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Anatomical Characterization and Evaluation of Starch Granules in Grain of Black Common Bean and Cowpea Raw and Cooked

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Bean is a popular name for a large variety of seeds from Leguminosae family. Beans are very important in human and animal diet because it´s a rich source of fiber, proteins, carbohydrates, vitamins B-complex and minerals, at relatively low cost. The *Vigna unguiculata* specie, known as cowpea-bean is widely consumed in the North and Northeast regions of Brazil, featuring the typical cuisine of these regions, in which the most famous dish is called Acarajé. This legume is also widely consumed in many African countries. The species *Phaseolus vulgaris*, the common-bean, alongside of rice is part of the daily diet in many parts of the world, composing one of the most nutritionally complete meals. This study aimed to achieve, by the optical microscopy techniques, the anatomical structure characterization of seeds of common-bean variety black, and cowpea-bean, as well as evaluation of starch granules present in grains in the forms raw, and cooked in a pressure cooker. Slides were prepared by the techniques of free-hand cut, and cut with the microtome in which the samples were previously embedded in paraffin. In the images of raw cotyledon samples under polarized light, the typical model of "Maltese cross" resulting by birefringence of the crystalline regions of the starch granule and the spherical structure was observed. In the images of cotyledon of the cowpea cooked samples, with polarized light, was not observed the starches granules, indicating a loss of molecular ordering due to heat greater than 120° F, but in the samples of cooked black bean, the granules of starch is present, may indicate a long time of grain storage. Three tissues layers can be observed in the images of hand-free-cut integument: epidermis, hypodermis and palisade parenchyma. The samples prepared by embedment in paraffin had their structures palisade disrupted, suggesting that the samples are suffering interference by technique.

1. Introduction

Popularly called beans are the grains from a diversity of plants belonging to several genera and species of the Leguminosae or Phabaceae family. Legumes are an important part of the human diet from many parts of the world because it´s a rich source of proteins, carbohydrates, vitamin B-complex, and minerals, at relatively low cost to the consumer. The bean is one of the main important food of the global population, containing a relatively high intake of protein (on average between 22 % and 26 %) reported by Barampama & Simard (1993), fiber, insoluble and soluble carbohydrates, vitamins; and minerals as potassium (25-30 mg / 100 g), phosphorus (about 0.4 %), iron (about 0.007 %), calcium, zinc and magnesium. Although the essential amino acids present in the grain, the supply of cysteine and methionine is limiting. Thus, the combination between bean and rice at the main meals can supply this deficiency, allowing that the protein intake can be compared to value of animal proteins (Pires *et al*., 2006). However, the digestibility of the beans is limited due to cell wall structure of the cotyledon and tegument, as well as the presence of some antinutritional factors, such as tannins, phytates, trypsin inhibitors and polyphenols (Bressani et al., 1983). Studies suggest some procedures

to eliminate toxicity and enhance digestibility, which include extrusion, steeping, germination and cooking (Sotomayor et al., 1999).
Phaseolus and Vigna are the mainly genera of the Phabaceae. The Phaseolus vulgaris L. species, populary called common-bean, represents 95 % of the overall legume cultivation. Brazil is the largest producer of this grain, accounting 16.1 % of global production, according to the Food and Agriculture Organization (FAO, 2009). Vigna unguiculata is a species popularly known as cowpea-bean. It is largely grown in the North and Northeast of Brazil, particularly in the semi-arid region of the Northeast, featuring typical cuisine of these regions, whose in which the most famous dish is called Acarajé. It is also used as animal feed, fertilizer and soil protection. Besides, cowpea is widely cultivated in Africa, where principal producing countries are Nigeria, Niger Republic, Burkina Faso. Being this grain a rich nutrient source, its flour can be used to prepare bread, cakes, pasta, noodles and snacks.
Several studies published in recent years show the importance of starch, related with technological processes of the industry as the attainment of plastic biofilms (Schmidt et al., 2013), as well as for metabolic processes in the human nutrition, as the glycemic response to ingested food. So, much more than just an energy component, the starch must be studied based on their chemical differentiations in order to manage and optimize their nutritional and technological application (Denardin and Silva, 2009). The starch content in dry basis of different bean cultivars is between 45 and 60 % (Bjorck et al., 1994). The applications of starch in food systems are mainly chosen for their solubility properties, gelatinization, paste viscosity, setback and digestibility. These properties are in turn, resulted from characteristics such as the size and shape of the granules, amylose and amylopectin contents, distribution of polymer chains, polymeric crystallinity degree and extraction residues (Bobbio and Bobbio, 1995). Such characteristics may be closely related to the events associated with gelatinization and retrogradation, such as swelling of the granule, leaching of amylose and / or amylopectin, loss of radial structure (birefringence), supra-molecular (crystalline), molecular and recrystallization (Denardin and Silva 2009). The size and shape of the granules varying between species, so for determining the size of the granules, microscopic methods have been applied (Leonel, 2007).
The present study aims, by optical microscopy, to characterize the anatomical structure of the seeds of common black-bean and cowpea, as well as evaluation of starch granules present in the raw grains and cooked in a pressure cooker, using two different techniques for preparing the slides. They are, freehand cut and cut with a microtome, being the second from the previously paraffined material.

2. Material and methods

2.1 Material

The experiments was performed in uncooked grains of Phaseolus vulgaris variety black, and uncooked grains of Vigna unguiculata variety cowpea, the both in triplicate. All there was purchased in a foodstuff wholesale store located in the Metropolitan Region of Rio de Janeiro. The supplier has no information about the harvested period because are heaps of different producers.

2.2 Methods

Anatomical studies were conducted in order to observe the structure of the seed. Slides were prepared by two different techniques, by free-hand cut, and microtome cut in which the samples were previously embedded in paraffin and sliced with microtome. In the first method the material were previously hydrated in a solution containing ethanol and glycerin (1:1 v/v) overnight, and sectioned by a razor blade. Then, the samples were stained with dyes blue-astra and safranin.
The technique of embedding in paraffin allows the serial sections of materials with a rotary microtome (Sass, 1951). The method was performed as described by Kraus and Arduin (1997), and began with the fixing process with a glycerin and water (1:1 v/v) overnight, in order to preserve the cellular structure without altering the chemical cell. Then was performed the ethanol dehydration process, in which the material is subject to increasingly concentrated solutions of ethanol 50, 70, 90 and 100 % for removal of the water present in this tissue. Thereafter, the matrix undergoes by the ethanol-xylene series. This batch consists in embedding the material in this solvents in the respective ratios 3:1; 1:1; 1:3 and pure xylene, making it ready to receive the molten paraffin. In this last phase the material has been placed in the mold. After drying, the formed block is properly trimmed and placed on the support to the microtome and sectioned. The tape formed by the cuts was applied to the slides using Haupt adhesives and placed over to 167º F overnight. Once dried, the removal of wax is performed by the xylene-ethanol series, this batch is opposite of the previously described and consisting in embedding the material in the solvents in the respective ratios 1:3; 1:1; 3:1 and 100 % ethanol. Then, was the descending ethanol series with this solvent in the concentrations 100, 90, 70 and 50 %. With the slides in 50% ethanol were applied the dyes blue-astra and safranin, and then continued the ascending ethanol series (50, 70, 90, 100 and 100 %). Finally, was carried out the ethanol-xylene series again (3:1; 1:1;

1:3 and pure xylene, respectively). The histological coverslips were attached using synthetic resin. The morphological study was done in stereo Taimim TM 99000777 and documented by photographic equipment Olympus PM-PBK-3 coupled with Kodak Gold ASA 100a. The images of the anatomical and histochemical analyses were obtained by microscope Quimis Q709ST-PLK, with capture system comprising Moticam 2300 camera and software Motic Images Plus ® 2.0. The starch granules were observed using polarized light. The images were edited in Adobe Photoshop 7.0.1 software and the boards assembled using PowerPoint® 2007.

3. Results and Discussion

Figure 1 below shows the optical microscopy of cross-sectional cowpea. Image A represents the integument of raw grain prepared by free-hand cutting, with 50 X optical magnification. Image B represents the integument of raw grain with 50 X optical magnification, prepared by the technique of embedding in paraffin with the serial sections of materials with a rotary microtome. Image C refers to raw cowpea cotyledon, prepared by free-hand cutting, obtained with the use of polarized light microscopy and 10 X optical magnification. Image D refers to cotyledon, also obtained with 10 X optical magnification, of cowpea cooked in pressure-cooked. The image E shows a cross section of cotyledon of raw cowpea taken through polarized light with 50 X optical magnification, prepared by free-hand cut. Image F shows cross section of cotyledon, whose material was previously paraffined, obtained by optical microscopy with 50 X optical magnification, using polarized light.

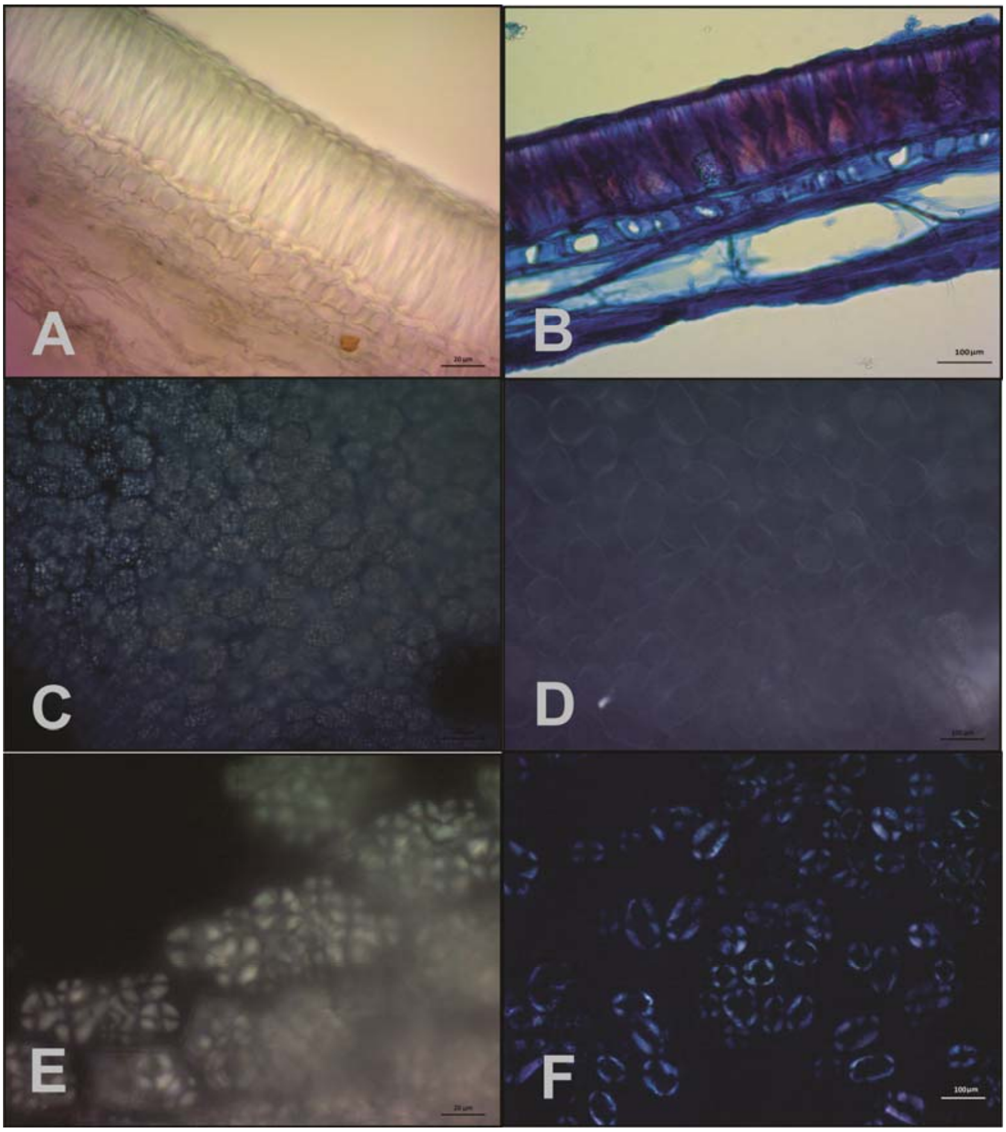

Figure 1 - Cowpea seeds. A) Raw cowpea tegument by cut-hand. B) Raw cowpea tegument parafined. C) Raw cotyledon 10 X. D) Cooked cotyledon 10 X. E) Raw cotyledon 50 X. F) Raw cotyledon 50 X.

Figure 2 below shows optical microscopy of black bean cross-section. The image A represents tegument of raw grain free-hand cut, obtained through optical microscope with 50 X optical magnification. In B, the slide was prepared using the technique of embedment in paraffin, also with 50 X optical magnification. Image C presents raw black-bean free-hand cut, obtained through optical magnification of 10 X, with polarized light. Image D shows the cotyledon of cooked black-bean, prepared by free-hand cut, with 10X optical magnification using polarized light. Image E presents raw black-bean free-hand cutting, 50 X optical magnification with polarized light, the picture shows starch granules with the typical model of the "Maltese cross". Image F shows raw black-bean with 50 X optical magnification and polarized light, whose sample previlousy paraffined.

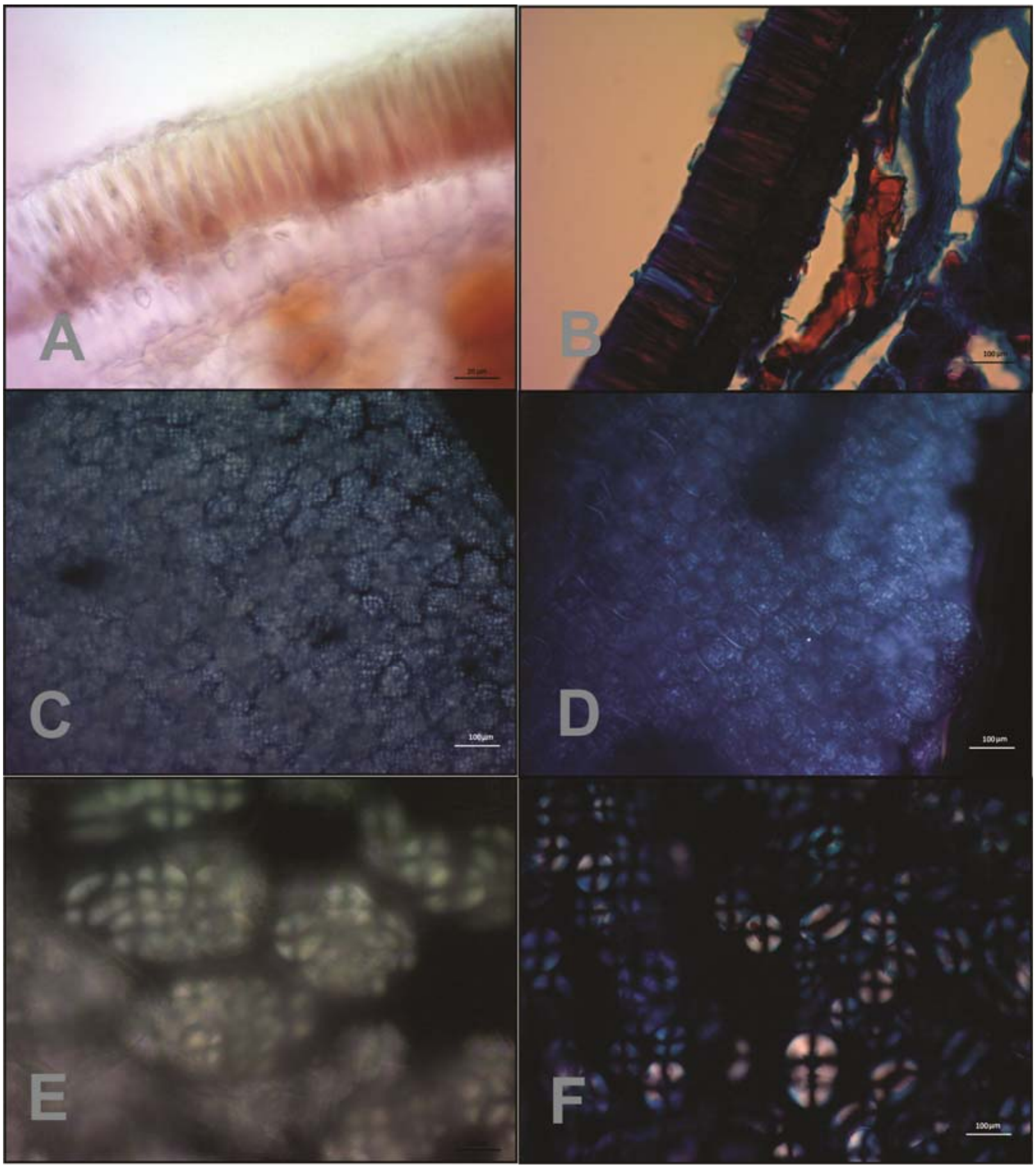

Figure 2 - Common black-bean seeds. A) Raw tegument by cut-hand. B) Raw tegument parafined. C) Raw cotyledon 10 X. D) Cooked cotyledon 10 X. E) Raw cotyledon 50 X. F) Raw cotyledon 50 X.

The seeds coats play an essential role in the germination process, with the regulating of water absorption factor (Cavariani et al, 2009). In images A of figure 1 is possible to observe the cell layers of the epidermis, hypodermis and spongy parenchyma. However, in image B, whose material was paraffined, the spongy parenchyma presents rupture, suggesting that the presence of the reagents used may have affected the structure of the sample, occurred due to the resumption of the metabolic activities of seed which were in dormant state. Therefore, the free-hand cut allowed better visualization of the structure. The large amount of starch granules observed in the cotyledone of cowpea (figure 1 - images C and D), is consistent with the function of this structure which is the energy reserves of seeds. Cotyledons are the first leaves that emerge from the embryo, and are directly related to nutrition during the seedling development, while the embryo still not can produce enough food by photosynthesis process (Esau, 1974). It´s also possible to observe, clearly, the typical model of the "Maltese cross" resulting from the birefringence of the crystalline regions of the starch granule, and it´s characteristic structure of spherical bead this legume. However, the presence of starch granule ("Maltese cross") was not observed in image D showing a lost of the structural organization with the fusion of crystals took place, characterizing the gelatinization phenomenon, that occurs when the starches are subjected to temperatures exceeding 120º F. Souza and Andrade (2000) had described, in studies with starch granules by microscopy under polarized light, which in more than 167° F the birefringence ("Maltese cross") can't be observed indicating loss previously existing molecular ordering. Biaszczak et al. (2007) had already described that the bean cotyledon cells, main organ responsible for the storage of seed, are round or elongated in shape with a mean size of 80 microns and have elongated bimodal starch granules firmly covered with protein material. The image F does not show major differences related to the sharpness of the starch granules morphology compared to the image in which the slides was obtained by the technique of free-hand cutting (figure 1 - E).

As in cowpea (figure 1 - A), in common-bean (Figure 2 - A) is also possible to visualize the presence of three cell layers: the epidermis, hypodermis and spongy parenchyma. According Peske and Pereira (1983), the epidermis consists in palisade cells called macrosclereids, which have columnar appearance and is important for the absorption of water by the seed. In the image B is possible to see the same break of integument tissue observed in cowpea that was subject to this same technique (Figure 1 - B) indicating that the presence of the reagents used may have affected the structure of the tegument. The image C show presence of starch in the cotyledon as observed in cowpea (Figure 1 - C). In this microscopy studies no differences in starch granules between black-beans and cowpea was observed. In D, the sample of black-bean was cooked in pressure-cooker. In this image of cotyledon is still observed the presence of the starch granule suggesting that the baking conditions were not enough for the occurence of starches granules gelatinization, possibly due to the long period storage of grains, features a phenomenon known as "hard-to-cook" (Reyes-Moreno and Paredes-López, 1993). Image E shows starch granules with the typical model of the "Maltese cross". Martínez-Preciado et al. (2012) had already described the morphological structure of common beans observed by scanning electron microscopy (SEM), noting that in the grains without presence of fat, the starch granules had irregular oval shape with sizes of 10-40 mm in length and 10-25 mm in width, as well as small spherical granules of 10 µm. In image F the slides was prepared with parafinned sample. No notable differences between the free-hand cutting and paraffined samples are observed respect of structure of the starch granules of the beans.

4 Conclusions

In the present study no major differences were observed between bean species Phaseolus vulgaris and Vigna unguiculata concerning the starches granules (typical model of "Maltese Cross"). Observing the cotyledons of cooked samples of cowpea is possible to observe a significant change in the structure of starches granules, a loss of birefrigence or the typical model of "Maltese Cross", characterizing a phenomenon called gelatinization, which occurs when the starch is subjected to temperatures exceeding 120 ° F. However, in the cooked samples of black-bean, under the same conditions, was not seen this loss, suggesting that the cooking conditions were not enough for occurrence of the gelatinization of starch granules. That may occurs possibly due a long period of grains storage, phenomenon known as "hard-to-cook". Although the incision by microtome often provides better structural visualization due the thinner sections, in this study the free-hand cutting technique was more suitable for preparation of integument slides, because no rupture of parenchyma occurs, and was not observed important differences in cotyledon between the pictures obtained by the two techniques. Furthermore, the free-hand cutting has the advantages of being simple, fast and does not require the use of organic solvents that are toxic and generate waste during preparation.

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