Ultrastructure of Cassava Root studied by TEM and SEM

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ABSTRACT

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.

KEYWORDS
cytology, resin embedding, TEM, SEM, LTSEM, Manihot esculenta Crantz.

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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 sclerenchymous 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]. Tysis occurs 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 [4-6]. 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

Figure 1:
(a) LTSEM of cross-section of cassava root. (b-e) Root wall structures prepared by infiltration method 1.
and 1% 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% osmium tetroxide in the rinsing buffer for 1 hour 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.

Infiltration Method 1. Half the total number of samples were infiltrated with a mixture of Taab premix embedding resin (Taab Laboratories Equipment Ltd, Aldermaston, Berks U.K.) with the following hard formulation - 50 parts Resin, 25 parts DDDA (dodecylsulphonanc acrylanhydride), 25 parts MNA (methyl nadic anhydride) and 3 parts BMDA (benzyldimethyamine). 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 at -25°C 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°C for 48 hours.

Infiltration Method 2. The second batch of tissue samples was immersed in solutions of Spurr’s epoxy resin [7] (Taab Laboratories Equipment Ltd). The following formulation was used: 10 parts of ERL 4206 (vinylcyclohexene dioxide), 6 parts of DER 736 (diglycidyl ether of propylene glycol), 26 parts of NSA (nonenyl succinic anhydride) and 0.4 parts of S-1 (dimethylaminomethanol). The ratios of resin to acetone were as follows: 1:3 overnight, followed by 1:1 and 3:1 for 4 hours 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°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 Reynolds’s lead citrate [8]. The method of Dad-dow 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. They were then transferred to the cold stage of a JEOL JSM 6310 scanning electron microscope (JEOL, Tokyo, Japan) and any frost visible on the surface of the sample was sublimed away at -85°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°C. The samples were returned to the SEM stage at -160 to -175°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 artifacts 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 TEM and LTSEM. 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 (phellogen) contain suberin formed by the inner side of the periderm cambium. 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 glycid-

![Table 1. The ultrastructural appearance of cell structures related to infiltration method.](image-url)
erol, 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, Aldermaston, UK) 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 Labs) 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 Table 1). 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 phellogen cell (pc) in Fig 1b shows the narrow walls (arrows) and an intercellular space (is) typical of this cell type. Figure 1c shows a parenchymatic cell (par) with a fibrous wall structure (w) and an amyloplast (a) containing starch granules, infiltration 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˚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 Fig 3). The benefit of this method in preserving membrane structure was due, most likely, to a reduced exposure of the cytoplasmatic 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 Fig 3b. A well-preserved dictyosome (d) and associated vesicles (arrows) and a mitochondrion (m) in addition to rough endoplasmic reticulum (er) are seen in Fig 3c. Fig 3d clearly shows the double outer membrane (arrow) and internal cristae (arrow-heads) of a mitochondrion. The intact membrane (arrows) and inner crystal (cr) of a microbody (mb) is depicted in Fig 3f. A phloem cell wall (w) in Fig 3a has numerous plasmodesmata (arrows) connecting the cytoplasm (cy) 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 Fig 3e and may be compared with that in Fig 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, starch and parenchyma cells undergoing tylosis. The methods described here will each have useful applications in research on cassava and other starch-filled tissues.