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Elisabeth Garcia
Universidade de Sao Paulo

Franco M. Lajolo
Universidade de Sao Paulo

Barry G. Swanson
Washington State University

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A COMPARATIVE STUDY OF NORMAL AND HARD-TO-COOK BRAZILIAN COMMON BEAN (*Phaseolus vulgaris*): ULTRASTRUCTURAL AND HISTOCHEMICAL ASPECTS

Elisabeth Garcia[1,*], Franco M. Lajolo[1], Barry G. Swanson[2]

[1]Depto. Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Caixa Postal 66355, CEP 05389, São Paulo, Brasil
[*]Present address: Dept. of Food Science and Technology, University of California, Davis, CA 95616-8598
[2]Dept. of Food Science and Human Nutrition, Washington State University, Pullman, WA, 99164-6376.

Abstract

Legume seeds stored under high temperature and humidity develop a texture defect known as hard-to-cook (HTC). Structural and histochemical characteristics of normal and HTC beans (*Phaseolus vulgaris*) were studied after storing them at 5°C/40% relative humidity (RH) or 40°C/75% RH for 60 days. Cotyledonary cells of HTC beans showed contraction of the cell content, whereas the cytoplasm of normal seeds occupied the total cell volume. Cell walls of HTC beans appear more compact, showing smaller intercellular spaces. In normal beans the cell walls of adjacent cells had larger spaces between them. Pectic material of sections of hard beans stained more intensely than cell walls of normal beans. Preliminary results suggest that cell walls of hard beans contain more calcium than normal beans. HTC-and-old beans (6 years of storage) were observed under SEM in comparison to irradiated beans. Irradiation of beans caused softening of the seeds. The results confirm involvement of the cell wall-middle lamella in the hardening of bean seeds.

Key words: Legumes, hard-to-cook, ultrastructure, histochemistry beans, *Phaseolus vulgaris*, textural defects.

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Direct inquiries to E. Garcia
Telephone number: (916) 752-5545
Fax number: (916) 752-4759

Introduction

The softness of cooked beans is an important condition for consumer acceptance. Beans stored at high temperature and humidity develop a texture defect known as hard-to-cook (HTC), because they do not soften during regular cooking. The deterioration of cooking and organoleptic attributes leads to economic losses resulting either from increased requirement for energy to achieve softening, or consumer rejection. In addition, HTC can decrease protein biological value of beans (Sgarbieri and Whitaker, 1982).

Involvement of different components of the cotyledonary tissue has been proposed to explain the development of HTC. Soft texture is associated with the ability of cotyledon cells to separate easily in cooked beans (El-Tabey Shehata, 1992). Some authors inferred the participation of pectin and phytin (Jones and Boulter, 1983a, b; Moscoso *et al.*, 1984), phenolic compounds (Hincks and Stanley, 1987; Srisuma *et al.*, 1989), and changes in starch and protein solubility (Molina *et al.*, 1975; Hohlberg and Stanley, 1987; Hussain *et al.*, 1989). Changes at the structural level have also been reported (Rockland and Jones, 1974; Varriano-Marston and Jackson, 1981; Shomer *et al.*, 1990). In spite of the knowledge accumulated on HTC, an adequate understanding of the physiological mechanism(s) has not yet been achieved.

The objective of this study was to compare structural and histochemical characteristics of normal and HTC seeds of the Brazilian Carioca cultivar of common bean (*Phaseolus vulgaris*) to obtain a clearer comprehension of the HTC phenomenon *in situ*. Specifically, we wanted to clarify the role of the middle lamella-cell wall in the HTC process.

Material and Methods

Material

Common bean (*Phaseolus vulgaris*, cv Carioca) seeds were provided by the Instituto Agronomico de Campinas-SP. Carioca beans have a cream background with tan stripes. Control samples were kept at 5°C/40%

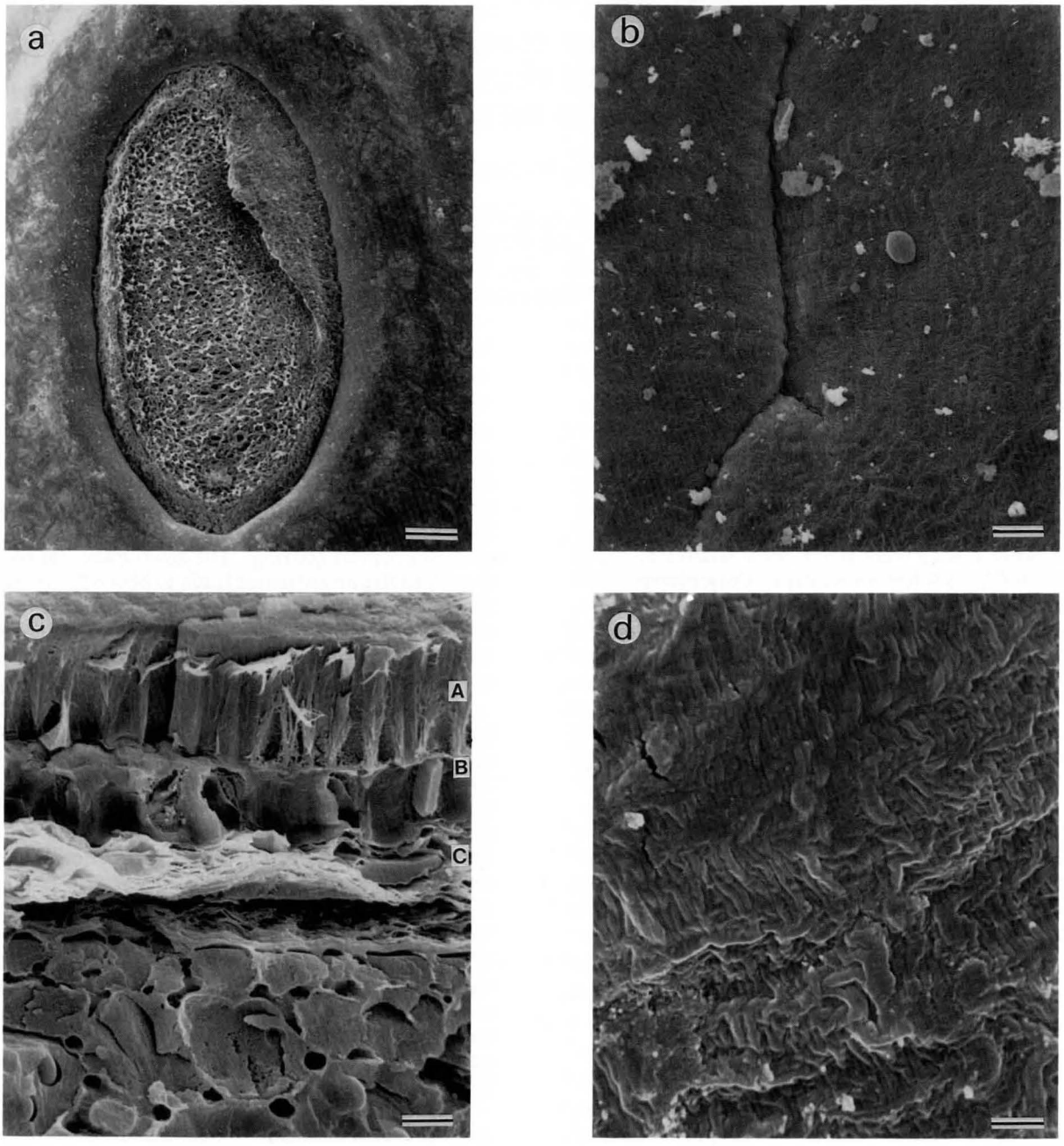

Figure 1. Scanning electron micrographs of *Phaseolus vulgaris* dry seeds showing hilum (**Fig. 1a**), micropyle (**Fig. 1b**), transverse section of seed coat (**Fig. 1c**) and seed coat surface (**Fig. 1d**). Figure 1c illustrates the palisade (A), subepidermal (B) and parenchyma (C) cell layers characteristic of legume seeds. Bar = 0.2 mm (a); 12.5 μm (b and c); and 2 μm (d).

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relative humidity (RH) and the HTC samples at 40°C/ 75% RH for 60 days (accelerated hardening; Vindiola *et al.*, 1986). Also a sample of beans referred heretofore as HTC-and-old was kept under room conditions for 5 to 6 years. A sub-sample of the beans stored for 6 years was irradiated with ^{60}Co at a 10 krad dose prior to storage.

Methods

Light microscopy. After removal of the seed coat, beans were fixed in ethanol:formaldehyde:acetic acid:water (30:10:10:10) for 7 days, with three substitutions of the fixative solution. The samples were then embedded in paraffin and sectioned (5 μm).

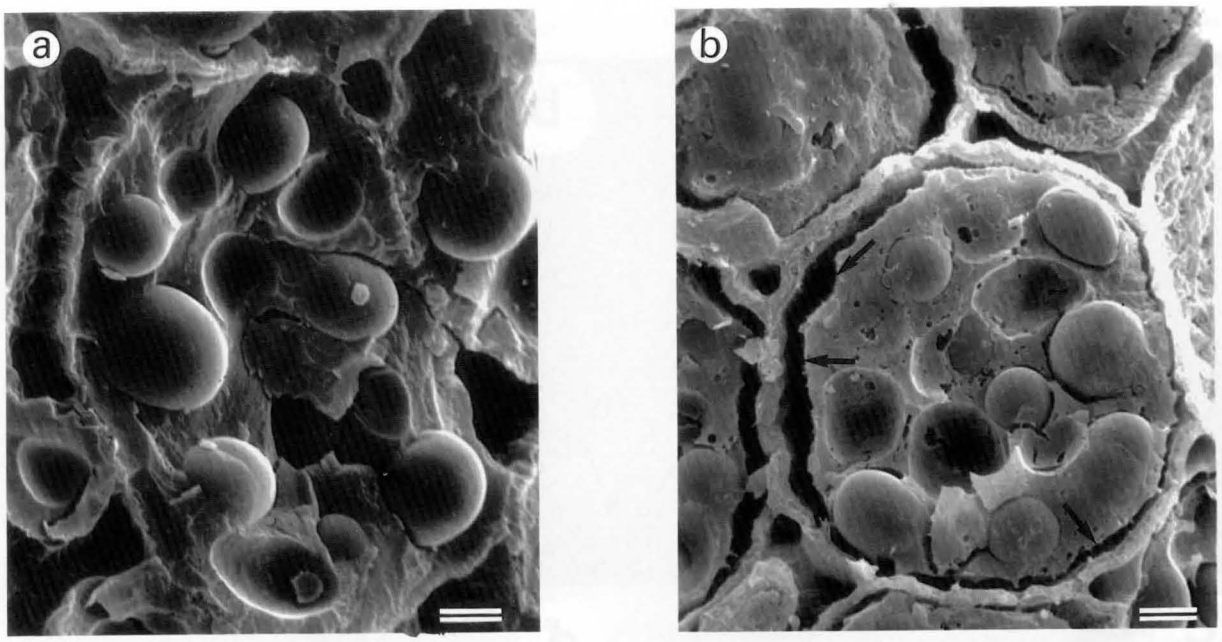

Figure 2. Scanning electron micrographs of cross-sectional view of dry common bean seeds: normal seed (**Fig. 2a**); HTC seed (**Fig. 2b**). Figure 2b illustrates the separation between the cytoplasm of HTC cotyledon cells and cell wall (see arrows). Bar = 6.67 μm (a); and 10 μm (b).

Sections were stained with a 1% methylene blue solution for 5 minutes to stain the pectic substances of the cell wall/middle lamella area (Kertesz, 1951). The excess dye was removed by successive washings with distilled water.

A 0.05% toluidine blue solution in acetate buffer pH 4.4 was also used for staining. After removal of excess dye by washing with water, the sections were air-dried and mounted in oil. Polycarboxylic acids (including pectic acids) are stained red and lignin and polyphenolics are stained green (Feder and O'Brien, 1968). Sections were viewed with a Nikon Labophot microscope.

Staining with 0.1% Congo red for 5 minutes was used to reveal β-glucans of the cell wall (Fulcher *et al.*, 1984). After washing the sections to remove the excess dye, they were air-dried and mounted in non-fluorescent immersion oil. Sections were viewed with a Nikon Fluophot microscope using IF 400-500 and 580 W filters.

Calcium was detected by treating sections with chlorotetracycline (CTC, Sigma C-4881), and viewing with a Nikon Fluophot microscope (IF 385-425 and 530 W filters) between 5 and 30 minutes after staining with CTC. A second slide prepared in the same way was subsequently treated with ionophore A23187 (Sigma C-7522) and the emitted fluorescence was observed as previously described (Powell, 1987).

Scanning electron microscopy. For the observation of the hilum, micropyle and seed coat surface, whole beans were mounted on aluminum stubs using colloidal graphite cement, and sputter coated with a 300 μm layer of gold. To fracture beans for cross-sectional examination, bean seeds were soaked in water overnight and then fixed in glutaraldehyde/formaldehyde (3%/3%) in 0.05 M, pH 6.8 phosphate buffer for 24 hours. The samples were then rinsed three times with phosphate buffer (0.05 M, pH 6.8), and finally with water. Subsequently, the samples were dehydrated using a graded ethanol series (10%; 20%; 30%; 40%; 50%; 60%; 70%; 80%; 2 X 95%; 3 X absolute ethanol), and finally critical point dried (Humphreys, 1974). The dried cotyledons were fractured under liquid nitrogen, using a razor blade hit with a hammer. The fractures were then sputter coated with gold (300 μm) after mounting on aluminum stubs (as described for the whole beans). The samples were examined with a Hitachi S-570 scanning electron microscope at an acceleration voltage of 20 kV. Pictures were taken with Polaroid 55 (P/N) film.

Results and Discussion

Despite the vast literature published on HTC, there is still controversy about the causes of this phenomenon. A comparison between HTC and normal bean seeds through microscopy techniques could reveal structural changes leading to an identification of the causes of the HTC phenomenon.

General structural characteristics

Preliminarily, an external characterization of the

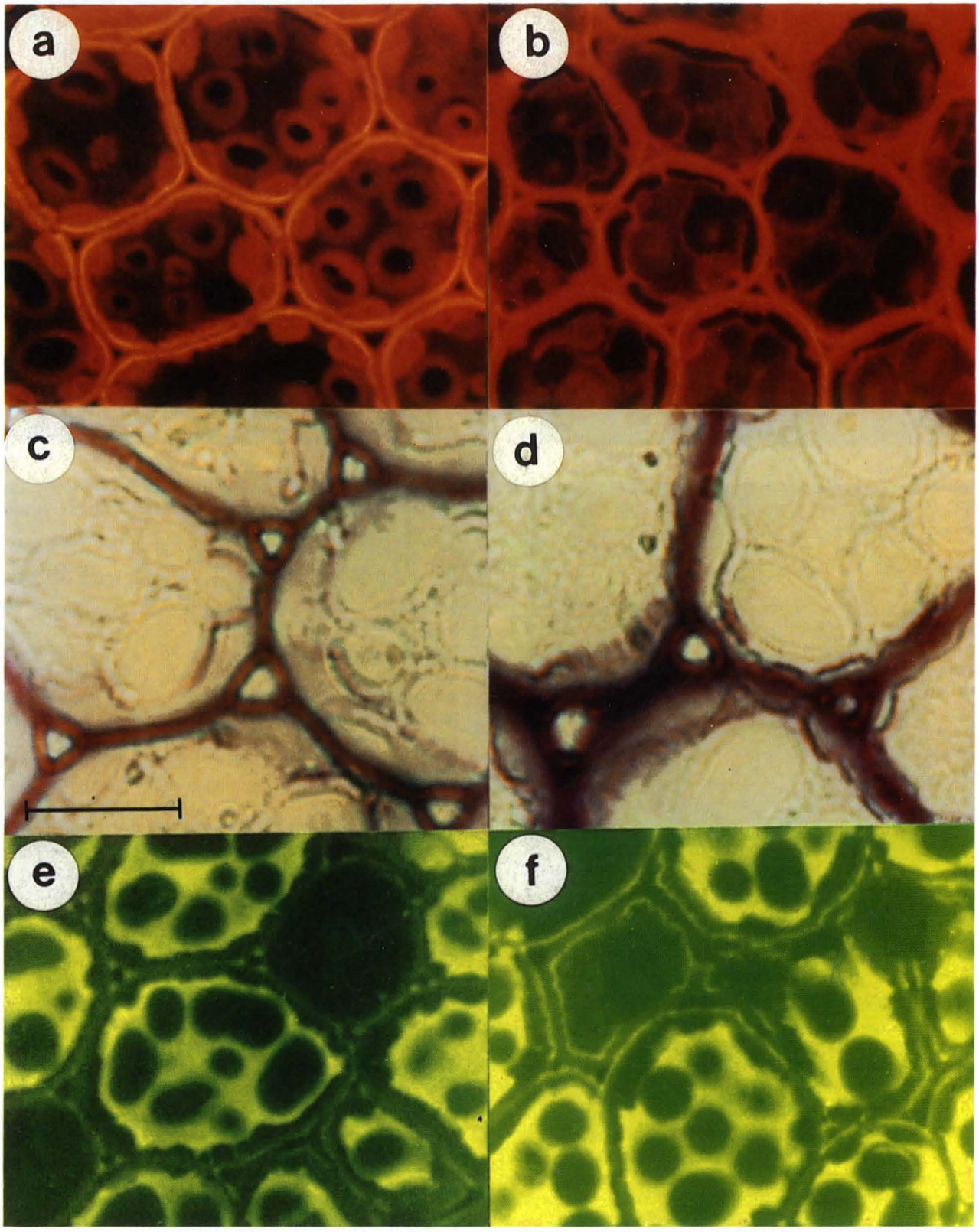
a
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Figure 3 (color plate, facing page). Longitudinal sections of dry common bean cotyledons observed under optical microscope after different staining procedures: with Congo red (**Figures 3a and 3b**), with toluidine blue (**Figures 3c and 3d**); with chlorotetracycline (**Figures 3e and 3f**) on normal seeds (**Figures 3a, 3c and 3e**) and HTC seeds (**Figures 3b, 3d and 3f**). Bar = 50 μm (a, b, e, f); and 25 μm (c, d).

Carioca beans was performed to compare this variety, widely cultivated in Brazil, with other common bean varieties described in the literature. Figures 1a and 1b show micrographs of the hilum and the micropyle of the Carioca beans. These structural features were similar to the varieties examined by Agbo *et al.* (1987). Observations of normal and HTC beans revealed no obvious differences in the external structural features. Even HTC-and-old beans (5 and 6 years old) did not reveal any external differences, except discoloration, which is a known characteristic of hard beans (Burr *et al.*, 1968; Garruti and Bourne, 1985; Vindiola *et al.*, 1986). The seed coat is shown in Figures 1c and 1d. A transverse fracture of a cotyledon (Figure 1c), showing the multiple layers (palisade, sub-epidermal and parenchyma) of the seed coat, is similar to the description of Hughes and Swanson (1985). Figure 1d shows the external appearance of the seed coat. The micrographs of the mature Carioca bean seed coat are similar to the black bean sample examined by Hughes and Swanson (1985) 19 days after flowering. The mature black seeds have a different seed coat pattern. No differences in seed coat were detected among normal (soft) and HTC Carioca beans.

Longitudinal sections of beans were viewed under light microscopy (results not shown). Cotyledonary cell size varied greatly. Vascular bundles were distributed through the entire cotyledon. Cells contained several starch granules, which were round- or oval-shaped and varied in size.

Viewed by SEM, the cytoplasm of normal seeds occupied the total cell volume (Figure 2a). In contrast, HTC bean cotyledon cells showed an accentuated contraction of the cellular contents (Figure 2b). This confirmed the observations of Varriano-Marston and Jackson (1981).

Staining with Congo red

Histological sections of beans were stained with Congo red and observed under fluorescence microscopy (Figures 3a and 3b). Hard beans showed a more compact packing of the cells, whereas normal beans showed a better definition of cell wall separation and larger intercellular spaces (Figure 3a).

Hardened samples also showed a more compact cytoplasm and cell wall (Figure 3b). Walls of adjoining cells were not well differentiated: in some areas only one stained line could be seen, instead of the two lines visible between two adjacent cells in normal seeds (Figure 3a).

Staining with toluidine blue

Longitudinal cotyledon sections were stained with toluidine blue which reacts with polycarboxylic acids giving a red color (Feder and O'Brien, 1968). Cell walls of HTC beans exhibited a stronger coloration of the cell walls (compare Figures 3c and 3d). Taking into account the composition of plant cell walls (Selvendran, 1983), our observations indicate that the pectins are reacting with the dye with different affinities in normal and HTC beans. It is possible that more carboxylic groups are free in the hard sample leading to the stronger coloration observed in Figure 3d, while in the normal seeds probably the methanol-esterified form predominates. Shomer *et al.* (1990) also detected an intense staining of the cell wall in HTC beans. The stronger affinity of the cell wall for the toluidine blue staining suggests that a higher number of free carboxylic groups are available for cross-linking between pectin molecules. Our observation is in agreement with the decrease in pectin esterification reported by Jones and Boulter (1983b) and decrease in pectin solubility (Jones and Boulter, 1983a, b; Hentges *et al.*, 1991).

Fluorescence with chlorotetracycline (CTC)

The participation of pectin in the hardening process could result from a prior de-methoxylation step following pectinesterase action (Jones and Boulter, 1983b; Stanley and Aguilera, 1985). An increased amount of calcium and/or magnesium at the cell wall/middle lamella level would be a further indication of formation of insoluble pectates. The presence of those divalent cations was investigated using a specific fluorescent probe, CTC. CTC binds to calcium and magnesium producing a fluorescent emission (Powell, 1987). If, after CTC reaction the samples are treated with the ionophore A23187, the Ca-CTC fluorescence is suppressed and the Mg-CTC emission can be detected. Using this technique, we observed that calcium was more abundant than magnesium at the cell wall/middle lamella (results not shown).

Histological sections of soft and hard beans treated with CTC are presented in Figures 3e and 3f. HTC bean sections showed a higher fluorescence over the entire cell wall contour, while soft bean sections revealed more intense fluorescence, predominantly at cell wall corners. Unfortunately, it was not possible to measure the fluorescence intensity to get a precise comparison of the samples. The Ca-CTC fluorescence was detected in the cell wall and not in the intercellular space (Figures 3e and 3f). This suggests that calcium was bound to the cell wall matrix pectin or was cross-linking outer chains of pectins of the middle lamella.

Fluorescence resulting from treatment with ionophore A23187 was comparable in both soft and hard beans (micrographs not shown). Residual fluorescence (Mg-CTC) was mainly restricted to the corners between cells.

The middle lamella appears to play an important role in the hardening process. Soaking of HTC beans

leads to mineral losses possibly as a consequence of increased membrane permeability (Varriano-Marston and Jackson, 1981; Jones and Boulter, 1983a; Hincks *et al.*, 1987; Srisuma *et al.*, 1989; Hentges *et al.*, 1991). Consequently, calcium ions released through the action of phytase upon phytic acid located in the protein bodies could cross the cell walls in higher amounts and eventually be bound by the free carboxyl groups of the pectins in the middle lamella. Carpita (1987) suggested that the cross-linking capacity of calcium favors the hypothesis that cell wall rigidity is controlled by the middle lamella pectin gel strength. Previous observations (Rockland and Jones, 1974; Varriano-Marston and Jackson, 1981; Plhak *et al.*, 1987; Shomer *et al.*, 1990) showed that the middle lamella of hard beans resists solubilization during cooking, thus preventing cell separation.

SEM of irradiated seeds

Because Mancini-Filho (1990) had reported a drastic decrease in cooking time of irradiated Carioca beans, an SEM comparison was then performed of HTC-and-old bean seeds (cooking time 520 minutes) and irradiated seeds (cooking time "zero" minute). HTC-and-old beans (Figure 4a) revealed the same characteristics already described in Figure 2b: compactness of the cells and contraction of the cellular content. The cells of the irradiated sample were not broken during cotyledon fracturing, and were well separated; intercellular cohesion material was not detected. These general observations were very similar to those reported for cooked beans where middle lamella was solubilized (Hincks and Stanley, 1986). Probably the high irradiation dose used was responsible for the hydrolysis of pectic substances resulting in a drastic softening of the seeds. This would be an additional indication of the important role of the middle lamella in defining texture of cooked beans.

Conclusions

The following observations support the hypothesis that middle lamella-cell wall is involved in the development of HTC in beans through the interaction of minerals with pectin: (1) stronger staining with toluidine blue (higher level of free carboxyl groups) in hard beans; (2) calcium seems to be more abundant in the cell wall of HTC beans; (3) disappearance of the middle lamella in irradiated beans. Nevertheless, this seems to be one of the several mechanisms involved. A more detailed study of the cell wall/middle lamella is still required for an in-depth comprehension of the texture problems observed in legume seeds.

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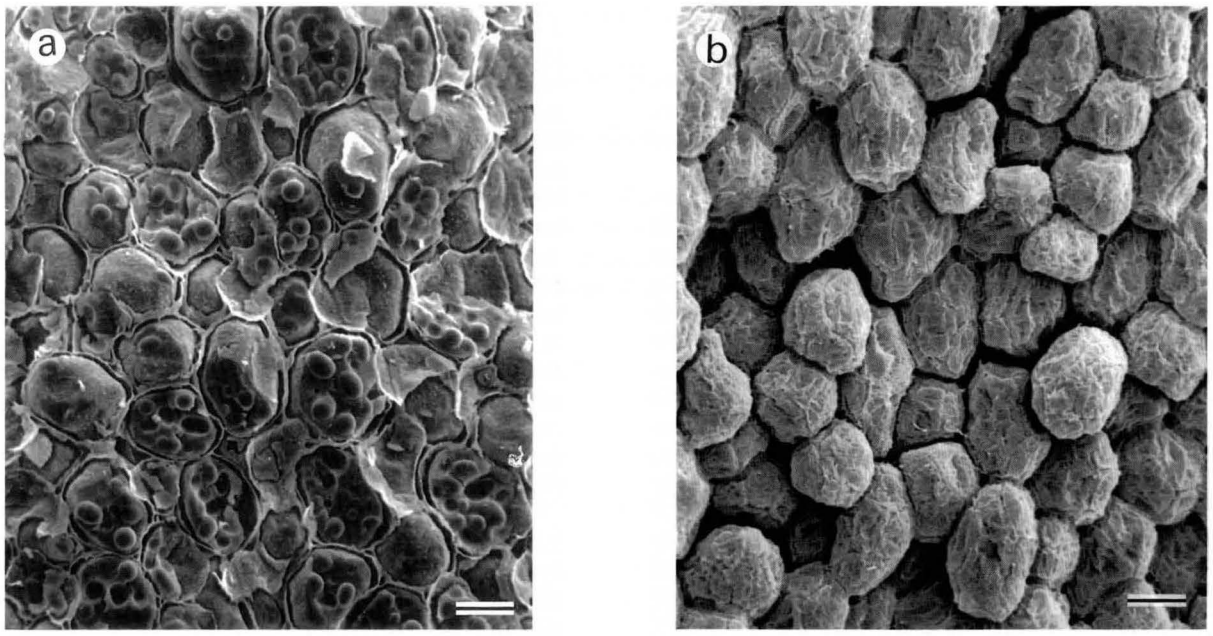

Figure 4. Scanning electron micrographs of cross-sectional view of dry common bean seeds. Fig. 4a shows HTC and old seed; Fig. 4b shows irradiated bean seed. Fracture of HTC and old bean seed exposes compacted cellular content and cell walls. Cotyledon cells of irradiated bean seed appear intact and separated. Bar = 50 μm.

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Discussion with Reviewers

W.J. Wolf: In your discussion of irradiated beans you refer to "a cooking time of zero minutes". If they were simply soaked in water would they be as soft as cooked unirradiated beans?
Authors: The reference to cooking time of "zero" minutes is related to the type of measurement adopted. We used a modified Mattson cooking device (Jackson and Varriano-Marston, 1981). Bean seeds were arranged in the holes of the cooker, while the tip of a plunger with calibrated weight was resting on each seed. When the cooking device was completely assembled, it was immersed in a container with boiling water. When the plungers penetrated the seeds, the beans were judged as cooked. Cooking time is determined when half of the seeds were pierced by the plungers. In the case of the irradiated beans, as soon as the cooker was placed in contact with the water, the seeds were perforated, giving a cooking time of "zero" minutes. However, we should point out that those seeds had a "gummy" consistency; they were soft, but when smashed they did not give a paste.

L.B. Rockland: It would be of interest to know if starch gelatinization was apparent in the irradiated beans.
Authors: We did not study the isolated starch of irradiated beans further.

J.S. Hughes: Is there any information to indicate that "hardness" or the "hard-to-cook" phenomenon in legumes is a problem commonly encountered by consumers in Brazil?
Authors: The climatic conditions (high temperature and humidity) in many areas of Brazil favor the development of HTC. It is a common practice to soak the beans overnight and cook them (whole seed) using a pressure cooker. Unfortunately, as in many other Latin-American countries, we do not have statistical data on the different causes of post-harvest losses related to legumes.

J.S. Hughes: Were any structural differences noted between the HTC beans (40°C/75%RH) and the HTC old beans (5-6 years old)?
Authors: We did not notice any obvious structural differences between these samples. The only striking difference is the dark color of the seed coat in HTC seeds, and even darker in HTC old beans, compared with soft seeds.

J.S. Hughes: Do you have any explanation for why you were unable to fracture the individual cotyledonary cells in the irradiated beans?
Authors: We believe that there was dissolution of the middle lamella, rendering the tissue very flexible. As a consequence, the tissue was deformed upon contact with the razor blade, and the cellular contents were not exposed.

E. Varriano-Marston: The supposedly more intense dye left on the sections shown in Fig. 3d than in 3c may be due to washing techniques/time after staining rather than any real difference.
Authors: The results presented were repeatedly observed; we confirmed that the washing technique and time adopted were appropriate.

Editor: How do the results of your work relate to the recent paper "Mechanism of hard-to-cook defect in cowpeas: verification via microstructure examination" by K. Liu, Y.-C. Hung, R.D. Phillips, Food Structure, Vol. **12**(1), 1993, pages 51-58?
Authors: The Liu *et al.* (1993) paper reported that decreased solubility and thermal stability of intracellular proteins during storage limited water availability in the cotyledon during cooking, and reaffirmed the role of the middle lamella in bean hardening. Our study focussed on microstructural and histochemical techniques on the middle lamella and cell walls of hard-to-cook beans, concluding that greater concentrations of free carboxyl groups and calcium in the cell walls may be responsible for hardening. Liu *et al.* (1993) observed no structural differences between dry control and dry aged cowpeas; we observed contraction of the cell contents of beans stored at 40°C and 75% RH. Both papers recognize the inherent involvement of multiple physiological and structural mechanisms in legume hardening.