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The morphological characterization of the dry seeds and reserve mobilization during germination in *Morinda citrifolia* L.[1]

Caracterização morfológica da semente seca e mobilização de reservas durante a germinação em *Morinda citrifolia* L.

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ABSTRACT - Information about the morphology, chemical composition and reserve mobilization is important in understanding the establishment of native and exotic species. The purpose of this study was to describe the morphology, chemical composition, and mobilization of reserves during germination of noni (*Morinda citrifolia* L.). Biometric and morphological analyzes were performed with 100 randomly selected dried seeds. Other seeds were treated with sulfuric acid PA and soaked in Petri dishes. Collected seeds of five different times of germination were used for cytochemical and chemical analysis. For cytochemical analysis, the cuts of 5µm were submitted to dyes TB at pH 4.0; XP at pH 2.5, reaction of PAS and Sudan IV. The seeds were crushed for chemical analysis with lipids, proteins and soluble sugars extraction that were determined gravimetrically, by the Bradford method and the Antrona method, respectively. The fatty acid composition of the dry seed was determined by gas chromatography. Noni seeds are albuminous and have a thick seed coat, rich in lignin. Lipid and protein bodies were observed inside the endosperm cells, representing 43.50% and 9.15% respectively, while the reservoir of soluble sugars was less than 5%. Linoleic acid was the most prevalent with 68.1%. The lipids were mobilized during germination, suffering a reduction of up to 38% of its total. Proteins, as well as lipids decreased by 25.78% during the germination period observed. The main reserves of noni seeds are lipids and proteins that are mobilized during germination to provide energy and matter to the developing embryo and synthesis of more complex compounds.

Key words: Cytochemical. Lipids. Fatty acids. Proteins. Germination.

RESUMO - Informações sobre as características morfológicas, a composição química e mobilização de reservas são fundamentais na compreensão do estabelecimento de espécies nativas e exóticas. O objetivo deste trabalho foi descrever a morfologia, a composição química e a mobilização das reservas durante a germinação das sementes de noni (*Morinda citrifolia* L.). As análises morfológica e biométrica foram feitas com 100 sementes secas escolhidas aleatoriamente. Para as análises citoquímica cortes de 5 µm foram submetidos aos corantes AT em pH 4,0; XP em pH 2,5; reação do PAS e Sudan IV. As determinações químicas para lipídeos, proteínas e açúcares solúveis foram realizadas por gravimetria, pelo método de Bradford e pelo método da Antrona, respectivamente. A composição de ácidos graxos da semente seca também foi determinada por cromatografia gasosa. As sementes de noni são albuminosas e apresentam um tegumento espesso, rico em lignina. Foram observados corpos lipídicos e protéicos nas células do endosperma, representando 43,50% e 9,15%, respectivamente, enquanto que as reservas de açúcares solúveis foram inferiores a 5%. O ácido linoléico foi o mais predominante com 68,1%. Os lipídeos foram mobilizados, sofrendo redução de até 38% do seu total. Como também as proteínas sofreram redução de 25,78% durante o tempo de germinação observado. Portanto, as principais reservas das sementes de noni são lipídeos e proteínas, que durante a germinação são mobilizados para fornecer energia e matéria para o desenvolvimento do embrião e para síntese de compostos mais complexos.

Palavras-chave: Citoquímica. Lipídeos. Ácidos graxos. Proteínas. Germinação.

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INTRODUCTION

Morinda citrifolia L., popularly known as noni, belongs to the Rubiaceae (Rubioideae) family and receives great attention due to its nutritional and medicinal value. Many health benefits have been attributed to this species, which has been one of the main medicinal plants used in Polynesia for over 2.000 years. Given its therapeutic potential, the fruit juice is highly sought after in popular medicine since it is used against several diseases such as diabetes, muscle pains, heart diseases, and it possess cancer preventive effect (BASAR *et al.*, 2010; JENSEN *et al.*, 2006; PAWLUS; KINGHORN, 2007; POTTERAT; HAMBURGER, 2007; WANG *et al*., 2013). There are many studies regarding the medicinal uses, but there is little information on the morphological and physiological characteristics of the noni seed.

Noni forms evergreen shrubs or medium-height trees 3 to 6 m tall (NELSON, 2005), but it may grow over 10 m. Native of Southeast Asia, noni grows in tropical and subtropical climates, being often found near the coast and in forests 400 m above sea level. It was spread by travelers and colonizers of Pacific islands besides ocean currents and migrating birds (MAcPHERSON *et al.*, 2007). It is found in saline, alkaline, low-fertility, shallow, sandy or rocky soils (MATHIVANAN *et al.*, 2005; NELSON; ELEVITCH, 2006; NUNES *et al.*, 2009).

The morphological characteristics of the plants in their early stages contribute to broadening the information, which is useful for understanding the physiology, ecology, phylogeny, and regeneration strategies of the species. Thus the external and internal morphology of the seeds are related to their dispersal and germination strategies. Likewise, the chemical composition can explain biochemical, physiological, cellular, and ecologic aspects, which aids in understanding the different adaptation strategies of the species to their natural environment (BEWLEY *et al*., 2013).

The information on which the seed morphological characteristics helps in the species dispersal, as well as which main reserves are stored and mobilized during germination are critical for increasing knowledge about the species. In face of the remarks on *Morinda citrifolia* L., this study aimed to describe the morphology of the dry seed, as well determine the chemical composition and mobilization of reserves of this specie during the germination.

MATERIAL AND METHODS

The experiment was carried out in the Plant Cell Biology laboratory of the Biology Department and the Seed Analysis laboratory of the Plant Science Department of the Federal University of Ceará - UFC, Brazil. Noni (*Morinda citrifolia* L.) seeds were provided by Embrapa Agroindústria Tropical in Fortaleza, CE, Brazil. After being processed, the seeds were packed in plastic bags and stored at 4 ºC and the following determinations and analyses were performed. In order to determine the weight of a thousand seeds, 16 sub-samples of a hundred seeds each were used according to the guidelines of the Rules for Seed Analysis (BRASIL, 2009).

To external morphology analysis a hundred seeds were randomly chosen. The length, width, and thickness of the seeds were measured with a digital caliper rule with 0.01 mm resolution. Other aspects were also analyzed, such as color, coat texture and consistency, embryo, hilum, and micropyle position, embryo type, cotyledons, as well as the presence of endosperm.

To seed germination, the seeds were sanitized with sodium hypochlorite 1% and then chemically treated with sulfuric acid. Next, they were soaked between germitest® paper sheets in Petri dishes with a water volume equal to three times the weight of the paper. The dishes were placed in a germination chamber at 35 °C ± 2 °C with a 10-hour photoperiod (ELAKKUVAN; MANIVANNAN, 2010) and the seeds were collected at the following times: T0 - quiescent seed; T1 - 8 hours soaking; T2 - radicle protrusion (up to 1 mm); T3 - 2 mm radicle; T4 - 3 mm radical (Figure 1E).

To internal morphology and cytochemical analysis the quiescent and germinated seeds were cut lengthwise and fixated in glutaraldehyde 1% and paraformaldehyde 4% in a phosphate buffer 0.02 M pH 7.2 (KARNOVISKY, 1965) for 48 hours. Next, the material was dehydrated in a graded ethanol series for a later embedding in Historesin (Leica Historesin Embbeding Kit - Jung). The 5 µm slices obtained with a semi-automatic microtome (Slee Mainz CUT 5062) were subjected to the following cytochemical dyes: a) Toluidine Blue (TB) 0.025% pH 4.0 (VIDAL, 1977); b) Ponceau Xylidine (XP) 0.1% pH 2.5 (VIDAL, 1977); c) Periodic Acid-Schiff (PAS) reaction (MAIA, 1979); d) acidified phloroglucinol (JOHANSEN, 1940). The slices were also subjected to polarized light, and free-hand slices were dyed with Sudan IV. The slides were examined in an OLYMPUS UC30 photomicroscope, coupled to a digital camera (model UC30) and a computer, we used the "CELL" *software* for image analysis.

To chemical analysis, the fresh seeds were macerated without the coat (only endosperm and embryo) in a mortar grinder for powder formation, then oven dried at 50 °C for 24 h. After this process, 50 mg of the powder was used for further analysis. The total protein was extracted with NaOH 0.1 M and determined by the method

from Bradford (1976) with Bovine Serum Albumin as a standard. The sugars were extracted with 80% alcohol and determined by the Antrona method (YEMN; WILLIS, 1954) using glucose (Sigma) as a standard. The lipid extract was obtained with hexane and the total content was determined gravimetrically. The fatty acids were analyzed as FAME by the method of Hartman and Lago (1973) in a GC-FID 3800 (Varian). The FAME aliquots (1 µL) were injected and the separation by the gas chromatography was carried out in a DB-5MS capillary column. The pressure of the carrier gas (hydrogen) was kept at 11.5 psi with a column flow of 1.5 mL min^{-1}. The oven temperature was initially kept at 120 °C for 1 min, increasing at 8 °C min^{-1} until 210 °C and then kept at this temperature for 45 min. The injector and detector temperatures were 250 and 280 °C, respectively. The samples were compared to standard FAME data purchased from Sigma in a QP2010 GC-MS system fat 70 eV. The heat and column conditions were the same as the ones used in the GC-FID system with helium as a carrier gas with a flow rate of 1 mL min^{-1}.

Experiments for the biochemical analysis were completely randomized, with four replicates to each treatment. Differences among treatments were tested for statistical significance using a one-way ANOVA followed by a Tukey's test. Data was expressed as a mean of four independent values ± the standard error (SE). All statistical analyses were carried out using the program ASSISTAT 7.7 ($P<0.05$).

RESULTS AND DISCUSSION

The weight of a thousand seeds was 31.77 g, which means that a kilogram of noni seeds contain approximately 31.476 seeds. The noni fruit is a syncarp, and from one fruit, 260 seeds can be obtained, and in 10 kg of fruit, there was reported 250 g of dry seeds, while one kilogram of seeds may contain approximately 40.000 seeds (NELSON, 2005). The length of the noni seed ranged from 7.96 mm to 12.06 mm and the width and thickness range from 3.85 mm to 7.15 mm and 1.57 mm to 3.36 mm, respectively (Table 1).

The seed coat is rough, woody and opaque, presenting a brown-reddish coloring (Figure 1A). Furthermore, the seeds have a slightly tapered base and a round top, with an air chamber in the end opposite to the micropyle, which is lined internally by a layer shiny as cellophane (Figures 1B and 1C). The hilum and micropyle are not well defined; the latter is located in the end opposite the air chamber, where the radicle protrusion takes place. According to Nelson (2005), noni seeds dispersion occur without a specific agent and can be realized by birds, bats, rats, and other mammals, being more commonly dispersed over water. The composition of this "cellophane" layer has not been determined, but it must have a hydrophobic character so the chamber becomes waterproof and fulfills its role. Normally, this structure has the role of making the seed float and be hydrophobic (PONNAIYAN; VEZHAVENDAN, 2005). The noni seed shape can be classified as oblong-triangular, ovoid to obovoid or reniform, bearing an air chamber in the end opposite the micropyle and without wings (MATHIVANAN *et al.*, 2005; PONNAIYAN; VEZHAVENDAN, 2005; SINGH *et al.*, 2006).

The coat protects and obstructs the visualization of the embryo, which is small (few millimeters) and, along with the endosperm, takes up half the seed length (Figure 1B). The embryo of the noni seed is axial and straight, of whitish-translucent color, with a well-defined hypocotyl-radicle axis, and with opposite leafy spatula-shaped cotyledons (Figure 1D). The noni seed embryo is also flat and oily, as described by Nelson (2005). The same type of cotyledon was found by Pietrobom, Paoli and Bieras (2010) when studying *Psychotria hoffmannseggiana*, which belongs to the same family. The seed is albuminous since after maturation it holds an endosperm, which involves the whole embryo. This tissue is oily, has a soft consistency, and is milky transparent, with a darkening on the edges and on the end where the radicle protrudes. The endosperm has an extension in the root axis end as an attached membrane (Figure 1D). This membrane fills the space between the micropyle and the embryo inside the coat.

Table 1 - Average, standard deviation, coefficient of variation (CV) and variation interval for length, width, and thickness (mm) and weight (g) of a thousand seeds noni (*Morinda citrifolia* L.)

Determinations	Mean	Standart deviation	CV (%)	Variation interval
Length (mm)	10.07	0.87	8.63	7.96-12.06
Width (mm)	5.22	0.53	10.22	3.85-7.15
Thickness (mm)	2.49	0.37	14.92	1.57-3.36
Weight of a thousand seeds (g)	3.18	0.14	4.39	2.92-3.31

Figure 1 - A) Noni (*Morinda citrifolia* L.) seed dried; B) Seed cut longitudinally, emphasizing inside of the air chamber and the space occupied by the endosperm and embryo (arrow); C) Cross section of the seed, emphasis the position of the endosperm and the air chamber (◆); D) Embryo surrounded by the endosperm, emphasis: cotyledons (co); embryo (em) e membrane winged (mb); E) Times collected the seed germination of noni with radicle protrusion in t2. Barr: 5µm

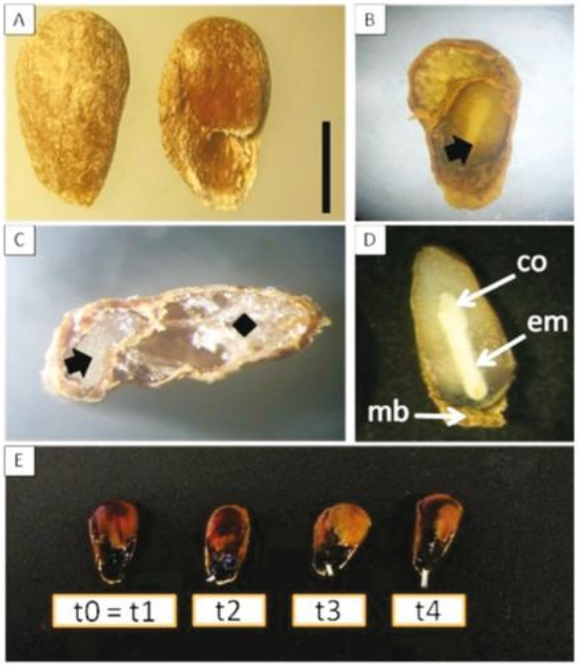
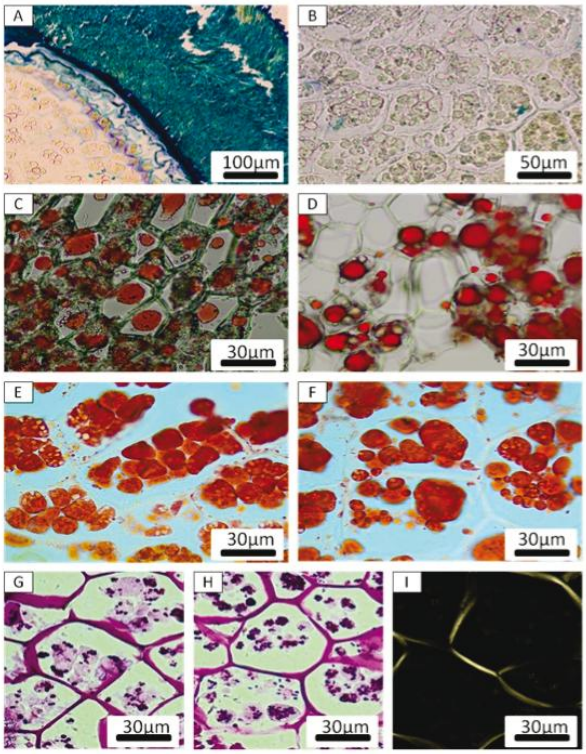


Figure 2 - Transversal noni (*Morinda citrifolia* L.) seed; A) Sections stained with toluidine blue pH 4.0, emphasizing seed coat; B) Endosperm cells; C and D) Endosperm cells stained with Sudan IV, emphasizing the lipid droplets in t0 and t4, respectively; E and F) Endosperm cells stained with Ponceau Xylidine, emphasizing the protein bodies in time t0 and t3, respectively; G and H) Endosperm cells stained by the PAS reaction, noting small grains of neutral polysaccharides in the times t0 and t4, respectively; I) endosperm cells submitted to the polarized light revealing starch grains (malta cross) at time t2



By the microscopy, was observed that the coat is stiff and made of several layers of cells with thick walls and fibers (Figure 2A), while the endosperm cells have thin walls (Figure 2B).

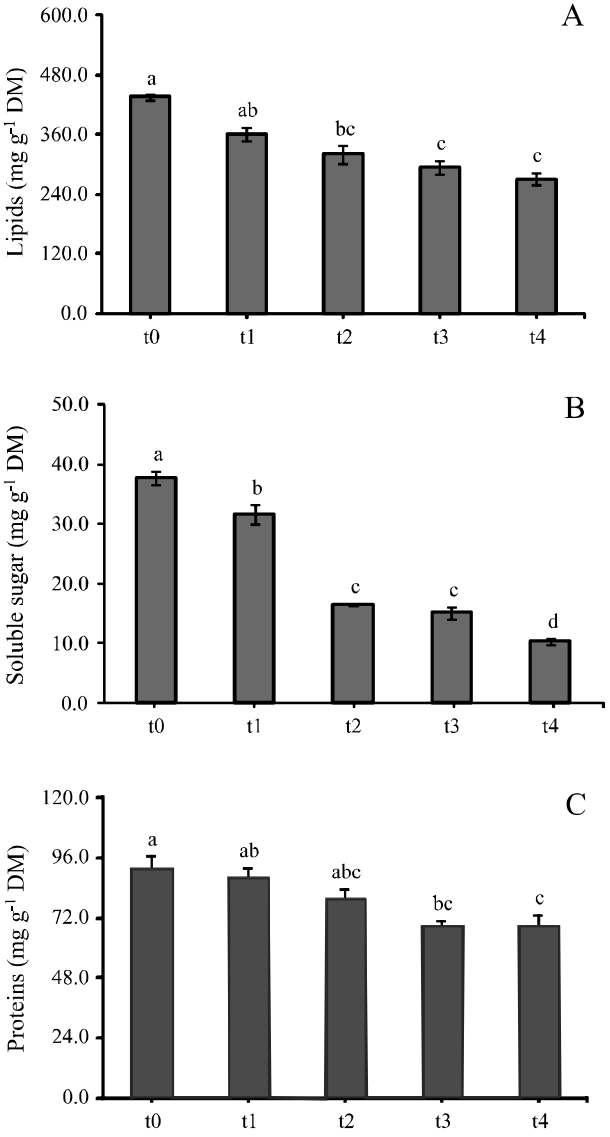
The greenish color seen with TB, which is a metachromatic dye for detecting anion radicals, suggests the presence of phenolic compounds such as lignin. The presence of this hydrophobic constituent was confirmed by the reaction with acidified phloroglucinol. As described by Yashaswini *et al.* (2014), the seed coat is tough, relatively thick and hydrophobic due to its composition. This rough consistence is the cause of the physical dormancy. According to Ponnaiyan and Vezhavendan (2005), the dormancy caused by the waterproof shell is responsible for limiting the cultivation of the specie; however, this resistance can be overcome by heating or chemical treatment. In several species, the presence of rough coat is fundamental to protect the embryo.

The results of the chemical analyses of quiescent seeds and of the mobilization during germination are shown in Figure 3. The noni quiescent seeds presented lipids as the main energy source, followed by proteins and soluble sugars.

The lipids content was around 43.50% (435 mg g^{-1} dry material), which is the main reserve in the oily endosperm. The obtained result was higher than what was reported by West, Jesen and Westendorf (2008), whose total oil content extracted from dry noni seeds was 124.9 g kg^{-1}. Seeds whose lipid percentages are high receive great attention in the pharmaceutical industry, in the production of lubricants, and in human and animal feed, as long as there are no toxic or allergenic substances in such seeds.

The lipid bodies were visualized by dying with Sudan IV inside the endosperm and cotyledons cells, being the endosperm the main storage tissue (Figures

Figure 3 - Changes in contents of lipids (A), soluble sugar (B) and proteins (C) in *Morinda citrifolia* seed during the germination, being T0 (quiescent seed), T1 (8 hours of imbibition), T2 (radicle protrusion), T3 (radicle 2 mm) and T4 (radicle 3 mm) the time in which the seeds were collected. The columns with different letters indicate significant difference at $P \leq 0.05$ among the means, according to Tukey's test. The standard error is represented by vertical bars



2C and 2D). The lipids reduction was significant since T2 when compared with quiescent seed (T0), having reduced in 38% until last period observed (Figure 3A). Furthermore, this effect was observed by citochemistry analysis by the reduction of the lipids bodies that could be related to the fact the lipase activity increase in the storage tissues during the germination (MARQUES *et al.*, 2013). These hydrolytic enzymes are activated to supply the growing seedling by the reserve depletion in the storage tissues, until they become autotrophic (BEWLEY *et al.*, 2013). In *Cereus jamacaru* seeds, the lipids are also the main reserve with approximately 55% of the dry mass for cotyledons (ALENCAR *et al.*, 2012). The authors obtained 66% reduction on the lipids content in 12 days after imbibition compared to dry seeds that were related with the increase of lipids content in the hypoctyl-radicle axis. In some cases, small seeds store lipid reserve that will be primordial to provide energy and matter to metabolic processes of the embryo, consequently, this reserve exhibits fast mobilization on beginning of the germination and in the initial seedling development.

The high proportion of lipids can represent a physiological characteristic of the noni seed that can make up for the small size of the reserve tissue since they have a greater energy-to-volume ratio. Triglycerides, the most common form of lipid storage, is hydrophobic, therefore it is stored without carrying the extra weight of hydration water. Additionally, the carbon atoms in triglycerides are more reduced compared to those in sugars, releasing twice as much energy compared to the oxidation of same quantity of carbohydrates (NELSON; COX, 2014). According to Kitajima (1996), this compensatory relation, besides preserving the amount of energy needed for the embryo, also favors seed dispersal since smaller seeds are easily dispersed due to its lower weight.Regarding the protein reserve, the analysis with Ponceau Xylidine, a dye used to detect total cationic radicals, revealed the presence of several protein bodies inside the endosperm and cotyledon cells (Figure 2E). Inside the cytoplasm of cotyledon and endosperm cells of quiescent seeds of sorghum (OLIVEIRA *et al.*, 2011) and jatropha (LOPES; GALLÃO; BERTINI, 2013), respectively, was also observed the presence of protein bodies. Although the noni seed stocks high energy quantity in the form lipid, the protein reserve is essential to all seeds as source nitrogen to embryo development, and for synthesis of new enzymes and raw material for the construction of new tissues and cells (BEWLEY *et al.*, 2013). It is possible to observe the mobilization of the protein bodies through of reduction their amount and size, when we compare the quiescent seed (Figure 2E) and the seed germinated with 3mm of radicle (Figure 2F). In the noni quiescent, the protein content corresponded to approximately 92 mg g^{-1} dry material (Figure 3C). This result was higher than the protein content found by West, Jesen and Westendorf (2008) that was 75 g kg^{-1} dry material.

In the present study, the protein reserves of noni seeds were significatively reduced from T3 and had a reduction of 26% during the observed period of germination. During the first hours of soaking, the difference in total protein content between the start time

and 8 hour soaking was not significant. This is because during this soaking period the proteins may be suffering degradation and biosynthesis simultaneously, which makes the variation of this reserve barely noticeable, mainly as result of synthesis of the hydrolytic enzymes. The beginning of the protein metabolism is marked by imbibition of the seed with water, resulting to produce free amino acids for biosynthesis and energy generation (TAN-WILSON; WILSON, 2012). Dantas *et al.* (2008) saw that during the early hours of soaking, in baraúna (*Schinopsis brasiliensis*) seed germination the protein values varied widely. In addition to the difference between species, the rate of mobilization of proteins may vary among genotypes, as observed by Tonguç *et al.* (2012) to assess changes in the reserve components of two variables safflower. In noni seeds, the soluble sugars represent only 3.74% (37.4 mg g^{-1}) of the dry material (Figure 3B). Therefore, they do not represent one of the main reserves for germination, being immediately used in the beginning of the germination (T1). However, according to Pontes *et al.* (2002), the use of sugars is variable, depending on the species, and they might be used immediately or only at the seedling stage. The PAS reaction stained the cell wall due to the presence of cell wall neutral polysaccharides (hemicellulose, cellulose, and pectin) and also revealed the presence of small granules inside the endosperm cells (Figures 2G and 2H). Through analysis with polarized light, it can be inferred that the granules shown by the PAS reaction were starch, which were identified by the birefringence effect. The polarized light also highlights the walls of endosperm cells, which look shiny due to the presence of cellulose, whose crystalline properties cause double refraction (birefringence) and make it shine when seen under this light (Figure 2I).

According to West, Jesen and Westendorf (2008), the noni carbohydrate content is essentially made up by fiber components, while there are no significant starch reserves in dry noni seed. However, during germination one storage component may be converted into another. In this case, the plants do not have fat transport mechanisms from the endosperm to the seedling tissues, therefore the lipids can be converted into sucrose, which, in high concentrations, can be stored as starch (BEWLEY *et al.*, 2013). Such an event occurs in dwarf cashew (*Anacardium occidentale*) seedlings, where the temporary increase of the starch reserve during germination and establishment seedling may be related to the degradation of the lipid reserve since the lipid catabolism pathway leads to sucrose synthesis (MARQUES *et al.*, 2013). The concentration of the different reserve components varies among species since they depend on genetics, physiology, and the main regulators that distribute the carbon storage in the seed (EKMAN *et al.*, 2008).

The noni seed fatty acids composition is shown in Table 2. Linoleic acid (C18:2) was predominant with 68.1%, followed by palmitic (C16:0) and oleic (C18:1) acids, which correspond to 12.7 and 12.3%, respectively. Other fatty acids are present in noni seeds, although in percentages below 5%. The fatty acids composition may vary among species due to the genetic load and climate conditions, and it may also vary among storage tissues. However, in oily seeds palmitic, oleic, and linoleic acids can reach higher amounts of up to 60% of the seeds mass (MELLO *et al.*, 2010).

Table 2 - Composition of fatty acids present in dry noni (*Morinda citrifolia* L.) seed

Fatty acids	Amount (%)
C8:0 octanoic (capric)	1.5
C16:0 hexadecanoic (palmitic)	12.7
C18:0 octadecanoic (stearic)	4.8
C18:1 octadecenoic (oleic)	12.3
C18:2 octadecadienoic (linoleic)	68.1
C20:0 eicosenoic (arachidic)	0.7

According to Graham (2008), linoleic acid (linoleate) is an important precursor of other polyunsaturated fatty acids and is essential in mammal diets. The results obtained were close to those found by Rios *et al.* (2009), who saw a prevalence of linoleic acid (68.89%) followed by palmitic and oleic acids at 12.28% and 11.79%, respectively. Whereas West, Jesen and Westendorf (2008) found lower amounts of linoleic acid (59%) and higher amounts of oleic acid (17%). Thus, the oil extracted from noni seeds is a good source of linoleic acid, a fatty acid that is crucial in diets. Linoleic acid is important for cell rigidity, blood coagulation, and in the inflammatory response to external aggressions.

CONCLUSION

Morinda citrifolia L. seed have irregular shape and is albuminous, being endosperm rich in lipid content followed by proteins, this classifies it as an oil seed. Our results suggest that lipids were the main reserve storage, beyond that they had the most intense mobilization when compared with another reserves to support energy and matter to embryo.

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