Ultrastructure of Cassava Root by TEM & SEM

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Page Banner: Cassava Ultrastructure

Keywords: Cytology, resin embedding, TEM, SEM, LTSEM, *Manihot esculenta* Crantz.

SUMMARY

Cytological investigations of cassava storage roots have been impeded by the difficulties of embedding, in resin, tissues that contain high amounts of starch and secondary metabolites. However, histological studies of this highly important crop are necessary in order to understand its biology and diseases. We present modified preparation techniques that provide good preservation of tissue to facilitate the study of important cassava cell structures with electron microscopy.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae, is a perennial bush whose centre of origin is the Amazon basin [1]. Cassava cultivation has now spread throughout the humid tropics from Latin America to Africa and Asia, where it is grown principally for its large starchy storage roots. The roots provide the staple food for over 500 million, and in 1991 world production was 162 million tonnes [2]. Cassava has the ability to grow on impoverished and marginal soils with the minimum of technological

input. As a result it is often the food of the poor and can play a major role as a famine reserve crop. However, cassava is valued as a starchy component in the diet by all social strata. In addition, cassava is increasingly being grown and processed as animal feed for export, or processed industrially into a range of products including starch [3]. Because of the importance of the crop much effort is directed towards improving cassava with respect to disease resistance, post-harvest traits and yield. This research requires cytological and histological work.

Transmission electron micrographs of Cassava root tissue structures are difficult to find among the current literature, as are images of storage damaged tissue. This dearth of ultrastructural information has been due in part to the difficulties in preparing Cassava root material for transmission electron microscopy (TEM). High levels of starch, lignin, suberin, tannin, lipid and phenolic materials in this type of tissue present problems in achieving adequate fixation and infiltration of resin into the cells necessary for successful ultramicrotomy and TEM.

The root of the Cassava plant is a thickened starch-filled tuber. In common with other plant structures the cell walls consist of polysaccharides formed by the condensation of monosaccharide units into chains of glucans, xylans and arabinans. The xylem tissue and schlerenchymous fibres are also composed of lignin, a hard variable material of cross-linked phenylpropane units, which adds stiffness to the cell walls. The outer layers of the Cassava tuber constitute the periderm: a tissue that replaces the epidermis in most stems and roots having secondary growth. The periderm is made up of an outer layer of cork tissue and an inner layer of living parenchyma cells. The outer cork layer contains suberin, a waxy substance characteristic of cork tissues and present in the thickened cell walls, and tannins, complex aromatic compounds such as glucosides, which provide protection for the plant and are also linked to pigment formation. At maturity this tissue is a non-living cambium layer. A secondary cortex of parenchyma cells termed the phelloderm is filled with suberin formed by the inner side of the periderm cambian.

Experimental samples of Cassava tuber, exposed to treatments designed to simulate harvesting damage, show an initial discolouration of the vascular tissue caused by pigmented deposits or gels made up of lipids, carbohydrates and phenolic material (4). Tylosis occurrs as a non-specific response to tissue damage in xylem vessels, resulting in the intrusion of parenchyma cells through pits into the secondary xylem. These callose protrusions are composed of a high content of lipid, carbohydrate and condensed tannins with lignin-like properties (4, 5, 6). Cytochemical investigations have been carried out at the light microscope level to further the understanding of changes that occur in Cassava roots following harvesting (4, 5), but little has been undertaken at the ultrastructural level. We have devised a protocol for the successful preparation of Cassava root including fixation with acrolein (acrylaldehyde), extensive vacuum treatments to draw fixative and resin into the cells and vessels, extended resin infiltration times and a prolonged freezing step or the use of low viscosity resin.

MATERIALS AND METHODS

Small pieces of Cassava tuber were immersed in a fixative solution of 2.5% glutaraldehyde and 1.0% acrolein in 0.05M PIPES (piperazine N, N, bis-ethanesulphonic acid) buffer at pH 8.0 with an osmolarity of approx. 450 mOsms. Further dissection of the tissue took place under the fixative and the resultant 1-2mm x 3-4mm pieces were subjected to a vacuum in order to draw the fixative into the cells and vessels. Fixation took place for 18 hours at room temperature with agitation. Rinsing of the samples to remove fixative was performed in 0.1M PIPES buffer pH 8.0 with added 0.1M sucrose to maintain a similar osmolarity to that of the fixative solution. Postfixation was achieved using 1.0% osmium tetroxide in the rinsing buffer for 1hour at room temperature. The tissue was washed in distilled water prior to slow dehydration through a graded acetone series. At this point in the preparation the material was divided into two batches to allow a later comparison of cellular ultrastructure between two different types of resin and infiltration methods:

Method 1. Half the total number of samples were infiltrated with a mixture of Taab premix embedding resin (Taab Laboratories Equipment Ltd) with the following hard formulation - 50 parts Resin, 25 parts DDSA (dodecenylsuccinic anhydride), 25 parts MNA (methyl nadic anhydride) and 3 parts BDMA. Tissue pieces were immersed in a 1:3 resin to acetone mixture overnight followed by an increase in resin to 1:1 for eight hours and a further increase to 3:1 for 16 hours. The tissue was subject to constant agitation at each of the infiltration steps and after 16 hours was placed in a 100% resin mixture under vacuum for 8 hours. At the end of this period the tissue was placed in a deep freeze and slowly frozen, remaining in this state for 1 year (two months is thought to be adequate – see discussion). It was then warmed to room temperature and agitated in a fresh 100% resin mixture overnight. The following day tissue pieces and resin were polymerised in moulds at 60^{o}C for 48 hours.

Method 2. The second batch of tissue samples was immersed in solutions of Spurr's epoxy resin (7) from Taab Laboratories Equipment Ltd. The following formulation was used: 10 parts of ERL 4206 (vinylcyclohexene dioxide), 6 parts of DER 736 (diglycidyl ether of polypropylene glycol), 26 parts of NSA (nonenyl succinic anhydride) and 0.4 parts of S-1 (dimethylaminoethanol). The ratios of resin to acetone were as follows: 1:3 overnight, followed by 1:1 and 3:1 for 4hours each. A 100% resin mixture was added to the samples and vacuum treatment was carried out overnight. The addition of a final resin mixture for 8 hours took place on the following day. These samples were orientated in moulds and the resin cured at 70^{o}C for 8 hours.

The resultant resin blocks were trimmed and faced with a glass knife before ultrathin sections of approx. 100nm were cut using a diamond knife. The sections were stained with 6% aqueous Uranyl Acetate followed by Reynold's lead citrate (8). The method of Daddow et al (9) was used to improve the staining of Spurr resin sections that often exhibit low contrast. Examination of sections was performed with a JEOL JEM1200 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80kv.

Samples prepared for low temperature scanning electron microscopy (LTSEM) were sliced with a razor blade into a suitable size, fixed to a sample holder and frozen in liquid nitrogen slush. They were then transferred to the cold stage of a JEOL JSM6310 scanning electron microscope (JEOL, Tokyo, Japan) and any frost visible on the surface of the sample was sublimed away at –85^{o}C. The sample holder was withdrawn from the SEM into the cryo-preparation chamber of an Oxford Instruments Cryotrans 1500 (Gatan, Oxford, UK) where it was sputter coated with gold at –172^{o}C. The samples were returned to the SEM stage at –160 to –175^{o}C for final viewing.

RESULTS AND DISCUSSION

Inadequate infiltration of liquid resin into Cassava root tissue has impeded ultrastructural investigation of cell structures. Tissue prepared by routine TEM methods has resulted in difficulties at the sectioning step and artefacts in the final image. As a consequence studies on the ultrastructure of cassava root tubers have been limited to easily prepared structures such as extracted starch granules [10, 11]. Here we present three methods of preparing cassava root tubers for analysis by SEM and TEM.

LTSEM is an easy and rapid method for the investigation of general cell and tissue structures and tissue formation (e.g. wound periderm) at low to medium magnifications. Figure 1a is a low magnification cross-section through the frozen-hydrated cassava root showing the periderm (Per); an outer layer of cork tissue (compressed cells) and an inner layer of living parenchyma cells. The cork layer and the secondary cortex of parenchyma cells (phelloderm) contain suberin formed by the inner side of the periderm cambian. This waxy substance contributes to problems arising during TEM preparation. The sclerenchyma (sc), a layer of thick-walled cells, and the cortical parenchyma (Par), filled with starch (arrow), also add to preparation difficulties. LTSEM proved to be an important tool in the study of wound response and storage disorders and may also be applied to quality assessment of starch produced by cassava.

In order to study the ultrastructure of cassava root tissue at higher magnifications, and to investigate changes and processes that occur after damage to the cells, it is necessary to prepare the tissue for TEM. In the early days of TEM Araldite, an epoxy resin based on the diglycidyl ether of bisphenol A mixed with DDSA in equal parts, was the most favoured resin in use due to its stable properties under an electron beam. The major disadvantage of Araldite resin was the high viscosity of the mixture and the resultant difficulties with infiltration of samples. Epon 812 (a shell product, no longer available) the triglycidyl ether of glycerol, and Spurr's resin formulation based on the cycloaliphatic diepoxide - vinylcyclohexene dioxide - were developed as less viscous alternatives to Araldite. Taab Embedding Resin (Taab Laboratories Equipment Ltd, Aldermaston, U.K.) is a resin formulated to counter the disadvantages of both Araldite and Epon 812. This mixture has a relatively low viscosity, compared with the former resins, and the same epoxide equivalent between different batches. Standardisation among batches of resin allows blocks of consistent properties and sectioning characteristics to be produced. Spurr's resin formulation (Taab Laboratories Equipment Ltd.) produces a mixture with an even lower viscosity. However, the resin blocks produced have less consistent sectioning properties and poorer staining qualities than the higher viscosity resins.

Embedding cassava root tissue samples into both Taab embedding resin and Spurr's resin proved effective as each resin type and infiltration method produced differences in the ultrastructure seen in the TEM (see table1.). Figure 1 depicts a range of cassava root wall structures prepared using infiltration method 1. The well-preserved walls of four different cell types achieved with this method are highlighted in the figure. A suberin-containing phelloderm cell (pc) in figure 1b shows the narrow walls (arrows) and an intercellular space (is) typical of this cell type. Fig 1c shows a parenchymatic cell (par) with a fibrous wall structure (w) and an amyloplast (a) containing a distinct lobed starch granule (s) also typical of this tissue. Routine preparative methods have not been successful for imaging cassava starch granules *in planta*. Bordered pits, seen in figure 1d showing wall (w) and pit (arrow) and the compressed walls of the periderm (fig.1e and top of fig.1a) have also resulted in unsuccessful thin sectioning and the occurrence of

artefacts associated with poor penetration of the resin. The infiltration methods described here allow a variety of wall structures to be studied.

A parenchymatic cell (par) adjacent to a xylem vessel, prepared by infiltration method 1, is shown in figure 2. The middle lamella (arrows) of the wall (w) is beginning to bulge into the xylem lumen (xl), note the fine detail of fibrous material being laid down on the cell side of the expanding wall (asterisk). This activity is indicative of an early stage in tylosis where initial intrusion of a parenchyma cell wall through a pit into the lumen of the secondary xylem (stage shown in figure 2) results in the eventual expansion of the whole cell into the vessel. These protrusions into the xylem are composed of a high content of lipid, carbohydrate and condensed tannins that cause problems for penetration of fixatives and resin into the structures. The combined use of glutaraldehyde and acrolein as the primary fixative coupled with deep freeze treatment has overcome these difficulties resulting in excellent ultrastructural detail depicting various stages in this important process (in preparation for publication). However, the lengthy deep freeze treatment was a frustrating disadvantage and the major drawback to method 1. The period of one year was not intentional and previous investigations of bacterial infection in cassava stems (12) indicate that one to two months at –25^{o}C is probably adequate for cassava root.

The parenchyma cell in fig 2 contains numerous organelles (nucleus n, mitochondrion m, endoplasmic reticulum er, and vacuole v) adequately preserved with method 1. However, infiltration method 2 using Spurr's resin formulation generally produced better images of cell organelles particularly membranes (as shown in figure 3). The benefit of this method in preserving membrane structure was due, most likely, to a reduced exposure of the cytoplasmic parts of the tissue to the resin mixture. A mesophyll cell (mc) with vesicle formation (arrow) taking place at the plasma membrane (open arrow) adjacent to the cell wall (w) is shown in figure 3b. A well-preserved dictyosome and mitochondrion in addition to rough endoplasmic reticulum are seen in figure 3c. The intact membrane and inner crystal of a microbody (mb) is depicted in figure 3f. Figure 3d clearly shows the

double outer membrane (arrow) and internal cristae (arrowheads) of a mitochondrion. A phloem cell wall (w) in figure 3a has numerous plasmodesmata (arrows) connecting the cytoplasm of neighbouring phloem cells. These structures were well preserved using method 2 in the thin cell walls between phloem cells only, whereas the alternative method allowed their study in a range of walls. A typical lobed starch granule (s) is seen in figure 3e and may be compared with that in figure 1c prepared with method 1. In the case of amyloplasts containing starch granules, infiltration method 1 proved the preferable method for detailed images of the lobed starch structure.

In conclusion, cell organelles (with the exception of amyloplasts) were best preserved using Spurr's resin formulation and infiltration method 2. Taab embedding resin coupled with the first infiltration method produced excellent images of the structure of cell walls, vessels, parenchyma cells undergoing tylosis and starch. The methods described here will each have useful applications in research on cassava and other starch-filled tissues.

Structure	Method 1	Method 2	Preferred Method
Cell Walls	**++**	**- / +**	1
Nuclei	**+**	**++**	2
Plasma Membranes	**- / +**	**+ / ++**	2
Mitochondria	**- / +**	**++**	2
Plastids	**+**	**++**	2
Amyloplasts	**+ / ++**	**- / +**	1
Dictyosomes	**- / +**	**++**	2
Endoplasmic Reticulum	**+**	**++**	2
Plasmodesmata	**++**	**++**	1
Parenchyma Cells (adjacent to vessel)	**+++**	**- / +**	1

Key: - = Poor: Broken membranes and/or uneven organelle matrix and staining. Compression/infiltration artefacts in walls.

+ = Good: Generally acceptable structural images.

++ = Excellent: Clear double membranes and/or even organelle matrix and staining. Details of wall fibres with few artefacts.

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Acknowledgement

This publication is an output from a research project funded by the U.K. Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of the DFID. Crop Post-Harvest Programme - R6983.

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Figure Legends

Figure 1. Cassava root wall structures. **a.** Low temperature SEM image of a cross-section through frozen-hydrated Cassava root tissue. Par: Starch containing outer (cortical) parenchyma, s: Sclerenchyma, Per: Periderm. Bar = 50µ **b. – d.** Tissue prepared using the standard epoxy resin infiltration method. **b.** Suberin containing cell showing narrow walls and intracellular space. Bar = 2µ **c**. Parenchymatic starch containing cell with an amyloplast and detailed wall structure. Bar = 200nm **d**. Bordered pits connecting vessels. Bar = 2µ **e**. Periderm cell wall layers from the region in the root of compressed cells (see top of fig.1a). Bar = 400nm.

Figure 2. Parenchymatic cell adjacent to a xylem vessel containing numerous organelles. The middle lamella (arrow) is beginning to bulge into the xylem lumen indicating an early stage in tylosis. Note the fine fibrous material being laid down on the cell side of the expanding wall (asterisk). The tissue was prepared with the standard epoxy method, n: Nucleus, m: Mitochondrion, v: Vacuole, er: Endoplasmic reticulum. Bar = 1µ.

Figure 3. Examples of cassava root cell organelles prepared using the low viscosity resin method. **a.** Phloem cell showing numerous plasmodesmata connecting the cytoplasm of neighbouring cells. Bar = 400nm. **b.** Mesophyll cell with vesicle formation taking place at the plasma membrane. Bar = 200nm. **c.** Dictyosome, rough endoplasmic reticulum and a mitochondrion in a mesophyll cell. Bar = 400nm. **d.** Mitochondria and cell wall. Bar = 200nm. **e.** Typical lobed starch granule structure. Bar = 1µ. **f.** A microbody in a mesophyll cell. Bar = 200nm.

Figure 1.

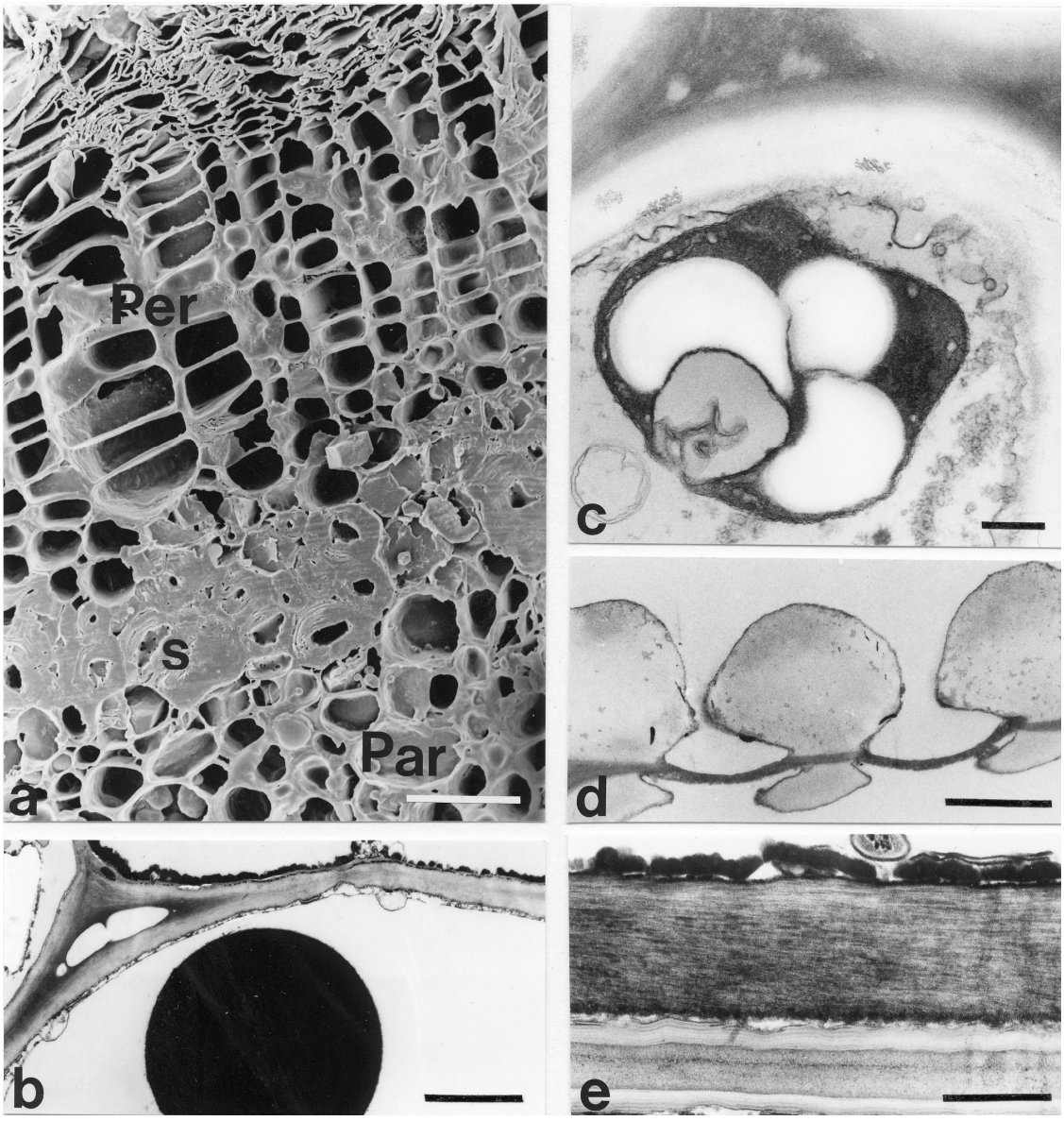

Figure 2.