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THE ROLE OF CELL WALL STRUCTURE IN THE HARD-TO-COOK PHENOMENON IN BEANS (*PHASEOLUS VULGARIS* L.)

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Abstract

Differences in structure, water uptake and efflux of solubles were found between normal and hard-to-cook beans (*Phaseolus vulgaris* L.). The staining characteristics of hard beans were different from those of the normal in both non-cooked and cooked tissue, especially in the cell walls. The three zones of the cell wall - the middle lamella, the primary and the secondary walls - were found in both normal and hard-to-cook beans. The primary cell wall of both normal and hard beans was partially degraded upon cooking, as seen by bright fields within the fibrillar pattern. The ultrastructure of the non-cooked secondary cell wall was significantly different between normal and hard beans. The middle lamella in normal beans disintegrated upon cooking, while that of hard beans was retained. Hard beans took up less water during soaking but released more solubles to the medium than normal beans.

It is concluded that the resistance of the middle lamella to solubilization upon cooking prevents cell separation, and this results in the hard texture of the hard-to-cook beans.

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KEY WORDS: Bean, Cell wall, Hard-to-cook, Middle lamella, Pectin, *Phaseolus vulgaris*, Ultrastructure.

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Introduction

The hard-to-cook (HTC) phenomenon in beans manifests itself in prolongation of cooking time, thus reducing the beans' nutritional and textural qualities. This phenomenon, which will be referred to as hardening, was found to be induced by storage at elevated temperatures and high relative humidities (Plhak *et al.*, 1987).

Several suggestions have been given to explain this phenomenon. Varriano-Marston and Jackson (1981) suggested that cytoplasmic changes during storage lead to enhanced cell adhesion, and hence hardening of the beans. Hincks and Stanley (1986, 1987) suggested that polymerization of phenols within the cell wall might lead to bean hardening. Based on the work of Wardrop (1971) and Esau (1977) suggesting that lignin monomers may be secreted into the region of the middle lamella, Varriano-Marston and Jackson (1981) assumed that hardening in beans stored under unfavorable conditions is caused by lignification of the middle lamella. However, no differences were found between normal and hard beans in the degree of lignification, content of tannins and specific activity of peroxidase (Plhak *et al.*, 1987). Histological studies (Varriano-Marston and Jackson, 1981; Jones and Boulter, 1983; Narasimha *et al.*, 1989) revealed differences in the packing density of the cotyledon cells between normal and HTC beans. No evidence relating these changes to specific cell components was presented.

The present study describes the structural changes occurring in the cotyledon tissue of beans as a result of hardening. The involvement of the cell wall, especially the middle lamella, in the hardening phenomenon is described and discussed.

Materials and Methods

Preparation of hard-to-cook beans:

Dry beans (*Phaseolus vulgaris* L. cv. 'Ivory') from local production, stored for about 3 months at ambient temperature and humidity (20-24°C, 50-60% RH) were used for the experiments. The moisture content of the

dry beans, as measured by oven-dry method (Anon., 1966), was 11.5%. Hardness was induced by incubating bean seeds at 42°C and 80% RH (in a desiccator, over a solution of saturated KCl), for 21 days. The incubated beans and a sample of non-treated beans kept at ambient room temperature and humidity were dried to a constant weight at 25°C under reduced pressure (250 mm Hg). The water content of the beans was determined by drying to a constant weight at 70°C at reduced pressure (250 mm Hg). The water content of both samples after drying was 12%.

Soaking and cooking of beans:

Ten g of beans was placed in 100 ml of distilled water in a 250 ml Erlenmeyer flask and shaken at a rate of 50 strokes/min for 18 h at room temperature. The weight of the beans and the volume of the liquid were then determined. The beans were cooked in boiling water at ambient pressure for 30 min; after cooking, distilled water was added to compensate for evaporation. The weight of the beans and the volume of the liquid were then measured. Water uptake was calculated as increase in weight after soaking or cooking, with a correction for the amount of solubles which leaked into the medium from the beans. Leakage of solubles was determined by weighing the residue after lyophilization of 10 ml of soaking or cooking liquid. Hardening was examined by pressing the cooked bean with the fingers; normal beans disintegrated, while the hard-to-cook retained their integrity.

Chemical analyses:

Nitrogen was assayed by the Kjeldahl procedure. Phosphate was analyzed in Kjeldahl digests by a Technicon Automatic Analyzer System (Technicon Corporation, Tarritown, NY). Trichloroacetic acid (TCA) coagulable nitrogen was measured in the precipitates formed by addition of TCA to a final concentration of 15%. Alcohol-insoluble solids (AIS) were precipitated by addition of two volumes of ethanol. The precipitate was washed twice with 70% ethanol, dissolved with 0.05 N NaOH and assayed according to the method of Blumenkrantz and Asboe-Hansen (1973). Total soluble sugars in the soak and cooking water were determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Light and electron microscopy:

Small slices (~1-2 mm^3) from the center of bean cotyledon tissue were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) for 2 h at 4°C. The slices were then rinsed several times with the same buffer, followed by washing with 0.1 M phosphate buffer (pH 7), postfixed with 2% OsO_4 in the phosphate buffer at 4°C for 2 h, and then washed several times with the phosphate buffer, followed by washing with distilled water. The fixed specimen was dehydrated gradually with ethanol and embedded in Agar 100 Resin (Agar Aid, Cambridge). For light microscopy, sections of ~3μm thickness were prepared by LKB Pyramitome, mounted on a glass slide, and stained with a 10 X diluted solution of toluidine blue and basic fuchsin (0.365 g and 0.135 g, respectively, in 60 mL of 30% ethyl alcohol). For transmission electron microscope (TEM), ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL-100CX TEM at 80 kV.

Results and Discussion

Characteristics of normal and hard-to-cook beans:

Hard-to-cook beans were found to be hard but springy after cooking, while normal control beans stored at room temperature were disintegrated easily when subjected to light finger pressure. The water uptake and efflux of solutes in beans upon soaking and cooking are shown in Tables 1 and 2, respectively. After 18 h of soaking in water, HTC beans took up 20% less water as compared with normal beans. From Table 1 it is seen that HTC beans release approximately tenfold more sugars and nitrogen, and about 45-fold more phosphorus to the soaking water than normal beans. Increased efflux of solubles and lower water imbibition upon soaking of HTC beans were reported by Jones and Boulter (1983). A

Table 1: Uptake of water by, and efflux of solubles from bean seeds soaked in distilled water for 18 h (mg/g dry matter).

Storage conditions	Water uptake	Efflux			
		Sugars[a]	Pectin[b]	Nx6.25[c]	P[d]
	±50	±1	±0.02	±0.5	±0.1
Control	1250	4.0	-	3.0	0.2
42°C at 80% RH[e]	1010	44.0	0.08	24.0	9.0

[a]Total sugars, as glucose, determined by the phenol - sulfuric acid method.
[b]As galacturonic acid
[c]No measurable amount of TCA-coagulable protein
[d]Phosphate
[e]Relative humidity

Figures 1-4. Light micrographs of cross sections of bean cotyledons, stained with toluidine blue and basic fuchsin (S - starch). Fig. 1 - normal bean. Primary cell wall is indicated by sharp arrows and the secondary one by blunt arrows; bar=10 μm. Fig. 2 - cooked normal bean. Primary cell wall is indicated by sharp arrows, and the secondary one by blunt arrows; bar=10 μm. Fig. 3 - non-cooked hard bean. Arrows indicate the cell wall; bar=10 μm. Fig 4 - cooked hard bean. Arrows indicate the cell wall; bar=10 μm.

disparity of views prevails concerning the extent of water uptake of HTC vs normal beans. Thus, Jackson and Varriano-Marston (1981) reported a similar extent of water uptake upon soaking by normal HTC beans. Small amounts of pectin were released from HTC beans upon soaking, while no AIS could be isolated from the soaking water of normal beans. From Table 2, it could be seen that after cooking for 30 min, there was twofold greater water uptake in normal beans than in HTC beans.

Table 2: Uptake of water by, and efflux of solubles from soaked bean seeds during cooking for 30 min (mg/g dry matter).

Storage		Water	Efflux				
					Nx6.25		
condition	HTC	uptake	Sug[a]	Pec[b]	Total	Prot[c]	P[d]
		±50	±1	±0.2	±0.5	±0.1	±0.5
Control	-	520	75	1.8	23.0	3.9	5.8
42°C at 80%RH[e]	+	260	7	t[f]	5.0	0.82	t[f]

[a]Total sugars, as glucose, determined by the phenol-sulfuric acid method

[b]As galacturonic acid.

[c]TCA coagulable protein.

[d]Phosphate

[e]Relative humidity.

[f]Traces

Efflux of carbohydrates, nitrogen and phosphorus was now higher in normal beans. Normal beans released pectin and TCA-coagulable nitrogen (protein) upon cooking. The increased efflux of solubles from HTC beans upon soaking indicates damage caused to the plasmalemma during the storage under adverse conditions. The reduced water uptake by HTC beans upon soaking may be ascribed to partial loss of semipermeability of the plasmalemma as well as to changes in high molecular weight components like pectic material or in intracellular storage proteins. Chemical changes leading to increased crosslinkage, or reduced hydrophilic character of these components will reduce their capacity to take up water during imbibition as well as during the cooking processes.

Light microscopy:

Light microscope observations showed that in cooked normal beans the cells of the cotyledon tissue tend to separate from each other (compare Figs. 1 and 2). However, in cooked HTC beans, cell adhesion and tissue integrity were retained (Figs. 3, 4). These findings are in agreement with those of other studies (Varriano-Marston and Jackson, 1981; Narasimha *et al.*, 1989 Plhak *et al.*, 1989), but the present results revealed distinct differences in the staining characteristics between tissue sections of normal and HTC beans. The most marked differences appeared in the cell walls. In HTC beans all of the cell wall regions were stained intensively (Figs. 3,4).

In the cooked beans, differences also were observed in the staining intensity of starch granules between normal (Figs. 1,2) and HTC (Figs. 3,4) beans. The integrity of starch grains and the residual birefringence observed under polarization microscopy of both normal and HTC beans suggest that starch was not entirely gelatinized upon cooking.

The differences in staining properties of the cell wall indicate a difference in the cell wall characteristics and composition between normal and HTC beans. A detailed study of the ultrastructure of the cell wall was therefore undertaken.

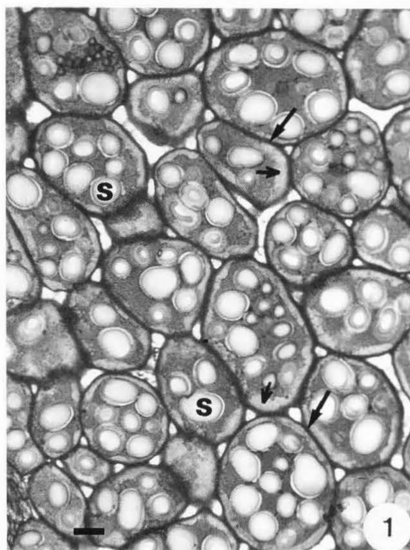
Electron Microscopy:

a. **Non-Cooked Beans:** The ultrastructure of cell walls in swollen normal beans before cooking is shown in Figures 5-9 Usually, the cell wall consists of three distinct regions: the outer region of the middle lamella, the middle region of the primary cell wall, and the innermost region of the secondary cell wall (Fig. 5). Facing the intercellular spaces the middle lamella is thicker, while it is thinner in the adhesion zone between the cells. A higher magnification of the middle lamella in the adhesion zone is shown in Figure 9. In many cases the middle lamella is so thin that it cannot be identified by ultrastructural observation.

The secondary cell wall is thick, adjacent to the intercellular space (Fig. 5), pits (Fig. 6) and disconnection zone (Figs. 7). The detailed structure of the secondary wall with three zones is shown in Figure 8. In cells of non-cooked normal beans the thick region of the secondary wall has a reticulated pattern, with an internal layer of dense structure (Figs. 5-8).

Higher magnification revealed that the structural elements of the reticulum are tubular. This reticulum seems to be embedded in a sparse microfibrillar matrix

Figures 5-9. Ultrathin sections of cotyledons of normal bean. The region of the cell wall includes the middle lamella (ML), primary cell wall (W) and secondary wall (S) with its outer, middle and inner layers (I, II, III, respectively). Figs. 5-7 - The secondary wall found usually adjacent to the intercellular space (ICS), pit (Pit) and disconnecting area (DC). Protein bodies (PB) seen in the cytoplasm (Cy); bar=1 µm. Fig. 8 - High magnification of the secondary cell wall shows reticulum (R), with its cross view seen as a tubular structure (arrows). The reticulum seems to be embedded in a fine fibrillar matrix (FM); bar=10 µm. Fig. 9 - High magnification of the middle lamella (ML) and the primary cell walls (W); bar=0.2 µm.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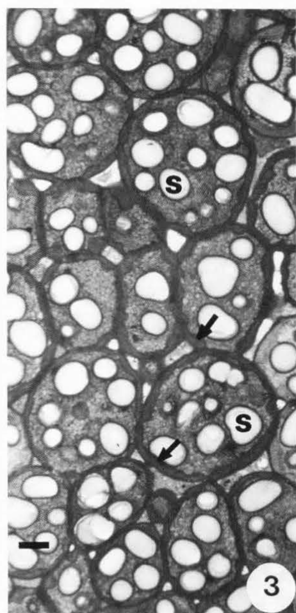
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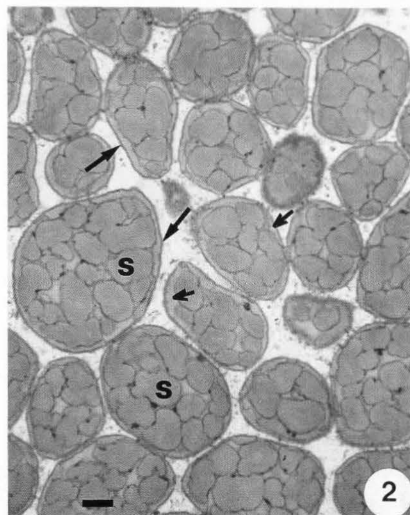
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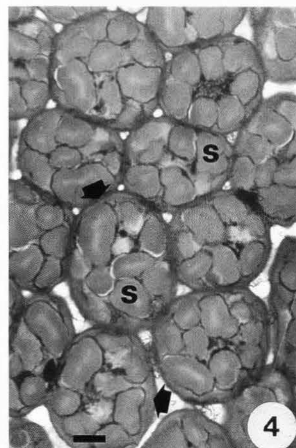
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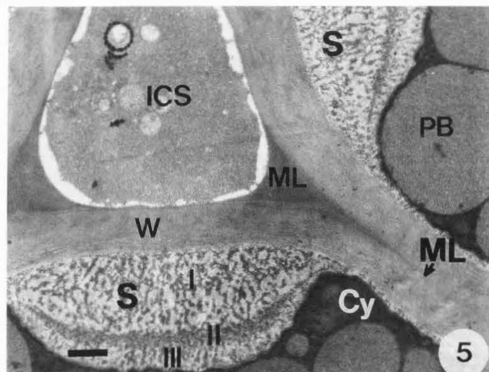
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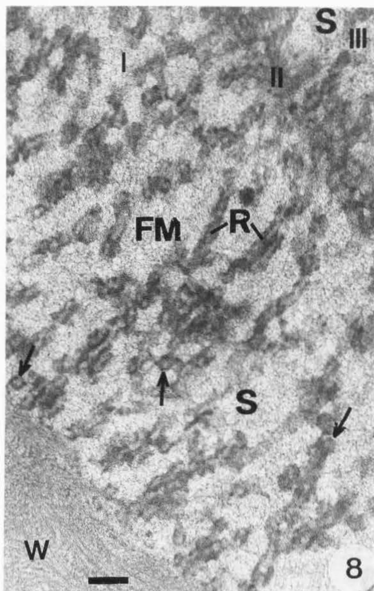
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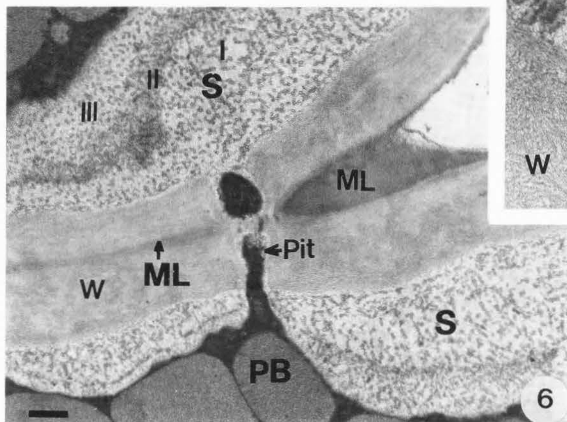
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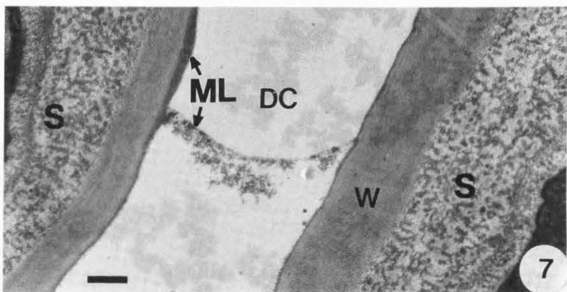
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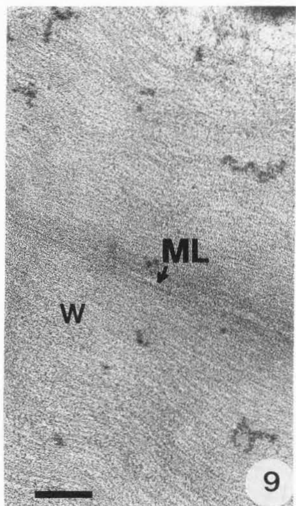
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(Fig. 8). The zone of the secondary wall shows densely packed tubules.

The cell walls of the non-cooked HTC beans differed from those of the normal beans in their ultrastructure, especially in the secondary cell wall (Fig. 10). The secondary wall is seen also adjacent to pits (Figs. 10,11). The typical reticular pattern of the secondary cell wall is not seen; instead, there appears a pattern of scattered tubuli with fibrillar connections (Figs. 11-13). A similar but less organized pattern is observed in the innermost region of the secondary cell wall (Figs. 13,14).

b. Cooked beans: The cooking process caused significant changes in the cell wall structure of normal beans. The primary cell wall is the most resistant part of the cell wall with respect to structural changes occurring upon cooking (Figs. 15-17), although many weakly stained locations are seen within its fibrillar pattern. It appears that in these locations the cell wall material was degraded upon cooking. The ultrastructure of the normal secondary cell wall (Figs. 5-8) changed dramatically as a result of cooking (Figs. 17,18). The middle lamella became loosened (Figs. 15,19,20). Detailed observations showed two typical zones from which the middle lamella was changed by the cooking process: (i) zones in which adjacent cells are attached to each other (Fig. 16), where the boundary between the cells is not seen clearly, because of the removal of the middle lamella; and (ii) zones in which cell separation is seen clearly and where the residues of the middle lamella are still observed (Figs. 19,20).

The most prominent change in the cell wall as a result of cooking appeared in the secondary wall. The typical reticular structure in non-cooked beans (Figs. 5-8) was lost, and replaced by an amorphous structure embedded within a fibrillar network (Figs. 15-18). The central zone of the secondary wall, where densely packed tubules were visible before cooking, are seen now as masses of dense amorphous matter (compare Figs. 15,18 with Figs. 5,8).

The secondary cell wall of cooked HTC beans (Figs. 21-23) is similar to that of cooked normal beans (Fig. 18). However, in cooked HTC beans the middle lamella retained its integrity (Figs. 21,24-26). In these specimens the primary cell wall contains weakly stained locations (Figs. 21,26), as was observed in the cooked normal beans (Figs. 15-17).

It appears that although part of the primary cell wall is removed as a result of cooking, the middle lamella is retained and still functions as a cementing agent which prevents cell separation upon cooking. This evidence supports the suggestion of Varriano-Marston and Jackson (1981) that the middle lamella is involved in reducing the cooking quality of beans. A possible mechanism by which the middle lamella becomes insoluble was suggested by Jones and Boulter (1983), who showed that during storage under conditions leading to HTC, phytate is partially hydrolyzed and pectin is de-esterified. This results in formation of calcium magnesium pectate, which may insolubilize the middle lamella.

The present study demonstrates in structural details the significance of the middle lamella in the HTC phenomenon. The results of Jones and Boulter (1983) suggests that HTC is due to changes in properties of the pectic components rather than deposition of lignin. Although Hincks and Stanley (1987) provided tentative evidence for lignification of cell wall in hard-to-cook beans, in a later study of Srisuma *et al.* (1988), no significant differences in lignin content were detected between normal and hard-to-cook beans.

Conclusions

The primary and the secondary cell walls respond to cooking similarly in both normal and HTC beans. In contrast, the middle lamella of normal beans is degraded. In HTC beans the middle lamella remains intact in spite of cooking, and hence it prevents cell separation and is thus responsible for the HTC phenomenon. However, the mechanism by which the middle lamellar components become insoluble during storage under unfavorable conditions is still unknown.

The involvement of other cellular components such as lignin, phenols, starch granules and proteins in the HTC phenomenon has not yet been elucidated.

Acknowledgements

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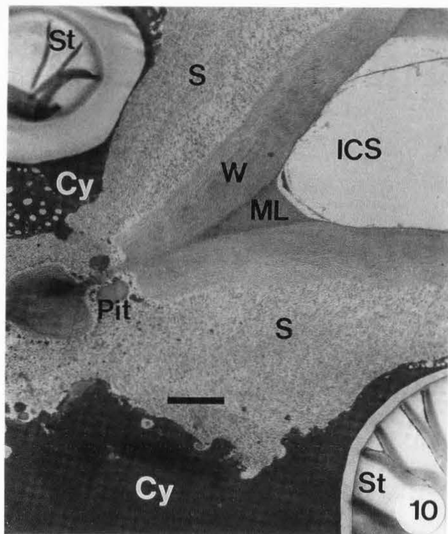
Figures 10-14. Ultrathin section of the cotyledon of a hard-to-cook bean. Fig. 10 - The area of intercellular space (ICS) with primary (W) and secondary (S) cell walls and pit (Pit), where the middle lamella (ML) seen clearly near the intercellular space. The cytoplasm (Cy) is seen as dark matrix with starch grains (St); bar=2 µm. Fig. 11 - High magnification of the primary wall (W) and the secondary wall (S) adjacent to the pit (Pit); bar=0.2 µm. Figs. 12-14 - High magnification of the primary cell wall (W) and the outer (OS), middle (S) and inner (IS) secondary cell walls, with tubuli (arrows) and the fibrillar connecting system (F). (Fig. 12 bar=0.2 µm; Figs. 13, 14 bar=0.1 µm.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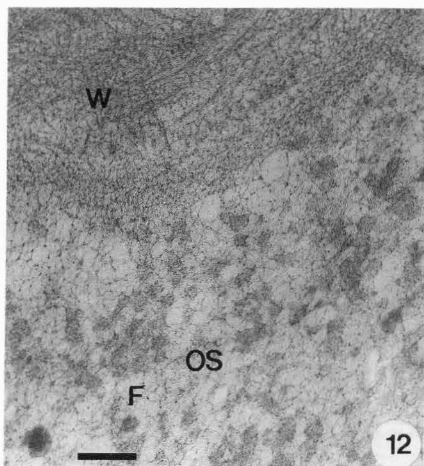
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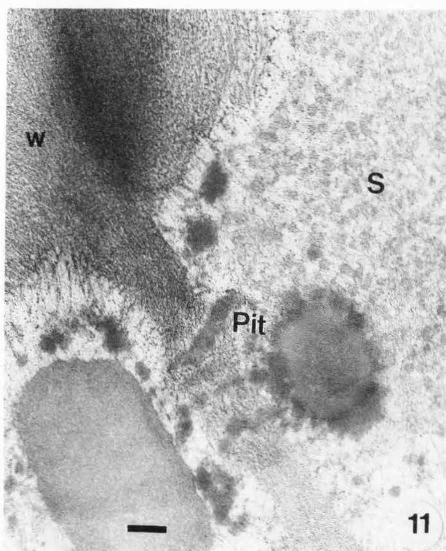
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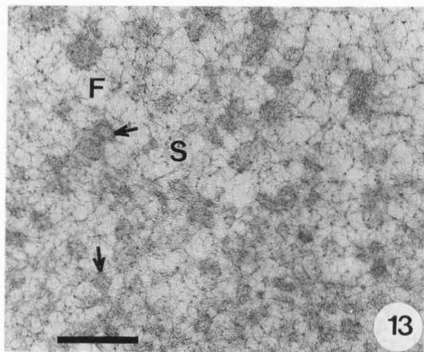
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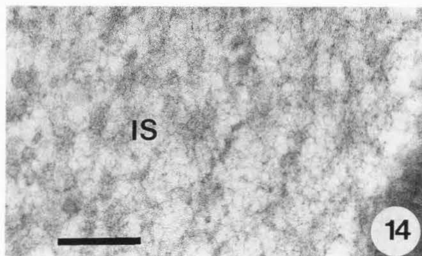
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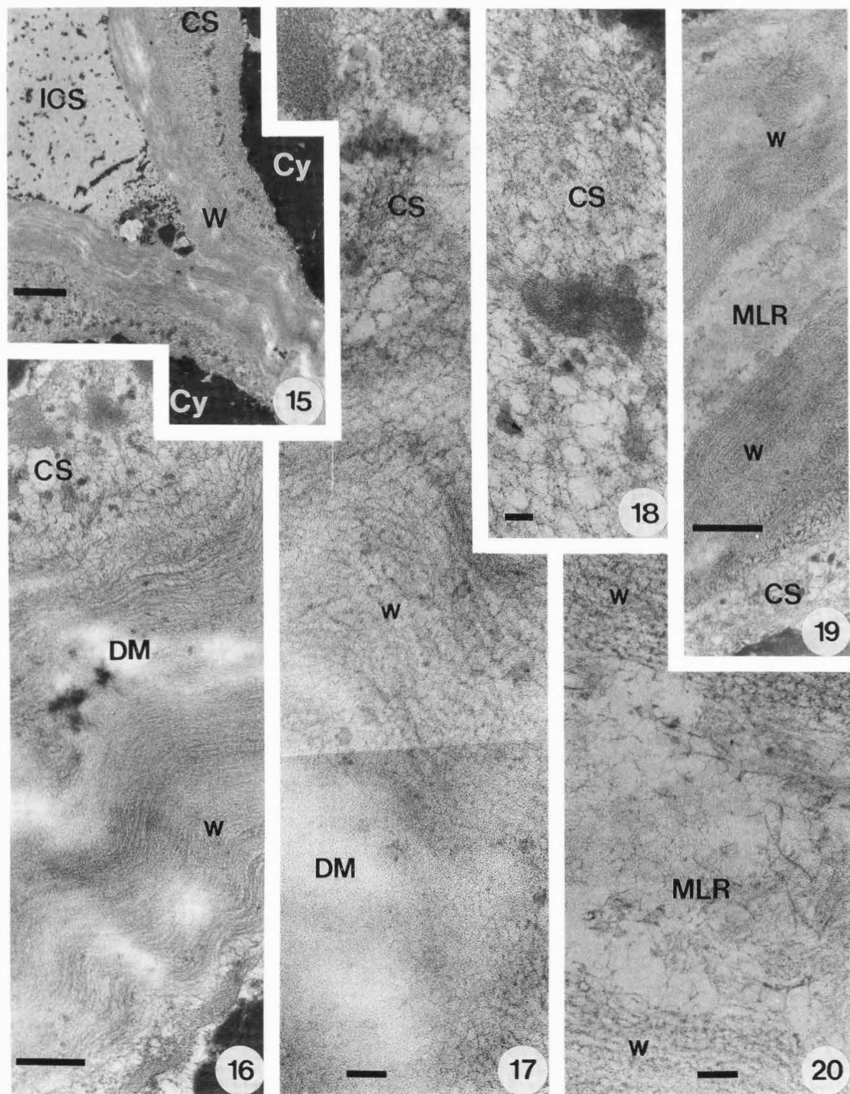
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Figures 15-20. Ultrathin section of cotyledon of a cooked normal bean. Fig. 15 - The primary cell wall (W) seen with weakly stained locations. The cooked secondary cell wall (CS) seen beside dark cytoplasm (Cy); bar=2 µm. Figs. 16-18. High magnification of the primary (W) and secondary (CS) cell walls. The weakly stained locations (DM) indicate partially dissolved primary cell wall (Fig. 16 bar=0.5 µm; Fig. 17 bar=0.1 µm; Fig. 18 bar=0.1 µm). Figs. 19, 20 - Primary (W) and secondary (CS) cell wall where residues (MLR) of the middle lamella are seen between adjacent cells (Fig. 19 bar=1 µm; Fig. 20 bar=0.1 µm).

Discussion with reviewers

M.C. Bourne: Do you believe that the skin (pericarp) of the bean seeds affects the hard-to-cook behavior?
Authors: Cotyledons cooked without the pericarp exhibit the HTC phenomenon as does the whole bean.

M.C. Bourne: What effects would you expect if the beans were stored at 42°C, 80% RH for periods much longer than 21 days?
Authors: We do not expect significant effects on the aspects studied by us. The beans would continue to be hard and the ultrastructural differences between normal and HTC beans would probably remain the same. The procedure to induce hardness was an accelerated one according to Varriano-Marston and Jackson (1981). The hardness induced shows properties of HTC which, according to the literature, were obtained by longer storage at high humidity but at a lower temperature, 30-35°C. However, all normal beans softened during 30 min of cooking.

M.A. Uebersax: What structural changes may have occurred during the initial drying process of the seed which could influence the observed results?
Authors: The initial drying process was not studied by us.

M.A. Uebersax: Do other studies exist to address these changes?
Authors: Other studies dealing with these changes are mentioned in the manuscript. However, these studies have not identified ultrastructural details as revealed by TEM in the present study.

M.A. Uebersax: Can the authors better describe the textural characteristics of these beans to enable better interpretation of the results and provide improved understanding of the extent or condition of hardening present in these beans.
Authors: The present study did not deal with physical measurements of texture. From our point of view it was important to determine whether the beans were soft or hard. The differences between HTC and normal beans were so obvious that the simple assay specified in the experimental section distinguished between them distinctly.

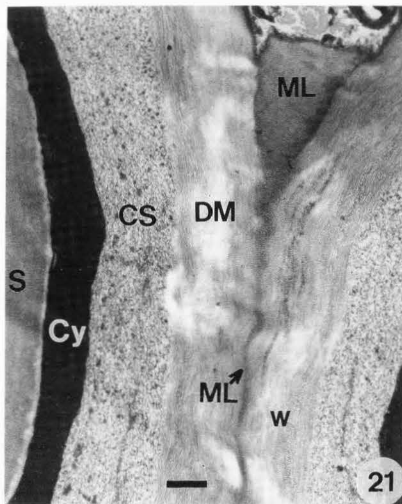
Reviewer III: Did the authors find any evidence for membrane disruption? No consistent trend has been shown in the literature regarding water absorption by hard and soft beans. The difference in water uptake between soft and hard beans however, was even more pronounced following 30 min cooking even though the cooking process disrupted plasma membranes to an even greater extent than storage at adverse conditions. This is supported by the increasing losses of soluble solids to the cooking water reported by the authors and would indicate that a factor other than membrane semipermeability may play an important role in the differences in water uptake between soft and hard beans. This factor may be related to the increased rigidity of cell walls and restricted swelling of hard-to-cook beans during soaking and cooking compared to nondefective samples.
Authors: Leakage of intracellular low molecular weight constituents from HTC beans into the soak water is an indication for damage to the plasma membrane. During cooking, the greater extent of leakage from normal beans compared to the HTC ones, indicates destruction of the plasmalemma of normal beans (the low molecular weight constituents having already leaked out from the HTC beans during the soaking before cooking). The inconsistent trend in the literature with respect to the extent of water imbibition of HTC beans is probably also due to the different ways it is reported. Not always are all the data necessary to correlate among various presentations reported unambiguously. Reduced water uptake by cooked HTC beans may also be the result of changes affecting the hydration capacity of the insoluble matrix of the bean tissue.

Reviewer III: What does the difference in staining ability mean?
Authors: A mixture of toluidine blue and basic fuchsin is known to be a multistain adsorbed by various cell components. The staining differences between the treatments indicate changes in the adsorption properties of some cell constituents, as can be seen in the light micrographs. For example, starch stained mainly after cooking, and this probably indicates adsorption of dye as a result of partial gelatinization and swelling. The secondary cell wall was stained intensively in HTC beans, and its ultrastructure was also distinctly different from that of normal beans. This indicates chemical and structural changes of these cellular components.

Reviewer III: What is the role of changes in secondary cell walls on the development and manifestation of the hard-to-cook defect?
Authors: We don't know. This has to be studied, as well as other aspects which can be elucidated by anatomical and ultrastructural work.

Reviewer III: It is difficult to agree with the identification of the middle lamella being as thin as claimed. The microscopic procedures used are well known to impart artifacts which could explain some of these results.
Authors: From the ultrastructural photomicrographs it can be seen that distinct differences exist in the middle lamellar zone after (rather than before) cooking between HTC and normal (control) beans. Furthermore, variable thicknesses have been observed along the middle lamella in the contact zones of adjacent cells.

Figures 21-26. Ultrathin sections of the cotyledon of a cooked hard-to-cook bean. Fig. 21 - The primary (W) and secondary (CS) cell walls are seen beside dark cytoplasm (Cy) and starch (S), where the middle lamella (ML) is seen undegraded in the adhesion zone between adjacent cells. Weakly stained locations (DM) indicate a partially dissolved cell wall matrix; bar=1 µm. Figs. 22, 23 - high magnification of secondary cell wall beside the primary cell wall (W) and the cytoplasm (Cy); bar=0.1 µm. Figs. 24-26 - High magnification of the middle lamella (ML) in several thicknesses between primary walls (W) of adjacent cells. While the middle lamella is seen undamaged, weakly stained locations appear in the primary wall matrix (Fig. 24 bar=0.1 µm; Fig. 25 bar=0.5 µm; Fig. 26 bar=0.5 µm).


ML
CS
DM
S
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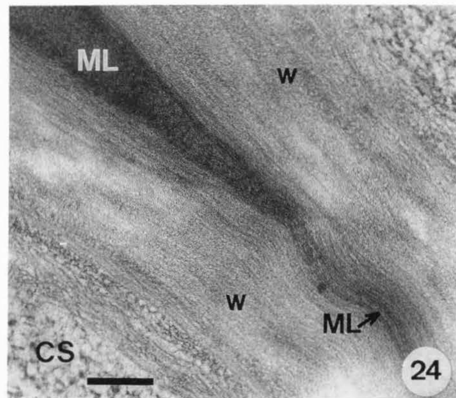
W
21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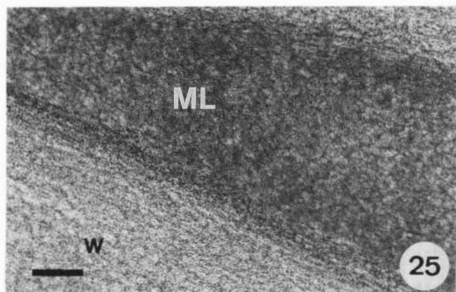
W
W
ML
CS
24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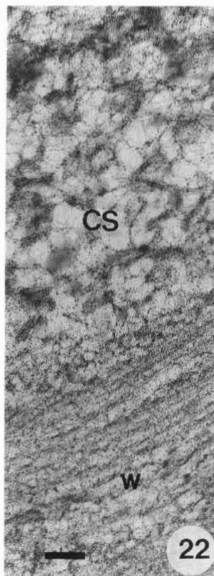
CS
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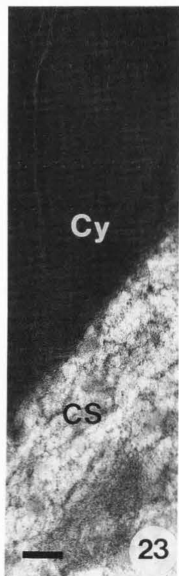
22


Cy
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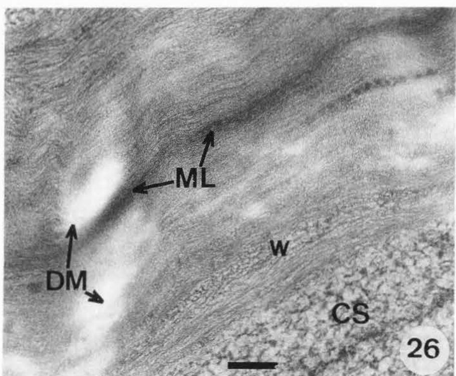
ML
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W
CS
26