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## Chapter

# Starch Granules from Cowpea, Black, and Carioca Beans in Raw and Cooked Forms

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## Abstract

Starch applications in food systems are mainly influenced by solubility, gelatinization, paste viscosity, digestibility, and retrogradation. These characteristics result from properties such as the size and shape of granules, amylose and amylopectin contents, distribution of polymer chains, degree of crystallinity, and extraction of waste. In beans, the percentage of starch contents on dry basis is between 45 and 60%, being 24–65% amylose. This chapter evaluated the structure of common beans starch granules (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) in raw and cooked forms, by optical microscopy (OM) and scanning electron microscopy (SEM). Thus it was possible to observe the gelatinization of the starch granules especially in cowpea and carioca beans, as well as the “hard-to-cook” phenomenon in the black beans.

**Keywords:** common beans, cowpea, starches, SEM, OM, X-ray diffraction

## 1. Introduction

Several studies published in recent years have shown that the source of starch can substantially influence various technological processes in the food industry, such as texture and water retention of food, as well as in vital metabolic processes of human nutrition, such as the glycemic response to food intake [1–3]. In addition, starch can also be applied industrially in the production of nanofilms and biodegradable plastics [4]. Therefore, rather than a simple energetic component, starch must be studied based on its chemical differentiation in order to direct and optimize their technological and nutritional application [1]. Starch is the major reserve substance of the Plantae kingdom and is synthesized by plastid organelles. In dry cereals grains are found 40–90% of dry weight, in legumes 30–50% of dry weight, in tubers 65–85% of dry weight, and immature or green fruits 40–70% of dry weight.

As in the other species of starchy legumes, the dry starch content in the various bean cultivars is between 45 and 60%. The granules contain ellipsoidal or spherical forms, with varying sizes, and contain high amounts of amylose (24–65%) [5].

Starches differ from the other carbohydrates due to granule form, built by the polymerization and by dehydration of amylose and amylopectin polysaccharides [6, 7]. Applications of starch in food systems are mainly governed by their properties of solubility, gelatinization, paste viscosity, retrogradation, and digestibility. These properties, in turn, result from characteristics such as the size and shape of the granules, amylose and amylopectin contents, the distribution of the polymer chains, degree of crystallinity of the granule, and the presence of extraction residues. These characteristics may be closely related to the events associated with gelatinization and retrogradation, such as granule swelling; amylose and/or amylopectin leaching; loss of radial (birefringence), supramolecular (crystallinity), and molecular structure; and recrystallization [1]. The size and shape of the grains vary between species, and for determining the size of the granules, microscopic methods have been applied [8].

The starch granule when observed microscopically under polarized light presents a typical “Malta Cross” model, resulting from the birefringence of its crystalline regions, which characterizes the radial orientation of the macromolecules. The center of the cross, called hilum, is considered the original growth point of the granule. The granule material is present in the form of concentric growth rings, which are arranged in alternating in the crystalline and amorphous regions. The fusion of these crystals and the inclusion of water cause rupture of this organized structure, characterizing the gelatinization of the starch granules [1]. The starch granules can be classified as simple when each plastid contains a granule or compounds when many granules are inside each amyloplast, as in the case of legumes [6, 7]. The shape can be spherical, oval, and polyhedral; the size is between 2 and 100  $\mu\text{m}$ ; and the particle size distribution is classified as unimodal, bimodal, or trimodal, being characteristic of the botanical origin. The surface is flat and relatively impermeable to large molecules such as amylases, due to the packaging of the amylopectin chains. By SEM, it is possible to observe the presence of some striations and fissures. Porosity to water and small soluble molecules occurs due to the reversible expansion of the amorphous regions, which may penetrate the entire granule during hydration, to form a continuous gel phase. However, the presence of pores or channels allows the entry of hydrolytic enzymes and other large molecules into the granules [9, 10].

X-ray diffraction patterns demonstrate that native (unmodified) starch grains contain between 15 and 45% crystalline material, corresponding to two polyphorm (A or B) and one intermediate form (C); the classifications are based in water content variations and in the double-helix packaging configuration. X-ray diffraction technique allows to distinguish the three types of crystallinity for the granules which, depending on their shape and crystalline structure, are called A, B, and C. These patterns of crystallinity depend in part on the length of the amylopectin chains, the packing density within the granules, as well as the presence of water. Starches with type A crystallinity exhibit intensity peaks at  $2\theta$  diffraction angles at approximately 15.3, 17.1, 18.2, and 23.5°; type B at about 5.6, 14.4, 17.2, 22.2, and 24°; and type C at about 5.6, 15.3, 17.3, and 23.5°. There is also a fourth type of crystallinity, type V, formed by the crystallization of amylose with lipids, which shows peaks of intensity at the  $2\theta$  diffraction angles at approximately 12.6, 13.2, 19.4, and 20.6° [11].

Type A crystallinity is described as a highly condensed and crystalline monocyclic cell unit, wherein 12 glucose residues from 2 chains in the counterclockwise direction harbor 4 molecules of water between the helices. Type A structure has

amylopectin of chain lengths of 23–29 glucose units. Hydrogen bonding between the hydroxyl groups of the amylopectin molecule is responsible for the formation of the outer helical structure, among which linear chains of amylose moieties are packed through hydrogen bonds with amylopectin outer chains. This polyphorm occurs in most cereals such as corn, rice, wheat, and oats. Type B crystallinity is more clearly defined as being composed of a basic unit of chains which are packaged in a hexagonal array, wherein the cellular unit has two double helices counterclockwise, aligned and arranged in parallel and has amylopectin chain lengths of 30–44 glucose molecules, containing 36 molecules of water for every 12 glucose residues, with half of that water being tightly bound to the double helices and the other half being concentrated in a screw shaft. In addition to being considered rich in amylose, these types of starch have similar shapes and sizes and are resistant to hydrolysis, both enzymatic and acidic. The C-type structure is an amylopectin-containing intermediate structure of chain lengths of 26–29 glucose units. It is common to some roots and legumes. Starches with type A crystallinity are more susceptible to hydrolysis due to the presence of surface pores permeable to certain enzymes, whereas type B grains have protective shells, called crystalline blocks. However, type B crystals have a lower melting temperature (77°C) when compared to crystals A, of 90°C [10–15].

Some structural features such as amylose content, length, and distribution of amylopectin chain and degree of crystallinity in the granule may be related to the events responsible for gelatinization and retrogradation, such as granule swelling, amylose or amylopectin leaching and loss of molecular structure, birefringence, crystallinity, and recrystallization (Rupollo, 2011). A lot of techniques have been used to evaluate the behavior of starch against gelatinization. Methods use employing polarized light microscopy, X-ray diffraction, small-angle neutron scattering, and differential scanning calorimetry (DSC) and the viscosity assessment of starch pastes using equipment such as Rapid Visco Analyzer (RVA) and Brabender Visco-Amylo-Graph [10, 12].

Figuerola et al. [16] carried out a study about the thermal property characterization of cowpea, carioca, and white and black beans using DSC. The analysis provides quantitative measurements of the heat flux associated with gelatinization, which is represented by means of endothermic peaks in a characteristic range for each botanical source [17]. According to the study, the enthalpies of gelatinization showed to be significantly different ( $p$ -ANOVA  $<0.05$ ) for all the samples; notice that the bean starch had the largest enthalpy, while the black bean had the lowest, being this property inversely proportional to the stability of the granule. The authors also conducted a study about the characterization of starch paste properties of the same bean varieties using an RVA, and they observed that the samples differed from each other, except for the minimum viscosity, for the carioca bean starches. According to the authors, this study assisted in a better structural and behavioral characterization of the starch granules these four different bean varieties, and the obtained results were taken as important parameters in the process and development of new food or technological products, with different applications of beans and their derivatives (flours and starch).

As time goes on for storage and cooling, gelatinized starch molecules are losing energy, and hydrogen bonds become stronger. In this way, the chains begin to reassociate in a more ordered state. This reassociation culminates in the formation of simple and double helices, resulting in junction zones between the molecules, forming crystalline areas. This phenomenon is called retrogradation and is influenced by factors such as temperature and storage time, pH, starch source, and presence of other components (lipids, electrolytes, sugars) and processing conditions. The amylose that had been exuded from the swollen granules forms a network

by association with chains surrounding the gelatinized granules. As a consequence, the paste viscosity increases by converting to a cloudy viscoelastic system, or to an opaque elastic gel, precipitation of insoluble starch crystals leading to phase separation. The main influence of retrogradation is observed in the texture and digestibility of food containing starch, such as bakery products, and in the loss of water (syneresis) of some desserts that use starch as a thickener. As for digestibility, the retrogradation can be related to the lower availability of nutrients to the digestive enzymes, resulting in a lower glycemic response [1].

In a study involving the modification of starch concentration in cowpea by heat treatment, storage, and freezing, Salgado et al. [18] observed that the type of cooking, degree of maturation, and storage time exerted visible effects in the production of this starch fraction. Thermal processing altered the original morphological appearance and crystallinity pattern. According to the authors, the phenomenon of gelatinization, followed by retrogradation, can be considered beneficial from the nutritional point of view because they increase the dietary fiber content. This fact confirms a functional property attributed to the food, especially considering the role played by the short-chain fatty acids produced during the fermentation of resistant starch by bacteria present in the human colon.

Legume pulses stored under high temperature and high humidity adverse conditions can develop an increasing cooking time, characterized by prolonging cotyledon softening, phenomenon known as hard to cook (HTC). Beans that have undergone this HTC effect require increasing the cost of energy (fuel) for the preparation and are less acceptable to the consumer due to changes in flavor, color, and texture, with decreased nutritional quality. Several hypotheses have been proposed to explain the cause of bean hardening; among them are oxidation or polymerization of lipids, formation of insoluble pectates, lignification of intermediate lamellae, and multiple mechanisms. Maurer et al. [19] studied fractions extracted from common black and red beans using Fourier transform infrared spectroscopy (FT-IR). The samples were stored under three conditions: control at 4°C, HTC at 29°C and 65% humidity for 3.5 months, and HTC-chilled at 2°C and 79%. Two isolated fractions of the beans, the soluble pectin fraction and the insoluble residue of the cell wall, were analyzed. The immersion water and the cooking water of the beans were also studied. The results showed that in general, phenolic compounds were more associated with the fraction of soluble pectin of HTC beans than in the control bean. The results also showed that HTC-chilled beans contained higher concentrations of phenolic compounds than the control beans. Regarding immersion water, the authors observed that HTC-chilled beans and HTC had higher concentrations of absorbent compounds than control beans, indicating that they lost more constituents to the water. In addition, the results indicated that the mechanisms of reversibility of the HTC defect may be different from those involved in the development of the phenomenon.

Oliveira et al. [20] evaluated the cooking quality and nutritional composition of black and red beans, with and without storage under refrigeration. The grains were evaluated immediately after harvest, and after 6 months of storage, they were previously dried in a greenhouse (65–70°C) to a mean humidity of 13%. They were then packed in polyethylene bags and stored in a cold room at 0°C and 50% relative humidity. They evaluated the quality for cooking, the coloring of the seed coat, and the nutritional quality (protein, potassium, iron, and zinc). The authors observed that the evaluated beans maintained the quality for cooking and nutritional quality after 6 months of storage under refrigeration and that the clarity of the carioca bean tegument was altered.

Kruger et al. [21] evaluated the role that minerals play in the development of the HTC phenomenon in cowpea and its effect on *in vitro* bioaccessibility. The mineral distribution in normal and HTC cotyledons of cowpea bean was analyzed

by proton-induced X-ray emission spectrometry (PIXE). The total phytate, tannin, and phenolic contents were analyzed together with the mineral bioaccessibility *in vitro*. The authors observed that in the HTC bean, Ca and Mg were more concentrated in the cell wall area of the medial lamella of the parenchyma cells. This, together with the reduction in phytate content, confirmed the phytase-phytate-mineral hypothesis as a mechanism for the development of the HTC phenomenon. Despite the reduction of phytate in stored cowpea, HTC decreased the bioaccessibility of Ca, Fe, and Zn in cowpea.

The goals of this work were to characterize the anatomical structure of common bean seeds (*Phaseolus vulgaris*) in the black and carioca varieties and cowpea (*Vigna unguiculata*), evaluate and compare the morphology of starch granules from these legumes by optical microscopy (OM) and SEM in raw and cooked forms, as well as to carry out the extraction of their starches and to characterize, by X-ray Diffraction, the models of crystalline structure.

## 2. Material and method

The beans were purchased from a retail food supply market in the city of Rio de Janeiro, RJ, and stored in a refrigerator with a temperature of approximately 4–8°C for about 12–18 months. The materials were subjected to the experiments, in the raw forms and cooked in a pressure cooker at 250°C for 25 minutes, and analyzed in duplicate.

For the characterization of the external morphology, samples of the abovementioned species and varieties were observed with the naked eye and with the aid of a magnifying glass 10X. Grains of both bean species were photographed using the Canon EOS Rebel T1i 15.0 megapixel camera. The images obtained were edited in Adobe® Photoshop® 7.0.1 software, and the boards were assembled using PowerPoint® 2007.

Anatomical studies on the raw materials were developed after hydration in a solution of 50% ethanol and glycerin (3:1), with the preparation of slides with transversal cuts in the freehand grains made in the middle region of the seeds. The cuttings were done with the help of blade stained with astra blue (C. not indicated) and safranin (C.I. 50,240) to prospect the structure of the organ under study. The slides were mounted in water. Subsequently, the samples were submitted to the paraffin infiltration technique, in order to obtain serial sections with the aid of a rotating microtome. The paraffined sections were affixed to the histological slides with Bissing adhesive and then dewaxed, dehydrated in an ethanolic series, and stained with astra blue (C. not indicated) and safranin (C.I. 50,240). After the ethanolic dehydration of the histological sections, the blades were assembled with synthetic resin (Entellan). Slides prepared from the included historesin material were stained with toluidine blue (C.I. 52,040), and the histological sections were adhered to the slides with water heated to 40°C. The images of the anatomical and histochemical analyses were obtained using a Quimis Q709ST-PLK microscope, with a capture system composed of a MOTICAM 2300 camera and Motic Images Plus® 2.0 software. Starch granules were observed using polarized light. The images obtained were edited in Adobe® Photoshop® software 7.0.1, and the boards were assembled using PowerPoint® 2007.

The cotyledon content of the raw and cooked bean samples was withdrawn for SEM studies. Prior to this procedure, the cooked beans were dehydrated at 40°C for 18 hours and then stored in a freezer at –10°C for 30 days and then were affixed in metal supports and coated with 30–35 nm of gold at  $6.10^{-2}$  atm in gold sputter FL9496 Balzers metallizer. The observations and documentation of the material

were carried out at the National Center of Structural Biology and Bioimaging (Centro Nacional de Biologia Estrutural e Bioimagem - CENABIO) of the Federal University of Rio de Janeiro (UFRJ), using Zeiss microscope, model EVO MA10, tungsten filament, working distance of 10 mm, and voltage of –15 Kv. The images were made with secondary electron detector.

Starch extraction was based on the method described by Wang and Wang (2004), with some modifications. The samples were ground in a laboratory mill (Perten, 3100) soaked in 0.1% NaOH solution in a ratio of 1:5 and allowed to stand for 20 hours. After dispersion, vigorous stirring in the blender was performed for 2 minutes. The resulting material was passed through a 63  $\mu\text{m}$  sieve and centrifuged (Sorvall® RC 6 Plus centrifuge) at 1200 RPM for 5 minutes at room temperature ( $25^{\circ}\text{C} \pm 2$ ). The supernatant was discarded, and the precipitate was resuspended in 0.1% NaOH solution and centrifuged again and the operation performed twice. The extracted starch was dispersed with distilled water and neutralized with  $1 \text{ mol L}^{-1}$  HCl to pH 6.5 and centrifuged. The sedimented material was resuspended in distilled water and centrifuged and the operation repeated twice. The resulting starch was oven-dried with air circulation at  $40^{\circ}\text{C}$  to  $11\% \pm 0.5$  humidity. The starch extraction yield was calculated on the difference between the dry flour masses before and after the starch isolation.

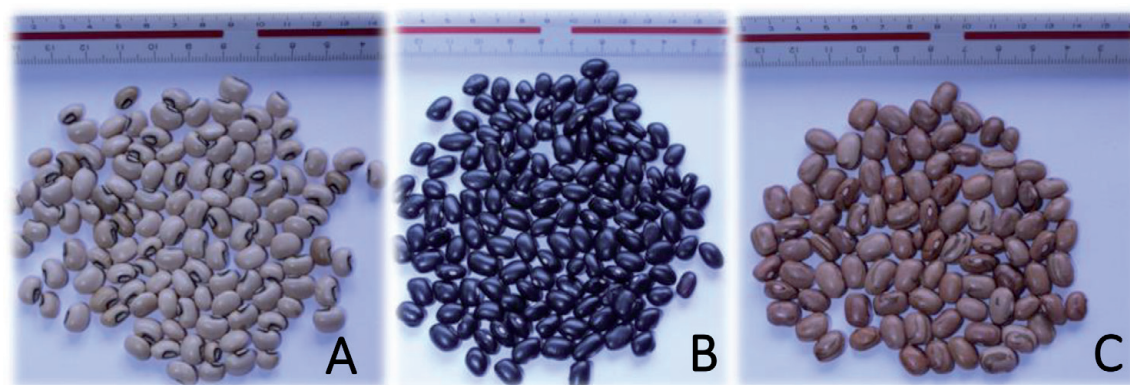
X-ray diffraction crystallinity tests were performed in the multiuser laboratory of the Chemistry Institute of the Federal University of Uberlândia (UFU). The diffractograms of the starches were obtained by an X-ray diffractometer (XRD-6000, Shimadzu, Brazil) in which the diffraction sweep region ranged from  $5$  to  $30^{\circ}$  with a target voltage of 30 kV and current of 30 mA. Scan speed was  $1^{\circ}\text{min}^{-1}$ . The relative crystallinity (RC) of the starch granules was calculated by the software XRD-6000 v. 5.2. The RC values of all bean samples were evaluated by GraphPad Prism® software, using the analysis of variance (ANOVA).

### 3. Results and discussion

#### 3.1 Morphological characterization

Common bean and raw cowpea grains can be visualized in **Figure 1**, being (A) cowpea, (B) black beans, and (C) carioca bean.

Seed format characteristics and tegument coloration were within the range expected for species and varieties. Measurements of the materials studied revealed that the measurements were approximately 0.5–0.7 cm wide and 0.7–0.9 cm long for the three varieties of beans.

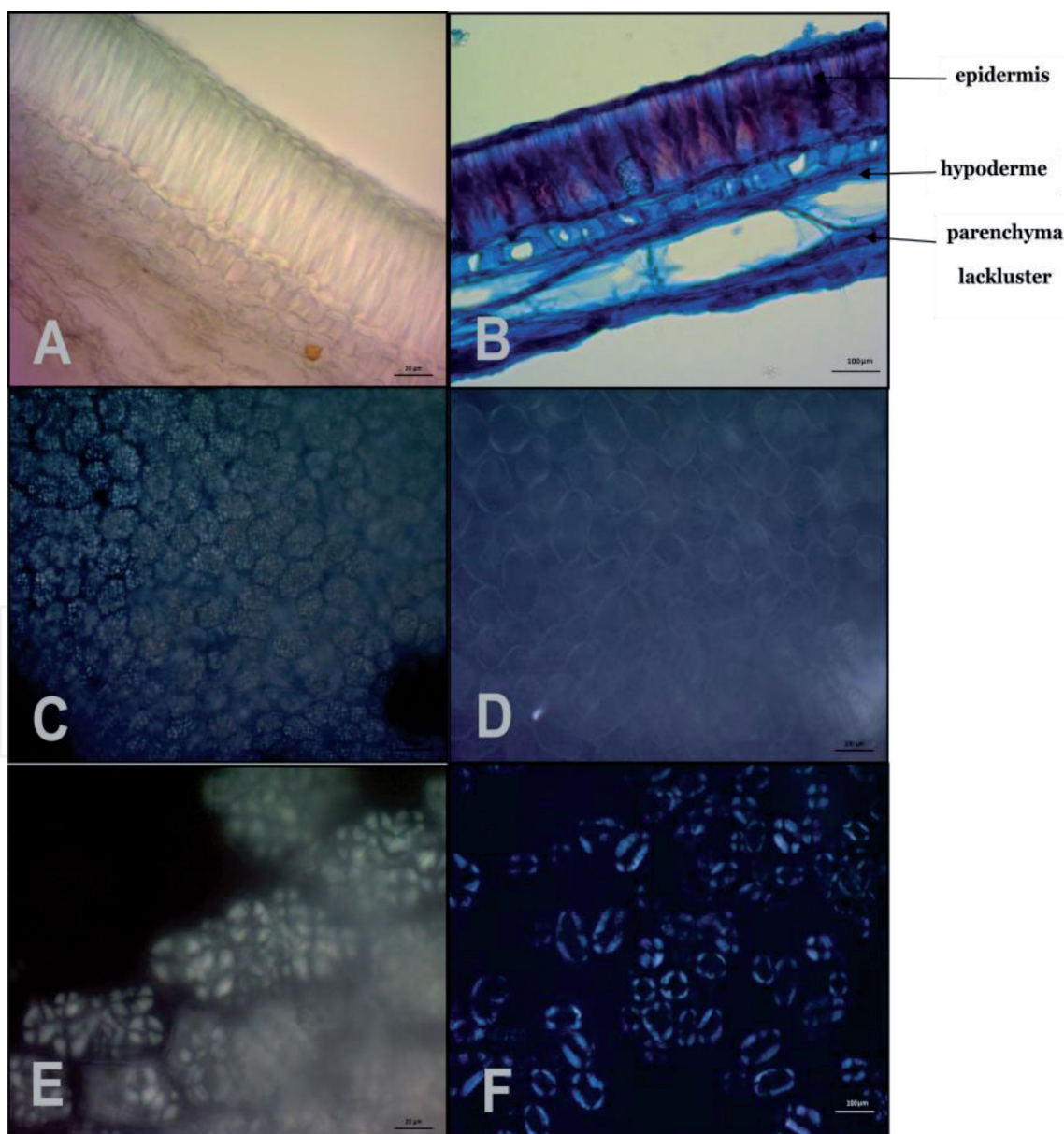


**Figure 1.** Anatomical structure of bean pulse (A) cowpea, (B) black beans, and (C) carioca bean.

## 3.2 Optical microscopy (OM)

### 3.2.1 Cowpea

**Figure 2** shows the optical microscopy of cowpea in cross section. Image A represents the integument of the raw grain prepared by manual cutting with an optical magnification of 50 x. Image B represents the tegument of the raw grain, prepared by the technique of infiltration in paraffin and serial cut with the help of a rotating microtome, both images with 50 x optical magnification. Image C refers to the cotyledon of the raw grain, and D refers to the cooked cotyledon under pressure; both images were observed using polarized light microscopy, with 10 x optical magnification, and slides prepared by freehand cut. Image E represents the cotyledon of the raw grain whose blade was prepared by freehand cut, and image F refers to cotyledon of the raw grain whose blade was prepared by serial cutting with the aid of a rotary microtome, preceded by paraffin infiltration; both images were obtained by optical microscopy with optical magnification of 50 x, using polarized light [22].



**Figure 2.** Cross section of cowpea: (A) seed coat of the raw seed cut by free hand (50 x), (B) seed coat of the raw seed, cut with microtome (50 x), (C) cotyledon of raw seed (10 x), (D) cotyledon of cooked seed (10 x), (E) cotyledon of freehand cut seed (50 x), and (F) cotyledon of the raw seed cut with microtome (50 x).



In the integument images (A and B), three layers of tissues can be observed: epidermis, hypodermis, and lacunar parenchyma. The epidermis consists of two layers of flattened cells, with thickened walls. The hypodermis is composed of osteosclereids that are hourglass-shaped. The cells of the lacunar parenchyma have a shape close to the cylindrical, arranged with gaps between them. The thickness of the tegument is highly correlated with characters that reveal the size and shape of the seeds and water absorption capacity [23]. The images from SEM of raw whole cowpea seeds from Biaszczak et al. [24] revealed integument with an average thickness of about 90  $\mu\text{m}$ , composed by palisade, glasshour and cells of lacunar parenchyma. In the tegument image whose material was previously infiltrated in paraffin (B), it is observed that the lacunar parenchyma presents rupture in the tissue, indicating that the presence of the reagents used in the embedding technique may have interfered in the sample structure, possibly due to the resumption of metabolic activities of seeds which broke their state of dormancy. Thus, the freehand cut allowed a better visualization of the structure, with the advantage of being a simple, efficient, and low-cost methodology, requiring no addition of organic solvents [23].

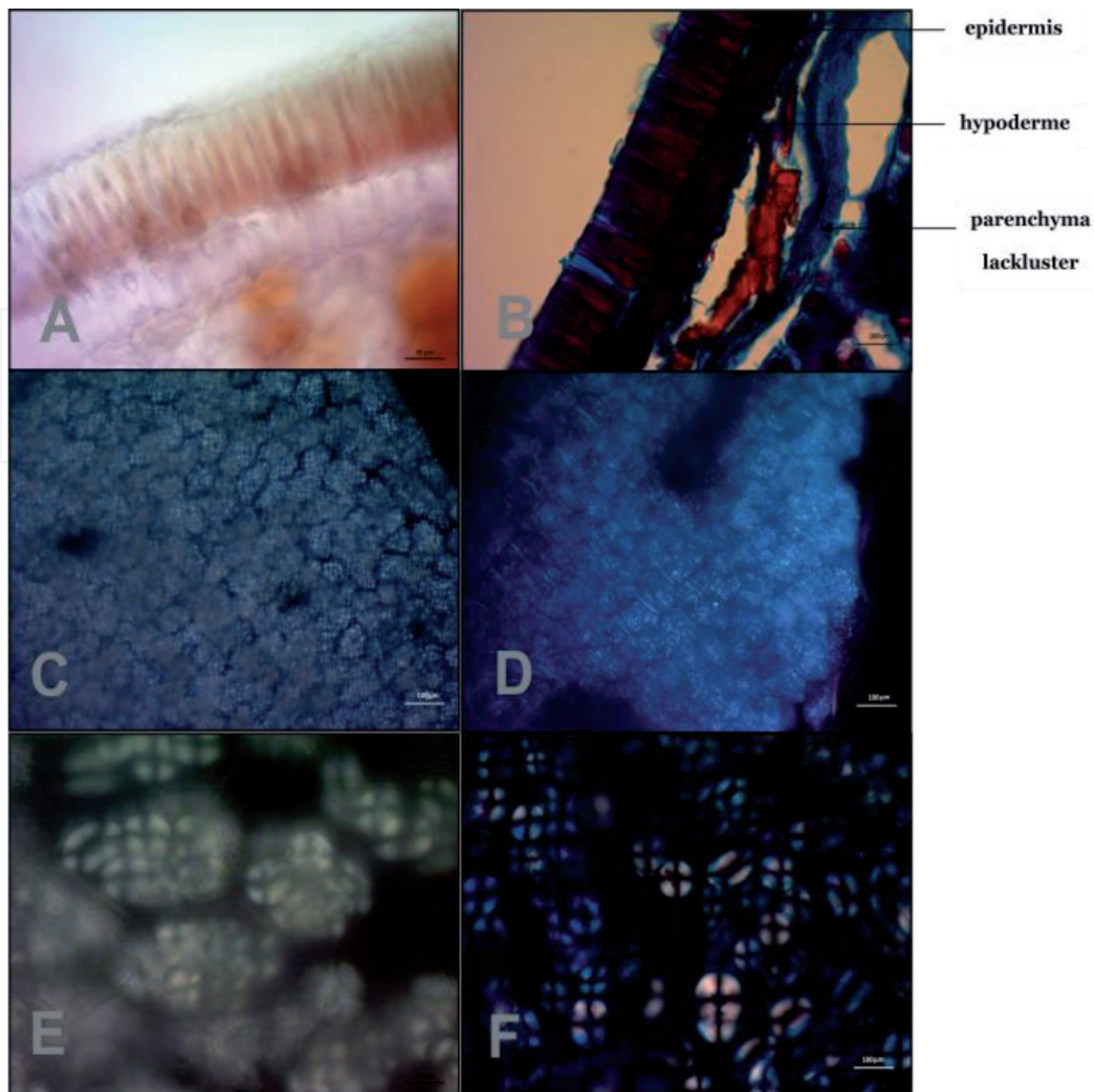
In the cotyledon images of the raw grain (C, E, and F) it is possible to perceive the great presence of amido within the amyloplasts, being coherent with the energy reserve function of this structure, since in beans as in other legumes, they constitute the main storage organs of the seed. The “Malta Cross” conformation of the starch resulting from the birefringence of the crystalline regions of the starch granule can be observed, as well as the characteristic spherical structure of the granule to that legume. Biaszczak et al. [24] reported that the cotyledon cells were rounded or elongated in the longitudinal axis, with an average size of 80  $\mu\text{m}$  and had bimodal, elongated starch grains, firmly covered with protein material.

In the cooked grain image (D) the presence of starch grains exhibiting the characteristic “Malta Cross” is not observed, which evidences the loss of structural organization with the melting of the crystals. Such alteration is characteristic of the gelatinization process that occurs when the starch is submitted to temperatures higher than 50°C. Souza and Andrade [25] reported that after submission to temperatures above 75°C, there is no birefringence of starch grains of corn by optical microscopy on polarized light indicating loss of previously existing molecular ordering.

In the cotyledon image whose material was infiltrated in paraffin (E), the sharpness of the morphology of the starch granules is smaller than the image whose blade was prepared by free hand cut (F), suggesting once again that the technique manual cutting is more advantageous for bean seed samples.

### *3.2.2 Black bean*

In **Figure 3**, optical microscopy of black beans in cross section can be observed. Image A represents the integument of the raw grain prepared by manual cutting with an optical magnification of 50 x. Image B represents the tegument of the raw grain, prepared by the technique of infiltration in paraffin and serial cut with the help of a rotating microtome, both images with 50 x optical magnification. Image C refers to the cotyledon of the raw grain, and D refers to the cooked cotyledon under pressure; both images were observed using polarized light microscopy, with 10 x optical magnification, and slides prepared by freehand cut. Image E represents the cotyledon of the raw grain, the blade of which was prepared by freehand cutting, and image F refers to the cotyledon of the raw grain, the blade of which was prepared by serial cutting with the aid of a rotary microtome; preceded by paraffin



**Figure 3.** Cross section of black beans: (A) seed coat of the raw seed, cut by free hand (50 x); (B) seed coat of the raw seed, cut with the help of a microtome (50 x); (C) cotyledon of raw seed (10 x); (D) cotyledon of boiled seed (10 x); (E) cotyledon of freehand cut seed (50 x); and (F) cotyledon of the raw seed, cut with the help of a microtome (50 x).

infiltration the images were obtained by optical microscopy with 50 x optical magnification using polarized light [22].

In the same way as in cowpea bean microscopy (Figure 2), three layers of tissues can be observed in the integument images (A and B): epidermis, hypodermis, and lacunar parenchyma. However, in the integument image whose material was previously infiltrated in paraffin (B), the lacunar parenchyma shows rupture in the tissue.

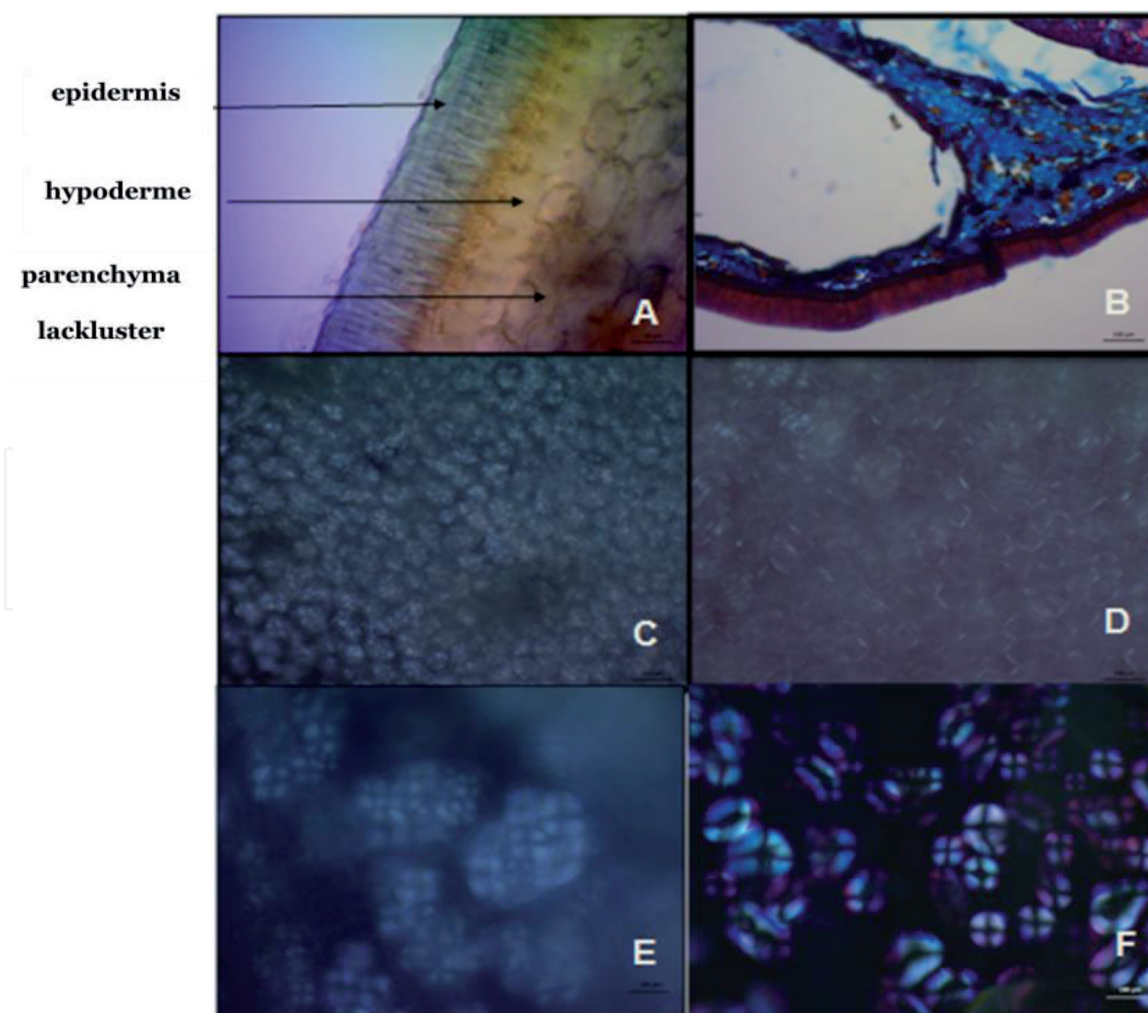
In the cotyledon images of the raw grain (C, E, and F), there is also a great presence of amido in the interior of the amyloplasts, its conformation of “Malta Cross,” and the spherical structure of the granule. In the study by Ambigaipalan et al. [26], all black and carioca bean starch grains exhibited a strong birefringence pattern under polarized light, indicating that amylopectin crystallites are arranged radially within the bead at right angles to their interface with single reducing end group for the yarn. Weaker patterns would be indicative of double amylopectin helices disorganized within the crystalline lamellae of these grains. Chigwedere et al. [27] investigated the relative contributions of cotyledons and seed coats toward hardening of common beans were and the rate-limiting process which controls bean softening during cooking was determined. The authors suggested that the

rate-determining process in bean softening relates to cell wall/middle lamella changes influencing pectin solubilization.

The presence of starch granules inside the amyloplasts is still observed in the cooked cotyledon image, suggesting that the cooking conditions were suitable for gelatinization, possibly due to a long storage period of the seeds, which characterizes an HTC phenomenon [19, 21].

### 3.2.3 Carioca bean

**Figure 4** shows the optical microscopy of carioca beans in cross section. Image A represents the integument of the raw grain prepared by manual cutting with an optical magnification of 50 x. Image B represents the tegument of the raw grain, prepared by the technique of infiltration in paraffin and serial cut with rotating microtome, both images with 50 x optical magnification. Image C refers to the cotyledon of the raw grain, and D refers to the cooked cotyledon under pressure; both images are observed using polarized light microscopy, with 10 x optical magnification, and slides prepared by freehand cut. Image E represents the cotyledon of the raw grain whose blade was prepared by freehand cut, and image F refers to cotyledon of the raw grain whose blade was prepared by serial cutting with the aid of a rotary microtome, preceded by paraffin infiltration; both images were obtained by optical microscopy with optical magnification of 50 x, using polarized light [22].



**Figure 4.** Carioca bean cross section: (A) seed coat of the raw seed, cut by free hand (50 x); (B) seed coat of the raw seed, cut with microtome (50 x); (C) cotyledon of raw seed (10 x); (D) cotyledon of cooked seed (10 x); (E) cotyledon of freehand cut seed (50 x); and (F) cotyledon of the raw seed, cut with microtome (2.5 x).

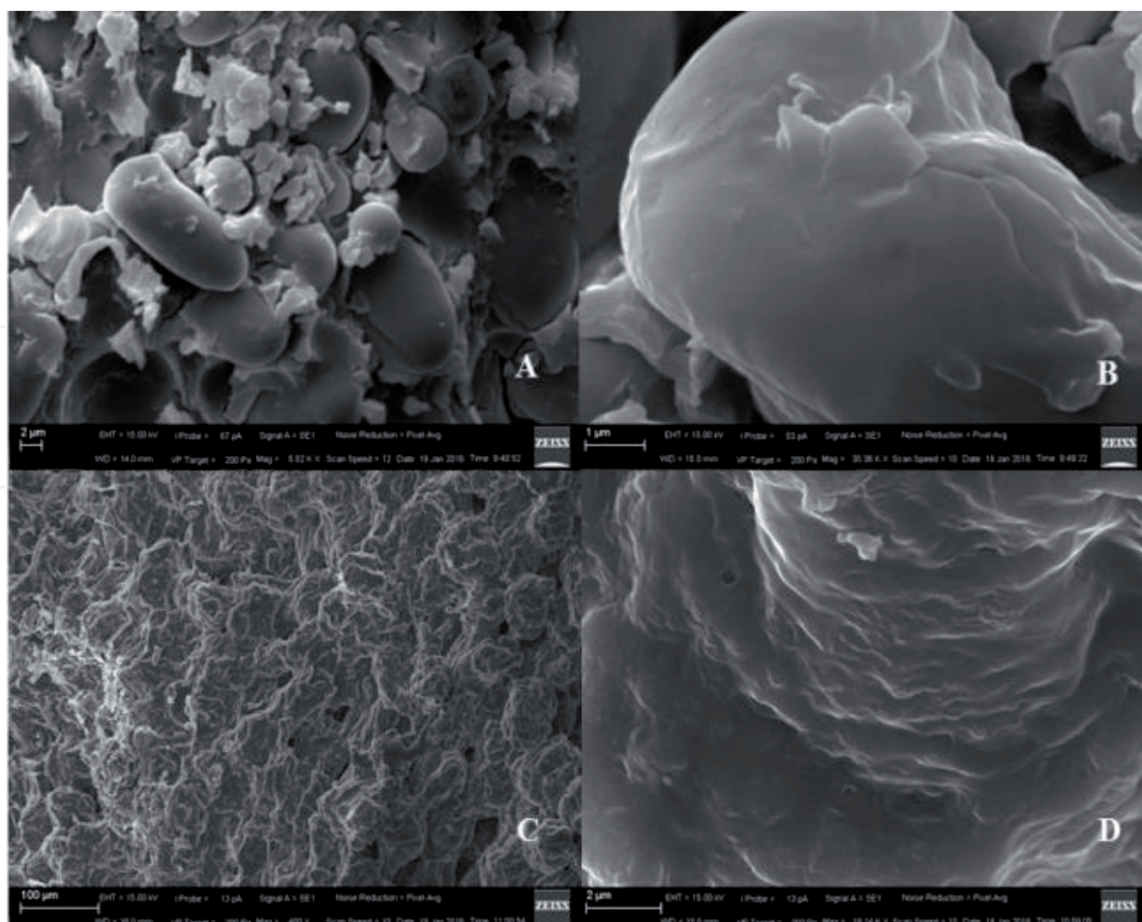
Similar to the cowpea (**Figure 2**) and black bean microscopy (**Figure 3**), the layers of epidermis, hypodermis, and lacunar parenchyma can be observed in the tegument images (A and B). In the image of cotyledon of raw bean (C), a large amount of starch granules is also observed inside the amyloplasts, and in the image of cooked cotyledon (D), the absence of this structure is observed due to the phenomenon of gelatinization, also observed previously in the cowpea image (**Figure 2**). With 50 x optical magnification of cotyledon (images E and F), no notable differences were observed in the starch granules, between the blades that were prepared by freehand cutting or rotating microtome.

### 3.3 SEM

#### 3.3.1 Cowpea

**Figure 5** shows a cowpea endosperm SEM. In image A granules of starch attached to the cotyledon cell wall and its reniform shape (magnification 5.52 Kx) can be visualized, and in B bulges on the surface of the granule (magnification 30.36 Kx) can be perceived, both refer to raw samples. In the images of cooked samples with different optical amplifications (C 400 x and D 19.14 Kx), it is noticed that the starch granules were grouped, losing their crystalline structure due to gelatinization [28].

The morphological aspect of the starch observed in **Figure 5(A)** is in accordance with the description of Agunbiade and Longe [29], which confirmed that grain lengths in all samples were mostly larger than their widths. With the enlargement



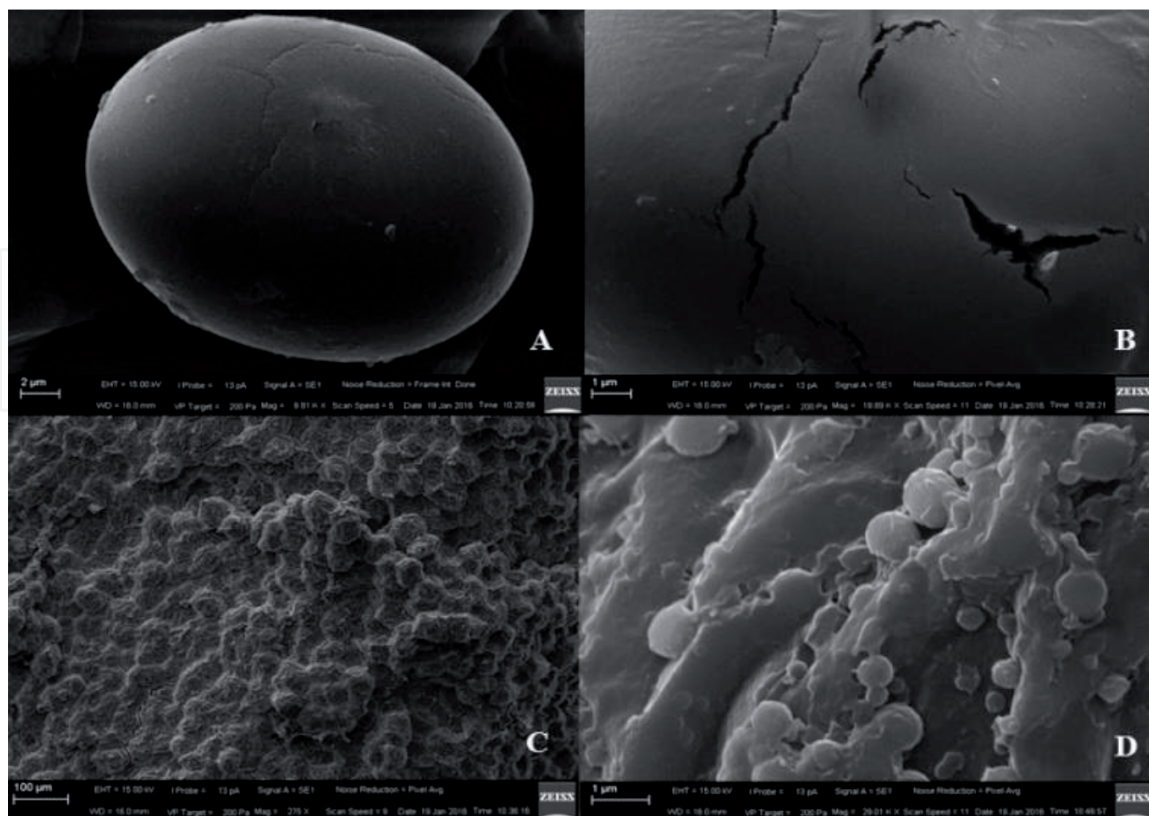
**Figure 5.**  
(A) Cowpea raw endosperm SEM (5.52 Kx), (B) cowpea raw endosperm SEM (30.36 Kx) granule surface, (C) cooked and gelatinized cowpea cotyledon (400 x), and (D) cooked cowpea cotyledon (19.14 Kx).

of the starch grains of the same **Figure 5(B)**, it is possible to perceive the presence of protrusions. The authors also compared the structure of cowpea, pigeon pea (*Cajanus cajan* L.) and yam bean (*Sphenostylis stenocarpa* L.), perceiving these grooves visible only in cowpea, being scarce in the yam bean, and almost imperceptible in pigeon pea. According to the authors, the morphological and legume starch characteristics are good indicators to identify their botanical origin and to detect if they are contaminated or adulterated with starches from other sources. They also observed that cowpea, pigeon pea, and yam bean exhibited appreciable shelf life stability, due to the low percentage of water and oil absorption.

Salgado et al. [30] observed that under the conditions in which their experiments were conducted, the morphological aspects of the starch grains were not influenced by the maturation stage of the grains. All presented a reniform shape, variable size between 11.8  $\mu\text{m}$  and 26.7  $\mu\text{m}$ , and smooth surface. Already the crystallinity pattern was higher in green beans than mature beans, as well as the percentage of resistant starch, whose test was based on the use of amylolytic enzymes.

### 3.3.2 Black bean

**Figure 6** shows, by SEM, the black bean endosperm. In image A, the ellipsoid format of the starch granule is visualized (magnification 9.81 Kx), and in image B it is possible to observe the presence of cracks on the surface of the granule (magnification of 19.89 Kx), both refer to raw samples. The images of cooked samples (C magnification of 275 x and D and 29.01 Kx) reveal the stable structure of the black bean seed, suggesting a relation with the difficulties in its cooking (HTC phenomenon), requiring a longer cooking time for complete gelatinization, compared to the other samples studied [28].



**Figure 6.** (A) Black bean raw endosperm SEM (9.81 Kx), (B) black bean raw endosperm SEM (19.89 Kx) surface of granule, (C) cooked black bean cotyledon (275 x), and (D) unaccomplished gelatinization in cooked black bean cotyledon (29.01 Kx).

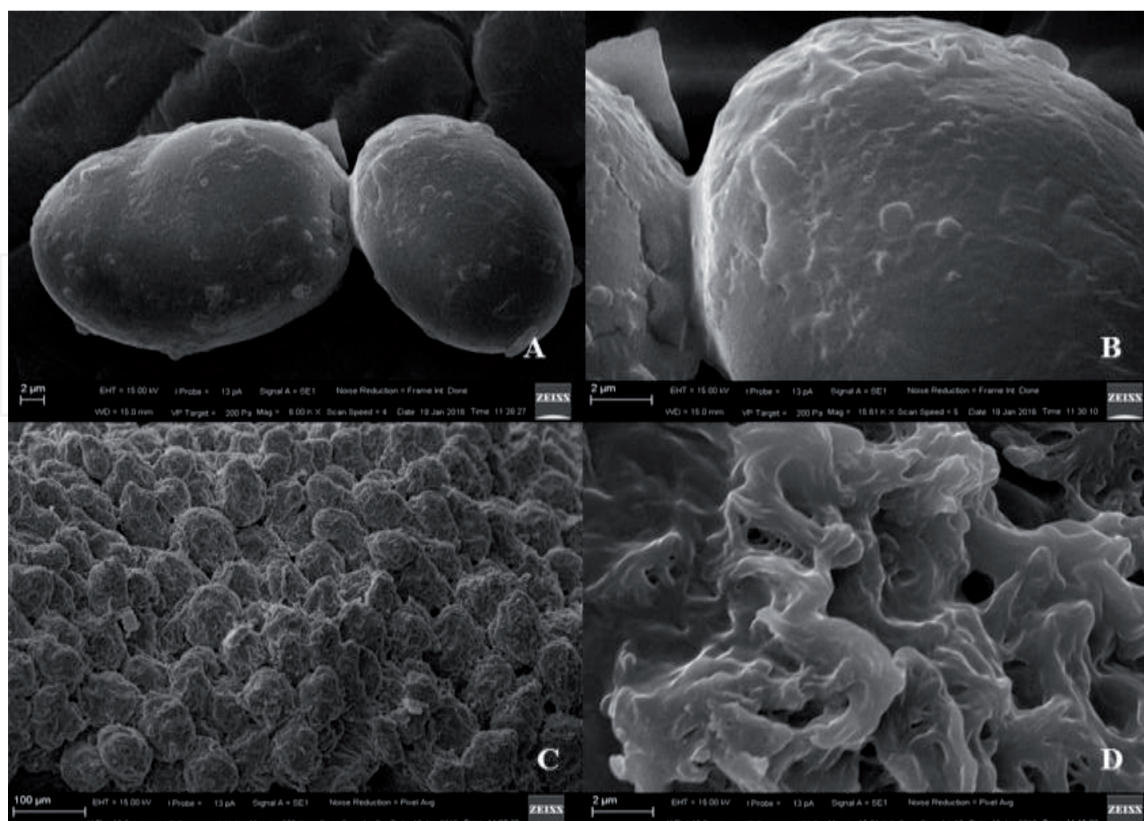
Ambigaipalan et al. [26], by SEM images, did not find the presence of cracks in black bean grains. Martínez-Preciado et al. [31] described the morphological structure of beans by SEM, observing that grains without the presence of fat had irregular oat-shaped starch granules with sizes of 10–40  $\mu\text{m}$  in length and 10–25  $\mu\text{m}$  in width, as well as small spherical beads of 10  $\mu\text{m}$ . It was also observed that the starch grains were well defined and did not suffer any damage.

HTC phenomenon is one of the main obstacles to the consumption of beans grown in countries of Latin America and Africa, where ambient temperatures and relative humidity are high throughout the year, conditions that increase the possibility of occurrence of this phenomenon. At the microstructural level, the visible result of HTC seems to be related to the inability of the middle lamella of cotyledon cells to soften or dissolve and separate the cells [19, 21].

### 3.3.3 Carioca bean

In **Figure 7**, the SEM of the carioca bean endosperm is observed. In image A, the ellipsoid formed of the starch granules (magnification 6.00 Kx) is observed, and in image B one perceives protrusions on the surface of the granule (magnification 15.61 Kx), both refer to raw samples. In the images of cooked samples (C 370 x and D 15.61 Kx), with different optical amplifications, it is noticed that the starch granules were grouped, losing their crystalline structure due to gelatinization [28].

In the isolated starch granule images by SEM from Wang and Ratnayake [31] study, there was no evidence of starch damage, with no visible cracks or notches in the surfaces, and the presence of foreign materials was also not observed. All cultivars presented spherical, oval, or elliptic forms with smooth surfaces. According to the authors, generally, *P. vulgaris* starch granules have similar morphologies



**Figure 7.** (A) SEM of the raw carioca bean endosperm (6.00 Kx), (B) SEM of the endosperm of raw carioca bean (15.61 Kx), (C) cotyledon of cooked and gelatinized carioca bean (370 x), and (D) cotyledon of cooked carioca bean (15.61 Kx).

between their varieties but are very different from other starches such as tapioca and banana. The shape of the starch granules and size influence their functional properties, such as paste viscosity. A high viscosity is desirable for industrial uses, in which the purpose is the thickening function. Ambigaipalan et al. [26] did not find the presence of cracks in starch granules of carioca beans, as well as in black bean granules, both raw and evaluated by SEM.

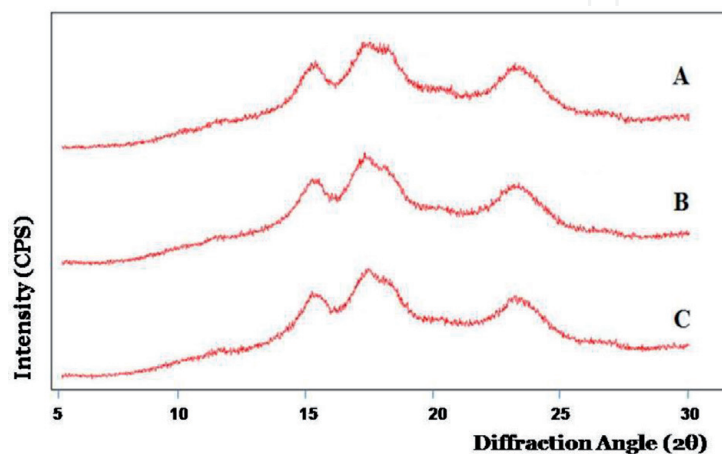
Rupollo [17] analyzed by SEM the starch grains isolated from carioca beans stored for 360 days under three conditions: hermetically sealed at 5°C and atmosphere modified by nitrogen at 15°C and in a conventional atmosphere at 25°C. The author observed great similarity between the granules, even in different storage conditions of the seeds. However, the starch granules of the seeds stored in a conventional atmosphere at 25°C appeared to be more aggregate than the others. The influence of storage conditions on starch properties was verified through a joint data analysis, which is a multivariate technique used to evaluate how consumers develop preferences for products or services. The bean starch stored in a nitrogen-modified atmosphere at 15°C did not differ in solubility and gel properties compared to beans stored in a conventional atmosphere at 25°C. However, the gel properties of these two conditions differ from the hermetically packaged at 5°C, which presented lower crystallinity, as well as the swelling and heat power required for gelatinization. The grain starch stored in a nitrogen-modified atmosphere at 15°C, in turn, demonstrated lower crystallinity, swelling power, and heat required for gelatinization than grain stored in a conventional atmosphere at 25°C.

Vanier et al. [32] characterized starches from four common bean genotypes to use in production of biodegradable films. The authors observed that depending on the common bean genotype, a great variation on starch properties was found, which, in turn, clearly impacted on the characteristics of the starch-based films.

### 3.4 X-ray diffraction

The X-ray diffraction properties provide evidence of an ordered structure of the starch granule. The difference between crystallinities is associated with amylopectin, while amorphous regions are generally related to amylose. **Figure 8** presents the diffractograms of beans starches.

The starch isolated from black bean shows the highest peak values for the three evaluated. The diffraction angles 15, 17, and 23° represent the highest intensity peaks detected in the X-ray diffractograms, being even higher in 17° for all the analyzed starches in this work.



**Figure 8.** Intensity of X-ray diffraction peaks of starch isolated from beans (A) cowpea, (B) black, and (C) carioca.

The yield of the starch isolation, the main peak intensities, and the relative crystallinity, verified in X-ray diffractograms, are shown in **Table 1**.

The relative crystallinity (RC) was in descending order, black bean (10.64%) > cowpea (10.57%) > carioca bean (10.50%), having varied significantly, considering the analysis of variance.

Gernat et al. [33] analyzed X-ray diffractograms of *Vicia faba* and *Pisum sativum* (bean and pea, respectively) compared to corn and potato starches, which are types A and B, respectively. The authors found, by means of a linear regression method, pea starch composed of 38.6% of type B and 61.4% of type A and 17.0% of bean starch of type B and 83.0% of type A. Garcia and Lajolo [34] analyzed the changes of HTC in starch grains and found a very strong birefringence in starch grains from HTC beans, suggesting that the starch isolated from these grains has a higher degree of crystallinity.

According to Hoover and Ratnayake [35], differences in relative crystallinity between starches are affected by crystal size, amount of crystalline regions (influenced by amylopectin chain content and length), and orientation of double helices in the crystalline domains and by degree interaction between double helices. In their work, all starches showed a pattern of type C X-rays, typical of legumes. The peak at  $2\theta = 5.54$  (characteristic of type B starches) was more pronounced in pinto bean and black bean starches. Relative crystallinity followed the order: pinto beans > lentil > smooth pea > pea > black beans > white beans.

Type A pattern has the shortest amylopectin chain. Its structure is orthogonal and contains only eight molecules of water with few irregular connections, and amylose is distanced from amylopectin by an amorphous region, which is less dense and absorbs water more rapidly and is more susceptible to chemical and enzymatic modifications. In relation to the C pattern, a higher intensity of the diffractogram peak suggesting strong internal bonds of the molecules and a higher degree of association between the starch chains is observed [36].

Lawal and Adebawale [37] analyzed the physicochemical characteristics and thermal properties of chemically modified porcine bean (*Canavalia ensiformis*) and observed, in addition to the conventional type C, an increase in the intensity of starch diluted in acid solution. The authors did not observe significant differences between the X-ray pattern of native starch and modified derivatives.

Rupollo [17], evaluating the effects of storage conditions and time on the quality of carioca beans, observed that the starch of grains stored in a conventional atmosphere at 25°C were more influenced than the starch isolated from beans stored in modified atmosphere with nitrogen at 15°C, certainly due to the development of the HTC effect on grains stored in the conventional system.

Pinto [38] evaluated carioca bean starch submitted to different treatments and observed the following sequence regarding the degree of relative crystallinity

Bean	Starch yield (%)	Intensity (CPS <sup>*</sup> )			RC (%)
		15°	17°	23°	
Cowpea	16.04	1863	2214	1822	10.57
Black	16.85	1938	2358	1834	10.64
Carioca	30.24	1922	2306	1806	10.50

\*CPS: counting per second.

**Table 1.**  
 Starch yield, main peaks intensity, and relative crystallinity of isolated starches from cowpea, black, and carioca.



obtained with the different procedures: enzymatic hydrolysis > native starch > heating > ultrasound > low humidity heat treatment.

#### **4. Conclusion**

No OM differences were observed between the morphology of the starch grains of raw samples of cowpea, black beans, and carioca beans. The cotyledons of cooked carioca bean and cowpea samples completely lose the structural organization of the starch granules. In the cotyledons images of black common bean samples, cooked under the same conditions as the others (180°C in a pressure cooker for 45 minutes), the presence of the starch granule is still observed, suggesting the occurrence of the HTC, confirmed in this study by SEM, phenomenon whose should be of great relevance in the inspection of grains put on sale for consumption. With regard to the crystallinity studies of starch granules, by XRD, the diffraction angles found in this work are more consistent with the classification of the standard polyphorm A, and the RC was in descending order: black bean > cowpea > carioca bean.

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