits with green epicarp (R=94, G=76 and B=49).
- Germination percentage, GSI, ARL, SDM, seed moisture content, fruit color and LEA protein are indicators of physiological maturity of *L. divaricata* seeds.
- Germination percentage, GSI, ARL, SDM, and seed moisture content showed the highest values in seeds from green fruits from the recently collected seed lot at all stages of physiological maturity.
- LEA protein showed greater activity in seeds obtained from fruits desplaying brown epicarp from both seed lots.
- The α-amylase enzyme showed greater activity in seeds from the stored seed lot.

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