Food Structure

Volume 4 | Number 1 Article 19

1985

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L. Sealy

S. Renaudin

D. J. Gallant

B. Bouchet

J. M. Brillouet

FOOD MICROSTRUCTURE, Vol. 4 (1985), pp. 173-181
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 U.S.A.

0730-5419/85$1.00+.05

ULTRASTRUCTURAL STUDY OF YAM TUBER AS RELATED TO POSTHARVEST HARDNESS

L. Sealy[1,2], S. Renaudin[2], D.J. Gallant[1], B. Bouchet[1], J.M. Brillouet[1]

[1] INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE
Centre de Recherches Agro-alimentaires
rue de la Géraudière - 44072 Nantes Cedex, France

[2] INSTITUT DES SCIENCES DE LA NATURE
Laboratoire de Cytopathologie Végétale
2, rue de la Houssinière - 44072 Nantes Cedex, France.

Abstract

Usually, parenchyma cell walls of monocotyledons do not develop secondary walls; however, a few days after harvesting, the yam tuber of Dioscorea dumetorum starts to harden. Two or three weeks later, hardness is so pronounced that the tubers cannot be eaten, even after a long cooking time.

Cytochemical studies using autofluorescence or some fluorescent dyes, such as phloroglucinol hydrochloride showed that the thin, and flexible cell walls of parenchyma tubers very quickly became fully lignified after harvesting. Ultrastructural studies of the hardened cell walls showed very thick secondary walls and very deep pit apertures. These secondary walls reacted strongly with lignin reactants such as potassium permanganate. The use of a radioactive (^{14}C) cellulose precursor, uridine-5'-diphosphate-glucose, confirmed the formation of such secondary walls. The lignification started from the corners of the cells around intercellular spaces and proceeded along the walls.

Initial paper received February 13, 1985.
Final manuscript received May 23, 1985.
Direct inquiries to D.J. Gallant.
Telephone number: (40) 76.23.64.

Key Words : Yam tuber parenchyma, postharvest, hardening, cell walls, scanning electron microscopy, transmission electron microscopy, autoradiography, cytochemistry.

Introduction

Tubers originating from tropical countries constitute the basic foods of most people in Africa, South America, India and Asia. Cassavas (Manihot utilissima Pohl), sweet potatoes (Ipomea batatas L.), taros or arums (Colocasia esculenta L.) and yams (Dioscorea sp.), respectively, are the principal cultivated crops (Gallant et al., 1982). Amongst the yam species, Dioscorea dumetorum (Kunth), a Cameroon and Nigeria crop, seems very interesting due to its crop yields (40 tons/hectare) as noted by Trèche and Guion (1979). Stakers are not necessary, the size and morphology of the tubers are generally homogeneous, and mechanization can be used; the protein content is relatively high (c.a. 10%/dry mass) and consumer acceptability is high after cooking (Onwueme, 1978). Even though these advantages exist, tubers of Dioscorea dumetorum present the disadvantage that they harden very quickly a few weeks after harvest, and cannot be eaten even after a long cooking time.

Only few studies have been made to explain such hardening. Delpeuch and Trèche (1978) described the presence of cellulose-like thickened areas in the parenchymatic cell walls. The same authors, and Brillouet et al. (1981) determined the biochemical changes that occurred during hardening. However, these studies, carried out on soft and hardened samples, did not show the right course of development of the phenomena. In the present study, microscopy, cytochemistry and autoradiography techniques were used to follow the ultrastructural changes which lead to hardening during six weeks postharvest.

Materials and Methods

Dioscorea dumetorum were cultivated at the experimental Station of Bambui (Cameroon West) in 1979-80 and 1980-81. After a growth of 8 to 9 months, tubers were harvested, and immediately covered with paraffin wax and then airmailed to Nantes (France). Paraffinized tubers could be stored several weeks at room temperature without any changes concerning size, ultrastructure, cooking behaviour and taste.

Sampling

Six tubers of the same size and weight were chosen for TEM and autoradiography. The hardening time started when paraffin was removed from the tubers. Tubers were stored in the dark (20°C) during 0, 3 and 6 weeks. Each tuber was punched in its middle part and perpendicular to the principal axis. Cylinders (6 mm diameter) obtained in this way were cut into two parts, each of them being equally divided into three equal parts: A, peripheral; B, medium and C, central parts of each tuber.

Microscopy

Light microscopy. Demonstration of the lignin has been done on hand sectioned samples: a) using the phloroglucinol-HCl stain, lignin components appearing red; b) or directly under UV light, lignin components as well as fluorescent phenolic compounds giving intense blue-white emission.

SEM. Samples from parts A, B and C were frozen in isopentane cooled to -160°C with liquid nitrogen, then fractured along the axis of the cylinders.

In order to see cell wall pit apertures, samples of outer cell walls were usually cleaned (24h, 40°C) with a solution of 20 µg/ml of alpha-amylase from Bacillus subtilis to hydrolyse the starch granules. Then, the samples were dehydrated in a graded acetone series and critical-point dried. Ten to 20 nm gold were deposited on dried samples using the JEOL ion sputter JFC 1100. Samples were examined in a JEOL 50A at 20 keV.

TEM. Samples (1 mm^3) were fixed in 3% glutaraldehyde (0.1M phosphate buffer, pH 7.2) for 2h at room temperature and postfixed for 1h in 1% OsO_4 (same buffer). Subsequently, they were dehydrated in a graded ethanol series and embedded in Epon 812. Silver sections were stained for 30 min in 2.8% uranyl acetate (50% methanol) at 48°C and for 5 min in lead citrate (pH 13). Sections were observed in a JEOL 100S at 80 keV.

Demonstration of the polysaccharides (TEM by the method of Thiery, 1967): Thin sections were oxidized on gold grids for 30 min in 1% periodic acid, treated for 48h in a saturated thiosemicarbazide solution, treated for 30 min in 1% silver proteinate and then rinsed several times in distilled water. This is the PATAg method.

Demonstration of the pectic substances (TEM by the method of Luft, 1971): During sample fixations 0.6% ruthenium red was added to the glutaraldehyde and 1% to the osmium tetroxide.

Demonstration of the lignin (TEM by the method of Hepler et al., 1970): Samples were fixed 90 min in 2% $KMnO_4$, then immediately dehydrated in a graded ethanol series and embedded on Epon 812.

Autoradiography (light microscopy): Small samples (8 mm^3) were immersed in a solution of the ammonium salt of uridine-5'-diphosphate-glucose (UDPG). UDPG radioactivity was 25 µCi/ml. After 6h incubation, the samples were fixed for 2h in 3% glutaraldehyde (0.1M phosphate buffer, pH 7.2), washed for 2h with non radioactive salted UDPG and for 12h in running water. The samples were embedded in Epon 812. Sections (2 µm) were deposited on glass slides and covered with the Ilford G5 emulsion. After 4 days, the emulsion was developed and the sections were stained with a 2% solution of methylene blue.

Control samples were treated with a radioactive UDPG solution to which $HgCl_2$ was added to inhibit the metabolic activity of the cells.

Results

SEM study of cell walls during hardening

When the paraffin was removed from a tuber, the parenchyma cell walls were very thin and presented a fold-like structure (Fig. 1a). After 3 weeks (Fig. 1b), cell walls in part A were already thin and flexible, but in parts B and C many pit apertures were seen, many more being visible near the corner of the cells. These pit apertures were regular (7 to 8 µm diameter). After 6 weeks (Fig. 1c), all the cell walls in parts A, B and C were pitted with apertures although they were more numerous in the corners around the intercellular spaces.

TEM study of cell walls during hardening

Immediately after paraffin removal, at low magnification (Fig. 2a) the cells of medullary region (part C) showed very thin and regular cell walls, a thin layer of cytoplasm containing several compound starch granules in plastids, and a large central vacuole, sometimes with a big hexagonal crystal. At higher magnification (Fig. 2b), it could be easily seen that the cell wall (800 nm thickness) consisted only of the middle lamella and the primary wall. The cytoplasm appeared dense and contained numerous ribosomes, mitochondria, rough endoplasmic reticulum, some dictyosomes (showing 3 to 4 cisterna but only few Golgi vesicles), and compound starch granules with small polyhedric faced units.

After 3 weeks, the cells showed important changes: a thickening of the cell walls that did not stain very well with uranyl acetate and lead citrate. Close to the secondary walls the dictyosomes secreted numerous Golgi vesicles, some of which (10 nm in diameter) were electron dense while others were larger and lighter in appearance. Between the cell wall and cytoplasm, the invaginations of plasmalemma formed pockets and the plasmalemmasomes (Fig. 3a, b) contained numerous spherical vesicles and elongated tubules. Many amyloplasts had been broken and the polyhedric units of the compound starch granules were dispersed into the cells (Fig. 4).

After 6 weeks, the cell walls had become much thicker and showed deep pit apertures traversed through by thin canals of plasmodesmata (Fig. 5a). In addition, the plasmalemma showed some invaginations of paramural bodies (Esau, 1977). In some cases, near to these formations, an irregular network of fibrils could be seen (Fig. 5b), presumably amorphous paraplasmic material associated to the cell walls. Most of the starch granules had burst and were dispersed into the cells.

Cytochemistry

Polysaccharides (PATAg): After 6 weeks only the middle lamella and primary walls were electron dense; the secondary walls were not stained

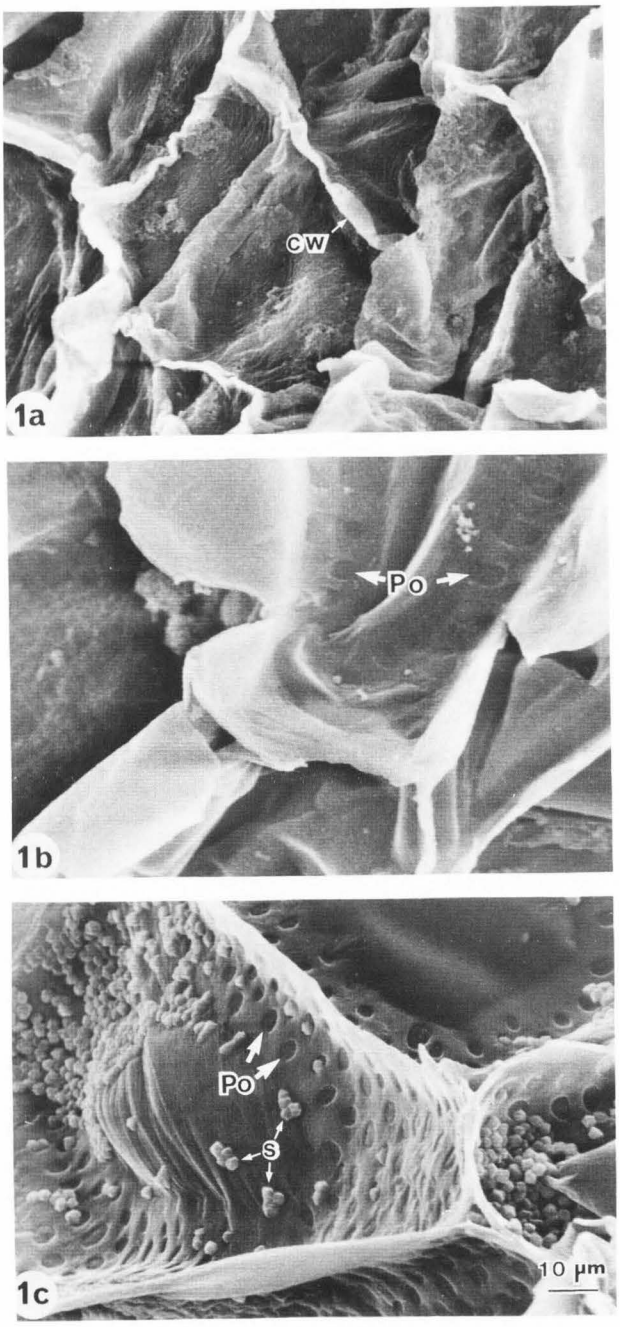


Fig. 1. SEM of the yam tuber parenchyma cell walls during hardening (part C). a) just after harvesting; b) 3 weeks and c) 6 weeks after harvesting. Cell walls (cw) are thin and flexible in figure 1a. Then they are more rigid. Pit apertures (Po) appear (figure 1b) and become very deep (figure 1c). Note the small starch granule units (s) on sample (Fig. 1c) incompletely cleaned. All figures at same magnification.

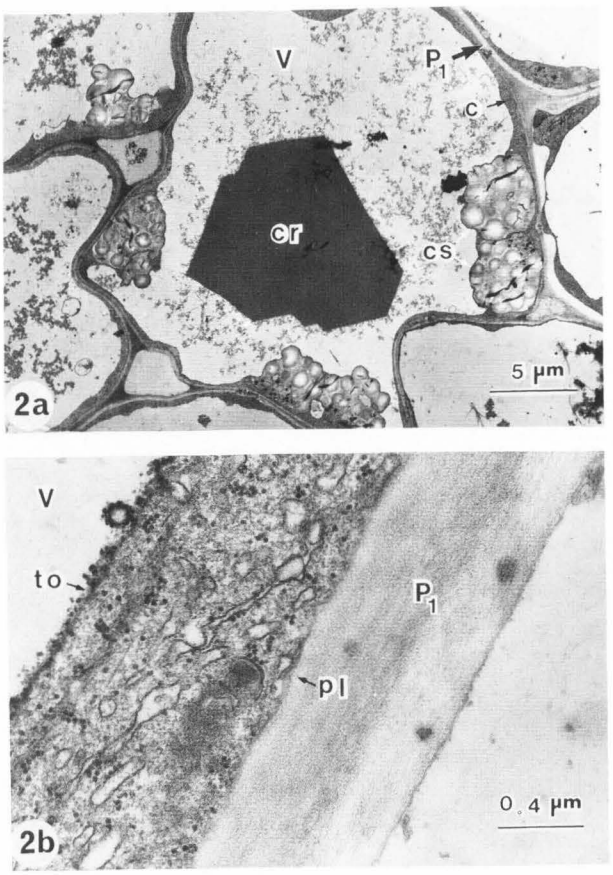


Fig. 2. TEM of the yam tuber parenchyma cells (part C) before hardening (uranyl acetate-lead citrate post-staining). a) just after harvesting, the parenchyma cells are surrounded by thin primary cell walls (Pl). A very large vacuole (V) contains a big crystal (cr) and compound starch granules (cs) are synthesized in a thin peripheral cytoplasma (c); b) at high magnification primary cell wall (Pl) shows stratifications in the carbohydrate deposits. Between the tonoplast (to) of vacuole (V) and the plasmalemma (pl) numerous ribosomes and endoplasmic reticulum are seen in cytoplasm.

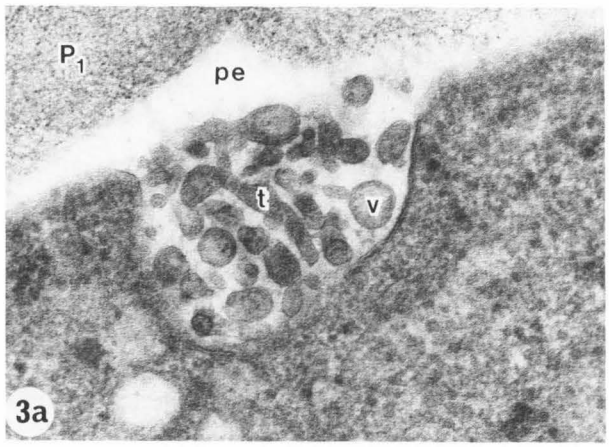
(Fig. 6a). Sometimes fibrillar formations were seen loosely bound to the plasmalemmasome vesicles and to the primary walls. These formations could correspond to neosynthesized cellulose or hemicellulose fibrils which constitute the network of secondary walls in growth (Fig. 7). Starch granules were stained on the periphery of each subunit but staining was very weak or absent on the center of these subunits.

Pectins (ruthenium red): The middle lamella was stained more than the other components of the cell walls (Fig. 8). Ruthenium red stain revealed a dense and fibrous network in the middle lamella. This network was already thicker than usually observed for the middle lamella. It could be concluded that the inner part of the primary wall also is rich in pectic fibrils.

Lignin ($KMnO_4$): The primary wall did not stain with $KMnO_4$ (Fig. 6b), as contrasted to the middle lamella and the secondary walls which were electron dense.

Autoradiography

Compared with the control sample in which



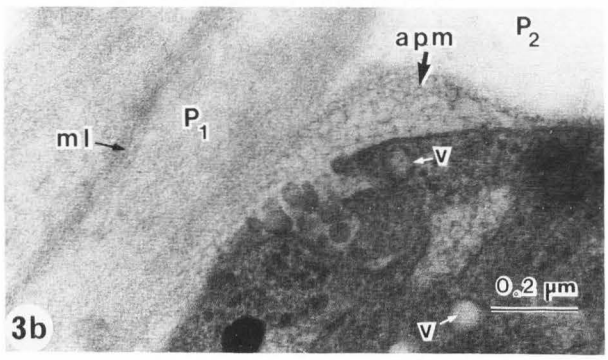


Fig. 3. TEM of yam tuber parenchyma cell walls during hardening (uranyl acetate-lead citrate post-staining). a and b) plasmalemmasomes. These formations are pocket-like plasmalemma invaginations containing spherical vesicles (v) and elongated tubules (t). a) plasmalemmasome opens towards the primary cell walls (P1) and vesicles (v) overflow their contents inside periplasm (pe); b) between such plasmalemmasome already described as a secretory pit (Roland, 1968) and the primary cell wall, amorphous paraplasmic material (apm) is incorporated into the secondary wall (P2). Note the well defined middle lamella (ml). Figures 3a and 3b at same magnification.

Fig. 5. TEM of the yam tuber parenchyma cell wall polysaccharides during hardening (uranyl acetate-lead citrate post-staining). a) cell wall is constituted of middle lamella (ml), primary (P1) and secondary (P2) cell walls. From place to place where transversal canalicles (the plasmodesmata, pd) go through the wall, secondary walls do not deposit, giving pit apertures (Po); b) dictyosomes (D) emit small, dense vesicles (arrows) and larger, clear vesicles (v) in cytoplasma near secondary wall (P2). Note the polyhedric unit (s) of the starch granule (Fig. 5a).

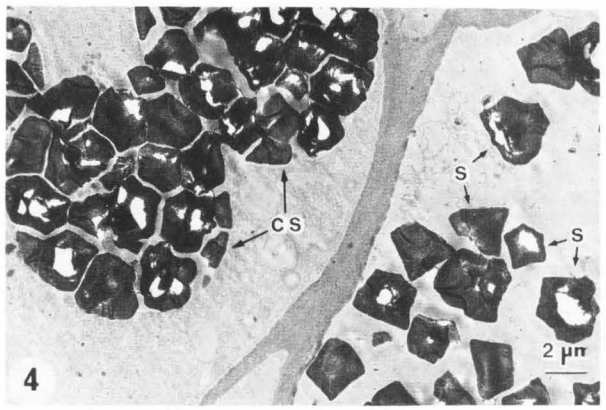
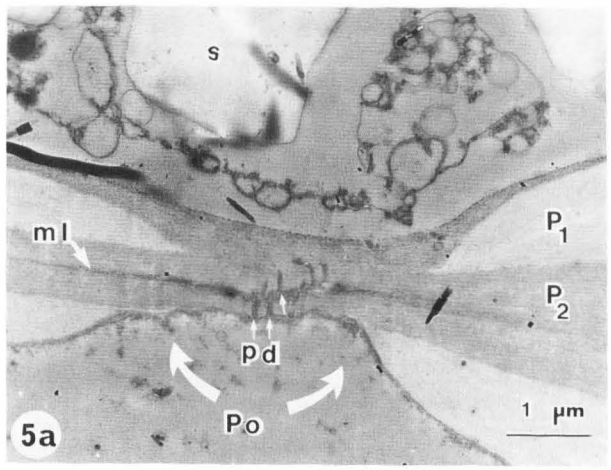
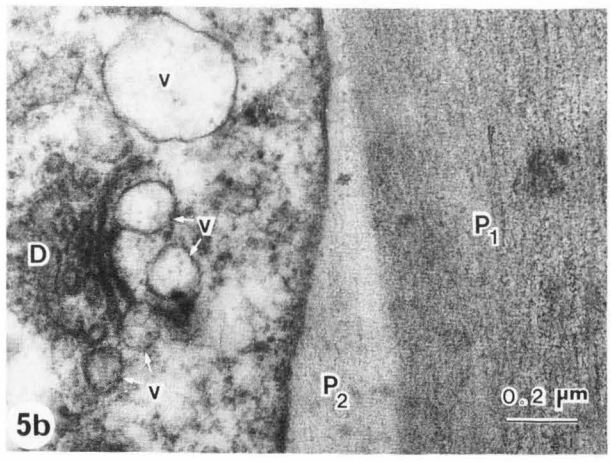


Fig. 4. TEM of the yam tuber parenchyma cells during hardening (PATAg post-staining). In some cells, compound starch granules (cs) are unmodified although in neighbouring cells compound starch granules are disintegrated, showing their polyhedric units (s) fully dispersed.





metabolic activity was inhibited (Fig. 9a), just at the beginning of hardening (3 weeks, part B) radioactive UDPG reacted in the corner of the cells around intercellular spaces (Fig. 9b).

After 3 weeks, all the cell walls had reacted, showing that postharvest hardness and cell wall thickening started around the intercellular spaces and then proceeded along the walls.

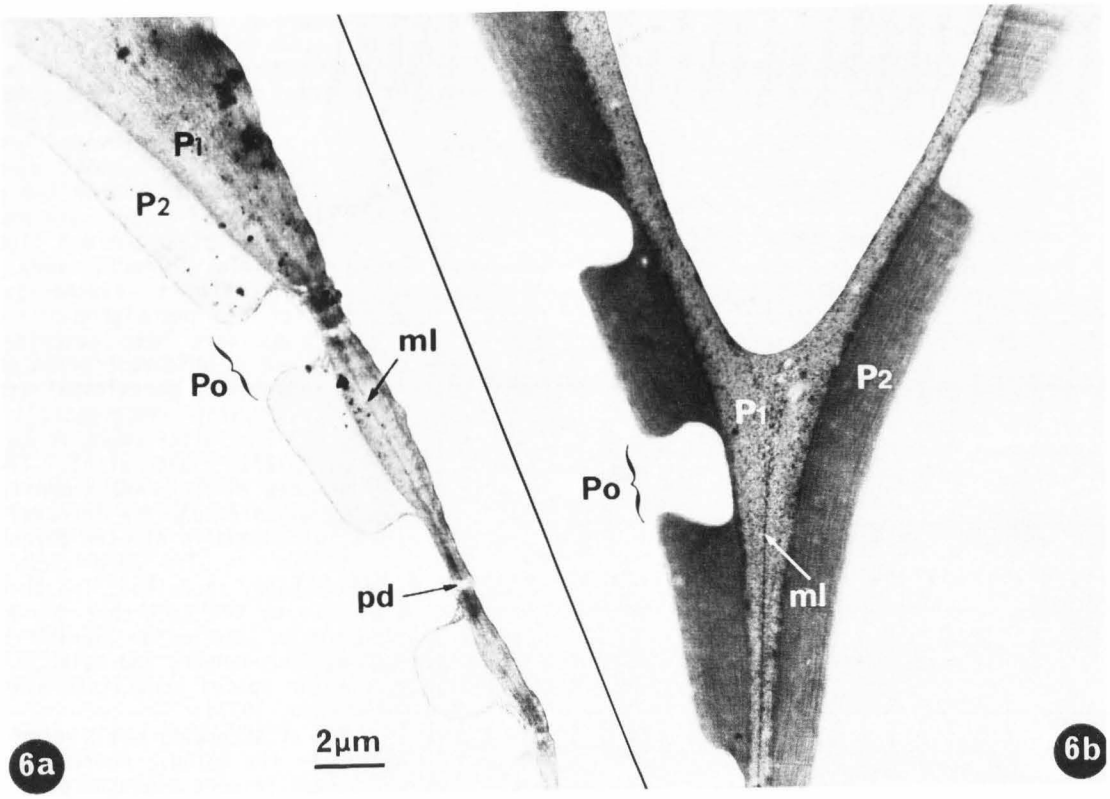


Fig. 6. TEM of the lignified yam tuber parenchyma cell walls. a) after the PATAg post-staining, primary walls (P1) and middle lamella (ml) are electron dense, whereas secondary walls (P2) remain clear. Note very deep pit apertures (Po) with plasmodesmata (pd); b) after $KMnO_4$ treatment, secondary walls (P2) and middle lamella (ml) are more electron dense than primary walls (P1). Figures 6a and 6b at same magnification.

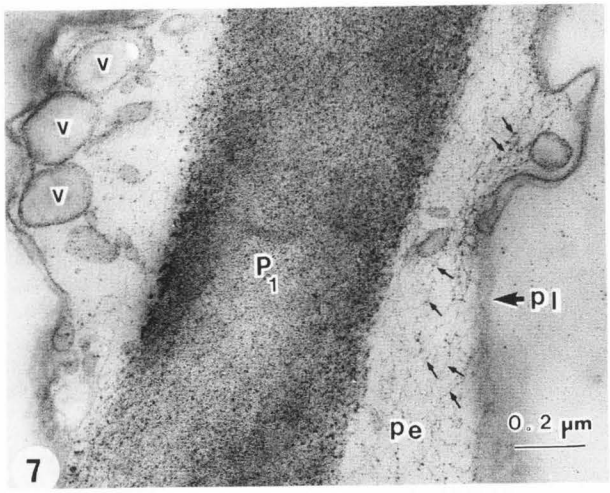


Fig. 7. TEM of the yam tuber parenchyma cell walls polysaccharides (PATAg post-staining). Neosynthesized cellulosic and hemicellulosic fibrils (arrows) constitute a network of the secondary walls in growth between primary wall (P1) and plasmalemma (pl) and vesicles (v) into the periplasm (pe).

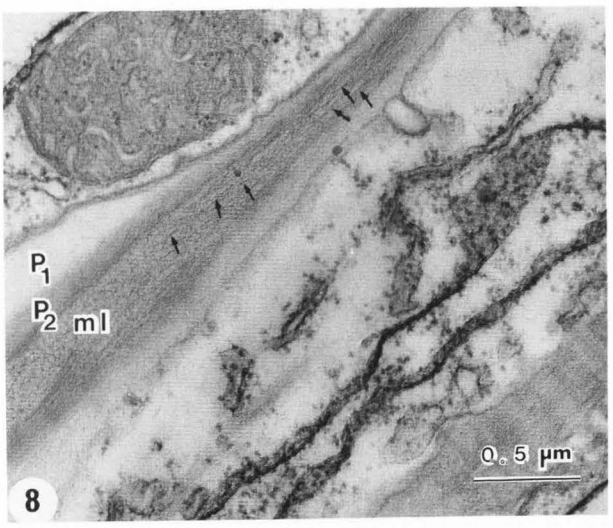


Fig. 8. TEM of the yam tuber parenchyma cell wall pectins (ruthenium red stain). Middle lamella (ml) shows fibrillar, disordered structure (arrows), nevertheless more ordered near the primary wall (P1). Secondary walls (P2) do not show such structure.

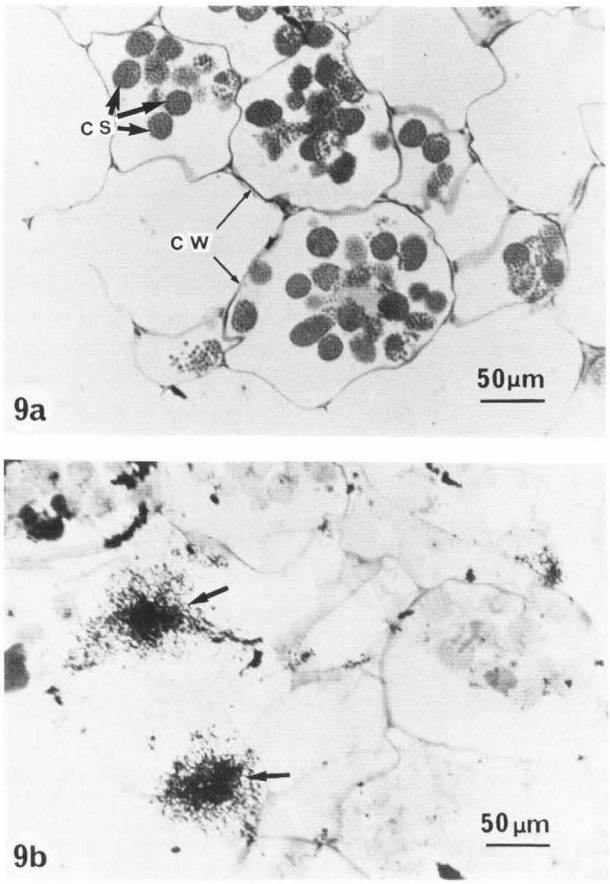


Fig. 9. Autoradiography of the yam tuber parenchyma cell walls (cw) during harvesting. a) control sample with metabolic activity stopped ($HgCl_2$). No activity appears in the cell walls. Note compound starch granules (cs) inside the cells; b) at the beginning, activity (arrows) appears in the corner of the cells around intercellular spaces. Then, activity goes along the walls.

Discussion

The results showed that the yam tuber postharvesting hardness is related to pronounced cell changes. Cell wall thickening was described by Delpeuch and Trêche (1978) in postharvested yam tubers as a thickening which contained "nodules" of undetermined nature and chemical composition. In fact, our SEM and TEM studies have shown that directly after harvesting, the cell walls were very thin, and consisted essentially of primary walls. After some weeks of storage, the cell walls thickened, except at the level of primary pit-fields and plasmodesmata in walls. The growth of secondary walls around the primary pit-fields led to the formation of deep pit apertures. It is likely that the "nodules" described by Delpeuch and Trêche (1978) correspond to the pit apertures.

The chemical composition of the thickening was said to be of cellulosic nature by Trêche and Delpeuch (1979). All the techniques we used ($KMnO_4$ staining by TEM, phloroglucinol-HCl staining observed by light microscopy, and autofluorescence by UV light) showed in contrast that these secondary formations contained lignified components. This lignification appears abnormal because in monocotyledon plants, only the cells in the xylem and sclerenchyma tissues are normally lignified, these cells clear out and lignification becomes irreversible. In fact, every plant cell possesses the potential to synthesize and polymerize monomers of the phenylpropane series (lignins). Some cases have been described in the literature, either in epidermic or in parenchyma cells after injuries or parasitical attacks (Vance and Sherwood, 1976; Rossignol, 1979; Perez-Rodrigues, 1981) or after exposure to pesticides (Carrasco, 1977). Saleh et al. (1967), Wardrop (1976) and Saka et al. (1980) demonstrated that lignification began around the intercellular spaces and was simultaneous in all the parenchyma cell walls. The same results were shown here using the autoradiography technique. The phenomenon may be explained by the fact that the oxidative polymerisation of the major lignin components needs oxygen (Freudenberg and Neish, 1968) and the intercellular spaces constitute a system of air channels (Esau, 1977).

It was shown that secondary walls were stained very weakly with the PATAg, whereas primary walls and middle lamella were electron dense. The absence of contrast could be explained by a particular structure of the cellulose (Freundlich and Robards, 1974). Nevertheless, we prefer Czaninski's (1979) explanation that explains this phenomenon as the encrustation of secondary walls by lignin attaching to the vicinal glycol groups of the cellulose chains and preventing the PATAg reaction.

The middle lamella reacted strongly with $KMnO_4$ and with the PATAg whereas the secondary walls reacted only with $KMnO_4$. This difference could be due to the fixation of lignin in the middle lamella by the pectin chains carboxyl groups (Czaninski, 1979), thus allowing the PATAg reaction.

During thickening, ultrastructural modifications were observed mainly in the dictyosomes and the plasmalemma. The dictyosomes secreted numerous Golgi vesicles. Indeed, their role appears to be the source of precursors to build up the walls. Despite extensive studies, we never observed any precursor-like elements in the Golgi vesicles near the plasmalemma. The presence of numerous lomasomes taking part in the formation of secondary wall identification is in agreement with the observations of Roland and Pilet (1971) on bramble cells, and Cox and Juniper (1973) on pectic and hemicellulose materials using tritiated myoinositol (a precursor of the pectic material and the hemicelluloses) where they found traces of radioactivity only in the plasmalemmasomes of celery leaf stalk cells.

During thickening the structure of the compound starch granules changed; amyloplasts burst, setting free all the starch units into the parenchyma cells. Possibly starch was hydrolyzed and used to synthesize the walls. This hypothesis is

supported by biochemical analysis (Sealy, 1982) which shows a decrease of starch content during hardening. This hypothesis is also in agreement with Juniper and Robert's results (1966) on zea mays root cap cells. They showed that polysaccharides precursors used by the Golgi vesicles are quantitatively limited in those cells, and that amyloplasts burst and the metabolites were liberated into the cells to be used by the dictyosomes.

Conclusion

Postharvest hardening of yam tuber is related to secondary wall formation. This begins around the intercellular spaces and progresses through the walls. The thickening follows an important activity of the Golgi system, the appearance of very numerous plasmalemmasomes and bursting of compound starch granules.

We cannot precisely state which stimulus starts this phenomenon. Further studies will be carried out on cellular areas and enzymes in order to inhibit the enzymatic activity that may be involved in the lignification of this yam tuber.

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Acknowledgements

The authors thank S. Trèche, ORSTOM, Yaoundé (Cameroon) for the yam tuber samples and gratefully acknowledge Mrs. Martin Chapeau for typing this paper.

Discussion with Reviewers

J.F. Chabot: In figure 2a, the cell on the right has a lignified wall, with pits. How frequently was this seen in control tissues? Although only "parenchyma" cells were discussed, it seems likely that there were other cell types, including some vascular tissue. Are all cell types altered during aging?

Authors: That is true. In the central part of tuber parenchyma (part C) other cell type, vascular-like tissues (vascular bundles) are lignified. Lignification of parenchyma cells generally begins in the corner of the cells around vascular bundle cells.

J.F. Chabot: What is the composition of the crystal seen in figure 2a?

Authors: We had not the opportunity to study the crystal composition using microanalysis in TEM. However, numerous bundles of needle-like crystals in parenchyma cells have been studied using microanalysis in SEM. Calcium rich, it may be possible that they were calcium oxalate crystals.

J.F. Chabot: How much of the aging phenomenon is related to a loss of water?

Authors: Loss of water has been studied on our samples. Before hardening, tuber water content was 82%. After 6 weeks storage, water content decreased to 75%. According to Trèche and Guion (1979) loss of water is about 60% after 20 weeks storage.

J.F. Chabot: Most of the cells in the aged tissues with lignified walls also have destruction of cytoplasmic contents. Is this the end result of a pathological process? Under natural conditions, do these yams resume growth when properly treated? (How much of the lignification is due to natural biological processes versus pathological changes?). Why is there no cytolasm in figure 6?

Authors: Destruction of cytoplasmic contents was essentially the result of very bad preservation of the cell structure and not the end result of a pathological process. Indeed, as reported by Trèche and Guion (1979), in Africa and ten months after planting (that corresponds to the beginning of the dry season) tubers are harvested and stored for 5 months (storage temperature varying from 18 to 31°C and humidity varying from 100 to 62% during night and day, respectively) tubers being under dormancy. During this period, about 40 to 50% of the yam tubers are generally lost owing to rottenness development. However, loss is about 6 to 10% only in case of Dioscorea dumetorum according to the hardening phenomenon. In Africa, dormancy ends practically after 8 weeks storage. In France, we observed during 3 successive years that germination of paraffined tubers (that were cultivated in Africa) always occurred about 5 months after they were harvested there. So, tubers of the yam species Dioscorea dumetorum resume growth whenever they have been stored.

Figure 6 is a picture mounting excluding cytoplasma in order to show exclusively the cell walls components.

J.F. Chabot: In figure 9 there was very little incorporation of label. Were only a few cells active? Was the cytoplasm normally so poorly preserved and retracted from the wall, or was this seen in the preparations for autoradiography?

Authors: As seen in figure 2a, cytoplasm was thinly layered along the walls and was not clearly visible under light microscopy. Concerning the cells' activity shown in figure 9b, incubation of sample with UDPG was short (6 h) and contact of labelled sample section with nuclear emulsion took only 4 days.

H.G. Fromme and M. Grote: What is known about the specificity of potassium permanganate for lignin considering the fact that potassium permanganate has long been used as a general fixative for plant tissues with a special affinity for the cytoplasmic membranes?

Authors: Potassium permanganate used for general fixation can also stain lignin as well as cytoplasmic membranes. Thus, it cannot be considered as a specific reagent. In our case, observation under light-microscope of samples stained with phloroglucine-HCl showed the presence of lignified walls at the same level as with potassium permanganate stain.

Absence of specificity on fern stele unlignified walls which were stained with potassium permanganate used as a fixative has been shown by M. Liberman-Maxe (Etude ultrastructurale et cytochimique de la différenciation des tissus de la stèle d'une fougère, Polypodium vulgare L.; ultrastructural and cytochemical studies of the fern stele tissular differentiation. Thèse de

Doctorat d'Etat. (1984). Paris VI University) who explained the stain she obtained on the fern stele by the presence of pectic acids.

Liberman-Maxe (1984) gave references of some authors who were in favour of the stain specificity for lignin: a) Hepler PK, Fosket DE, Newcomb EH. (1970) as cited in references; b) Kerr AJ, Goring DAI. (1975). The ultrastructural arrangement of the wood cell wall. Cellulose Chem. Technol. 9, 563-573; c) Ruel K, Barnoud F. (1981). Supramolecular aspects of wood constituents as seen by electron microscopic investigations. Int. Symposium on wood and pulping chemistry, Stockholm, 1, 11-15; d) Wardrop AB. (1965). Cellular differentiation in xylem. In: "Cellular ultrastructure of woody plants". W.A. Côté, Jr (ed), Syracuse University Press, 61-97; e) Wardrop AB. (1981). Anatomical aspects of lignin formation in plants. Int. Symposium on wood and pulping chemistry, Stockholm 1, 44-51.

References of authors who were not in favour of the stain specificity for lignin are: a) Czaninski Y. (1979). Cytochimie ultrastructurale des parois du xylème secondaire (ultrastructural cytochemistry of secondary xylem walls). Biol. Cellulaire 35, 97-102; b) Hoffman P, Parameswaran N. (1976). On the ultrastructural localization of hemicelluloses within delignified tracheids of spruce. Holzforschung 30, 62-70; c) O'Brien TP. (1974). Primary vascular tissues. In: "Dynamic aspects of plant ultrastructure". A.W. Robarts (ed). McGraw Hill Book Company, Maidenhead-Berkshire (UK), 414-440.

H.G. Fromme and M. Grote: Why were samples for SEM investigations not fixed before or after cryo-fractioning, in any case, before dehydration?
Authors: Preservation of cytoplasmic structures was not necessary because cell walls were generally cleaned and washed in order to see the pit apertures.

H.G. Fromme and M. Grote: Does the paraffin covering of the tubers affect the metabolism of the cells? (e.g., by interference with the gas exchange process)? Provided that this is the fact, might this explain the bad morphological preservation of the cells visible e.g., in Fig. 5a, 5b and 8?
Authors: Paraffin covering of the tubers does affect the metabolism of the cells (e.g., respiratory system and gas exchanges) but it cannot explain their bad preservation. After 6 weeks hardening, material was extremely hard and it was very difficult to obtain a good preservation of the cell structures according to very low fixative penetration.

H.G. Fromme and M. Grote: Do the authors anticipate practical hints from such investigations with regard to economic utilization of this food?
Authors: This study started on agronomical observations and showed that hardening was closely bound to parenchyma cell walls lignification. No practical hints were anticipated by authors regarding the economic utilization of this yam tuber. Further studies must be fundamental only.