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OBSERVATIONS ON THE STAINING OF PECTIC SUBSTANCES AS EXTRACTS AND IN THE TRANSFUSION TRACHEIDS OF *WELWITSCHIA*

JUNE E. BEAN and CHRIS H. BORNMAN

(*Department of Botany, University of Natal, Pietermaritzburg*)

ABSTRACT

A series of staining-time sequences was used to stain extracts of pectin and pectic acid with alkaline hydroxlamine-ferric chloride, specific for these substances, in an attempt to find an improved, reproducible and, if possible, a quantitative staining technique at the electron microscope level. The unfortunate heterogeneity and variability in composition of such pectic preparations made this impossible.

However, good differential staining was obtained using the stain *in vivo* on *Welwitschia mirabilis* one-year-old leaf sections. The distribution of the pectic substances within the cell walls of differentiating transfusion tissue, particularly of transfusion tracheids, was studied.

UITTREKSEL

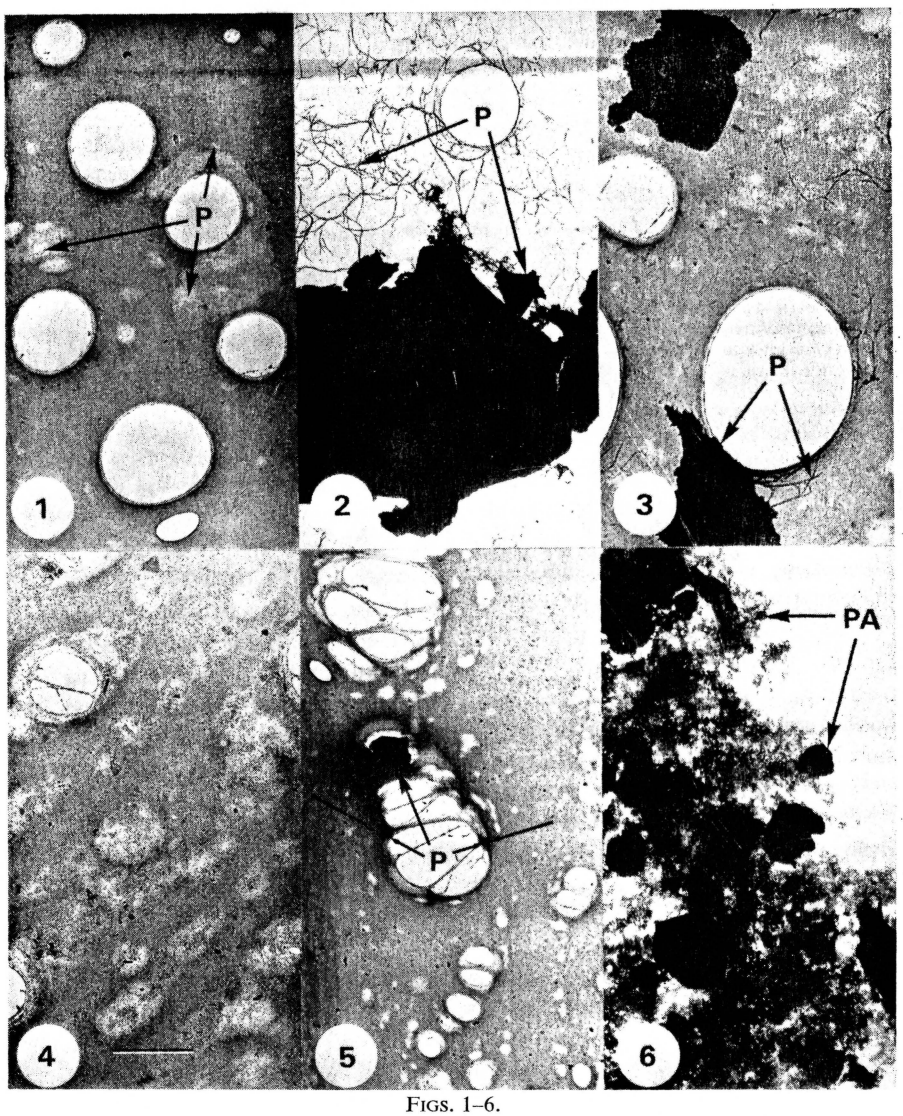
WAARNEMINGS OP DIE KLEUR VAN PEKTIESE STOWWE AS EKSTRAKTE EN OOK IN DIE TRANSFUSIE-TRAGEÏEDE VAN *WELWITSCHIA*

In 'n poging om 'n verbeterde, herhaalbare en moontlik kwantitatiewe kleurtegniek vir pektiese verbindings in plantweefsel op elektron mikroskopiese vlak daar te stel, is 'n aantal kleur- en tydsekwensies met pektien en pektiensuur in samehang met alkaliese hidroksielamien-ferrichloride uitgevoer. Die variasie in en heterogene aard van die samestelling van die pektiese preparate het egter die pogings grotendeels verydel.

Goed-gedifferensieerde kleuring is egter *in vivo* verkry met ultradun sneë van eenjarige blare van *Welwitschia mirabilis*. Die verspreiding van pektiese stowwe in die selwande van die differensiërende transfusie-weefsel, vernaamlik die transfusie-trageïede, is bestudeer.

INTRODUCTION

A number of attempts have been made to find a stain specific for pectic substances and which also makes possible their histochemical localisation with the electron microscope. The heavy metal dye, ruthenium red, often used in light microscopy as a pectin stain, does not have the required specificity (Kertesz, 1951). Gee, Reeve and McCready (1959) found that alkaline hydroxylamine-ferric chloride was specific for pectic substances and used this stain in conjunction with the light microscope. The insoluble iron complex formed with the reactive methoxy groups of pectin is also electron dense, a property required for stains used in electron microscopy. Therefore this stain was tried at the electron microscope level as well (Albersheim *et al.*, 1960, 1963; Bornman *et al.*, 1969). Albersheim *et al.* used a prefixation staining technique on onion root tips, while Bornman *et al.* stained after fixation and sectioning of abscission zone tissue in *Coleus* and *Gossypium* petioles.



FIGS. 1–6.

Fig. 1. Pectin (P) dusted onto formvar-coated grid. STAIN (control): Sol*n*. B (0,75 min.) + Sol*n*. C (2 min.). Pectin particles unstained. Fig. 2. Pectin (P) dusted. STAIN: Sol*n*. A (15 min.) + Sol*n*. B (0,75 min.) + Sol*n*. C (5 min.). Pectin stained intense black. Fig. 3 Pectin (P) dusted STAIN Sol*n*. A (2 min.) + Sol*n*. B (0,75 min.) + Sol*n*. C (1 min.). Pectin stained intense black. Fig. 4. Pectin (P) incorporated into formvar coating. STAIN (control): Same as in Fig. 1. Pectin particles unstained. Fig. 5. Pectin (P) incorporated. STAIN: Same as in Fig. 2. Pectin stained intense black. Fig. 6. Pectin acid (PA) dusted. STAIN: Same as in Fig. 3. Particles stained intense black.

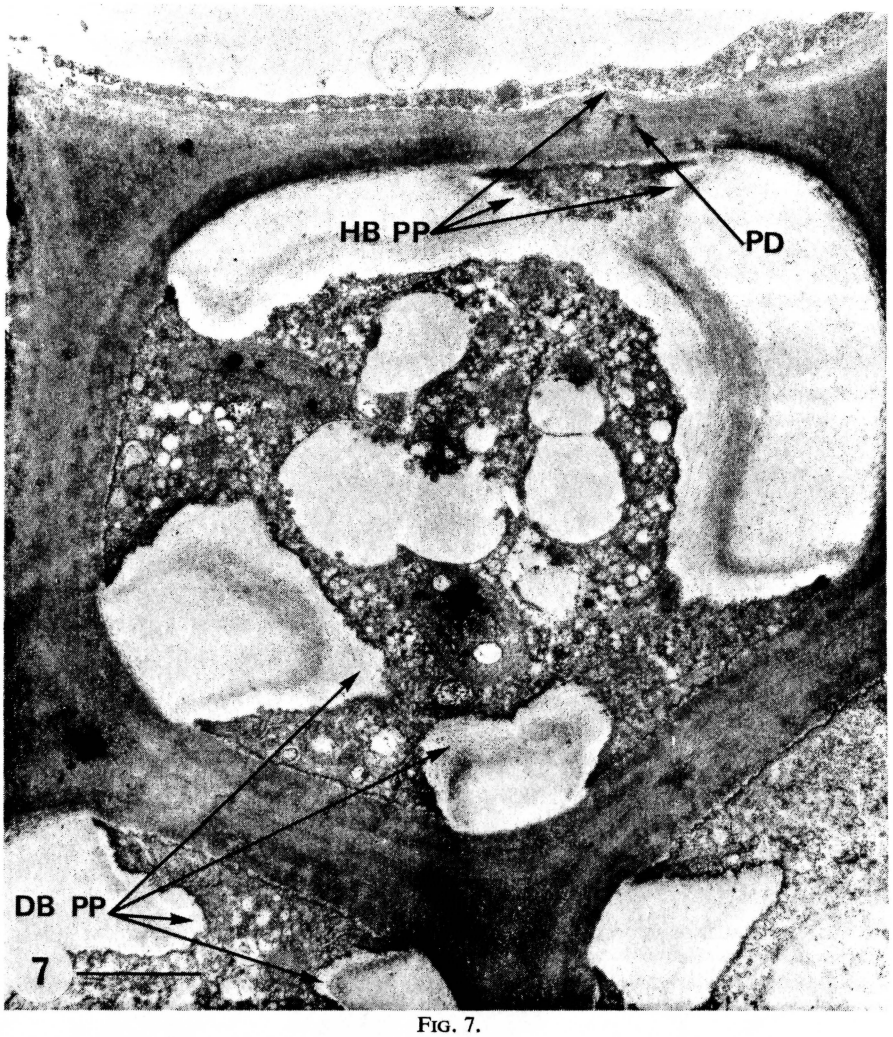


FIG. 7.

TS through transfusion tracheid. STAIN (control): Sol*n*. B (0,5 min.) + Sol*n*. C (1,5–2 min.). There is no real difference in stain intensity between compound middle lamella and cytoplasmic contents. DB PP, bordered pit pair; HB PP, blind pit; PD, plasmodesma.

This paper reports firstly on attempts to stain extracts of pectic acid and pectin differentially at the electron microscope level by using different staining-time sequences. It was hoped that, in the results of the study, there would be found a basis either for a quantitative stain for pectic substances *in vivo*, or a method of staining which would give more reproducible results for pectic

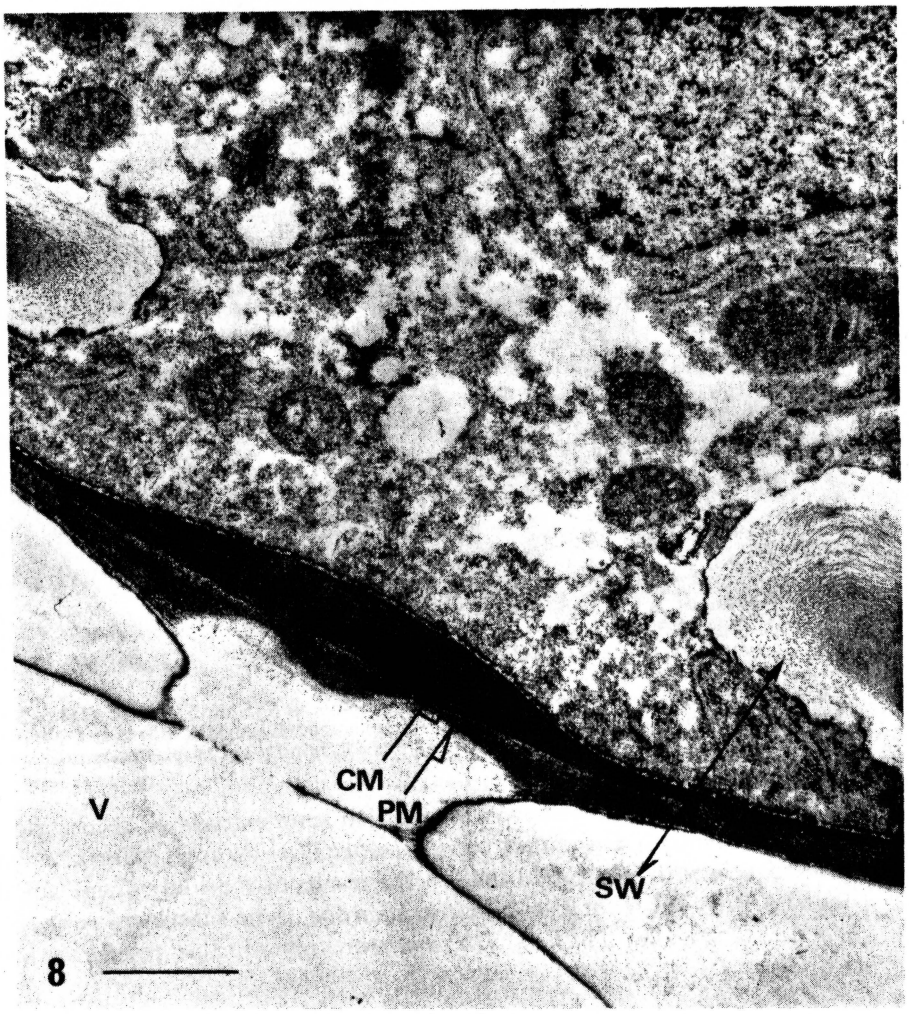


FIG. 8.
TS through pit region between two transfusion tracheids. STAIN: Sol*n*. A (15 min.) + Sol*n*. B (0,5 min.) + Sol*n*. C (1,5–2 min.). Compound middle lamella (CM) stained intensely; pit membrane (PM) very lightly stained as a result of pectic substance breakdown. Very few pectic substances in secondary wall (SW) of younger transfusion tracheid but none in secondary wall of older cell. V, vacuole.

substances in plant tissue than those obtained hereto. Secondly, *Welwitschia* leaf sections were also stained in order to test the specificity of the hydroxylamine-ferric chloride stain *in vivo*, and a subsequent examination of the distribution of pectic substances in the differentiating transfusion tracheids was made.

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Materials and Methods

Samples of powdered pectic acid and *Citrus decumana* pectin extracts, as well as *Welwitschia* one-year-old leaf sections, were stained. *Staining solutions:* (Albersheim *et al.*, 1960)

Solution A: Alkaline hydroxylamine (equal volumes of 14 g NaOH per 100 ml and 14 g $NH_2OH.HCl$ per 100 ml) 60% with respect to ethanol.

Solution B: 95% ethanol: conc. hydrochloric acid (10:1). Wash to acidify.

Solution C: 10% ferric chloride (anhydr.) solution in 60% ethanol containing 0,05 N HCl. Centrifuged before use.

Wash: Distilled water.

Staining procedure

Three different combinations of staining times were used for each specimen (Table 1), and examined under the electron microscope.

Controls were treated with solutions B and C only, the basic hydroxylamine treatment being omitted.

The staining sequences of the *Welwitschia* sections had to be modified slightly as a result of distintegration of the copper grids in Solution C.

Table 1
Different staining sequences used on specimens mounted in various ways on electron microscope grids*

METHOD: (i)—(iii) Pectin, Pectic Acid (iv) *In vivo*	STAINING TIMES (MIN.)			
	Solution A	Solution B	Solution C	
(i) Dusted (Ni grids)	— 15 10 2	0,75 ,, ,, ,,	2 5 2 1	Control
(ii) Spread (Ni grids)	— 15 10 2	0,75 ,, ,, ,,	2 5 2 1	Control
(iii) Incorporated (Ni grids) . . .	— 15 10 2	0,75 ,, ,, ,,	2 5 2 1	Control
(iv) Leaf Sections (Cu grids) . . .	— 15	0,5 ,,	1,5—2 ,,	Control

* Wherever possible, nickel (Ni) grids were used. However, when the leaf sections which had already been mounted on copper (Cu) grids were being stained, the grids tended to disintegrate after about 2 minutes in the ferric chloride solution. The uranyl acetate/lead citrate staining of these sections prior to their being stained for pectin probably accelerated this disintegration.

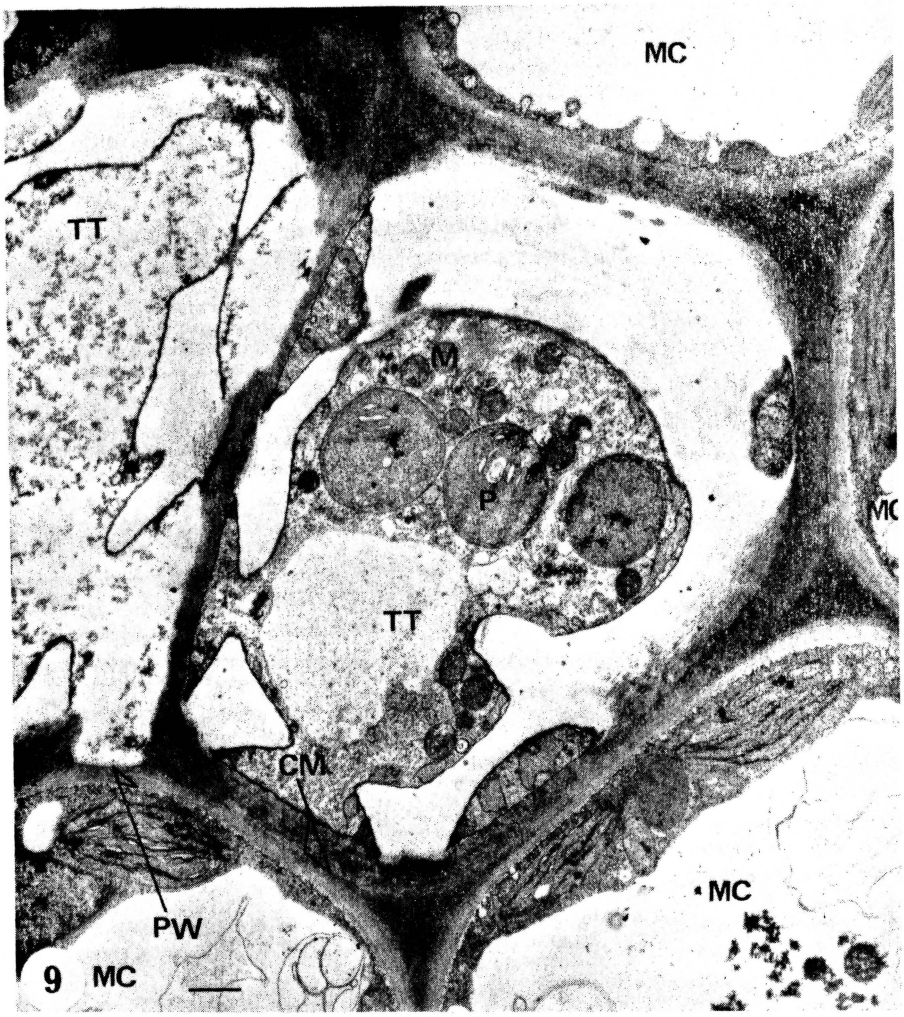

FIG. 9.
TS through two transfusion tracheids (TT) surrounded by four other mesophyll cells (MC). STAIN: Same as in Fig. 8. Breakdown of pectic substances in pit regions of compound middle lamella (CM). Primary wall (PW) of mesophyll cells still completely intact and distinguishable from compound middle lamella. P, plastid; M, mitochondrion. This stage is more advanced than in Fig. 8.

The extracts of pectic substances were applied to the grids either on or in a coating of formvar (0,3% in ethylene dichloride) in three different ways: by dusting formvar-coated grids with finer particles of pectic compound, by spreading a drop of the pectic substances suspended in 80% ethanol onto the coated

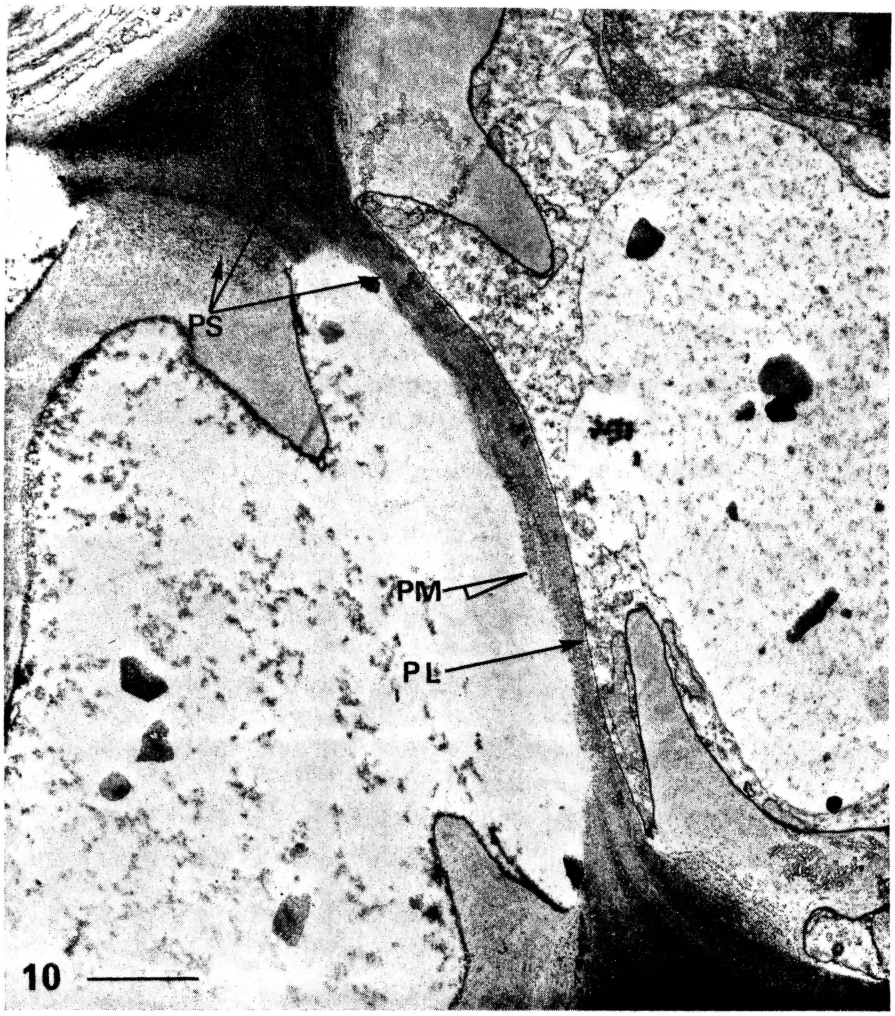

FIG. 10.

TS through two transfusion tracheids. STAIN: Same as in Fig. 8. Distribution of pectic substances (PS) in different layers of cell wall clearly illustrated; pit membrane (PM) is on side nearest the older transfusion tracheid and plasmalemma (PL) of younger cell is still intact. The dark specks are probably from the disintegrating copper grid. Stage of development approximately the same as in Fig. 9.

grid, or by incorporating the extracts into the formvar solution before using this suspension to coat the grids.

Good sections of one-year-old *Welwitschia* leaf tissue on copper grids which had already been stained with uranyl acetate/lead citrate were available. Only

those on which the compound middle lamella was clearly visible were selected for further staining with the hydroxylamine-ferric chloride stain.

Results and Discussion

Figures 1–3 illustrate a series of stain treatments on pectin dusted onto

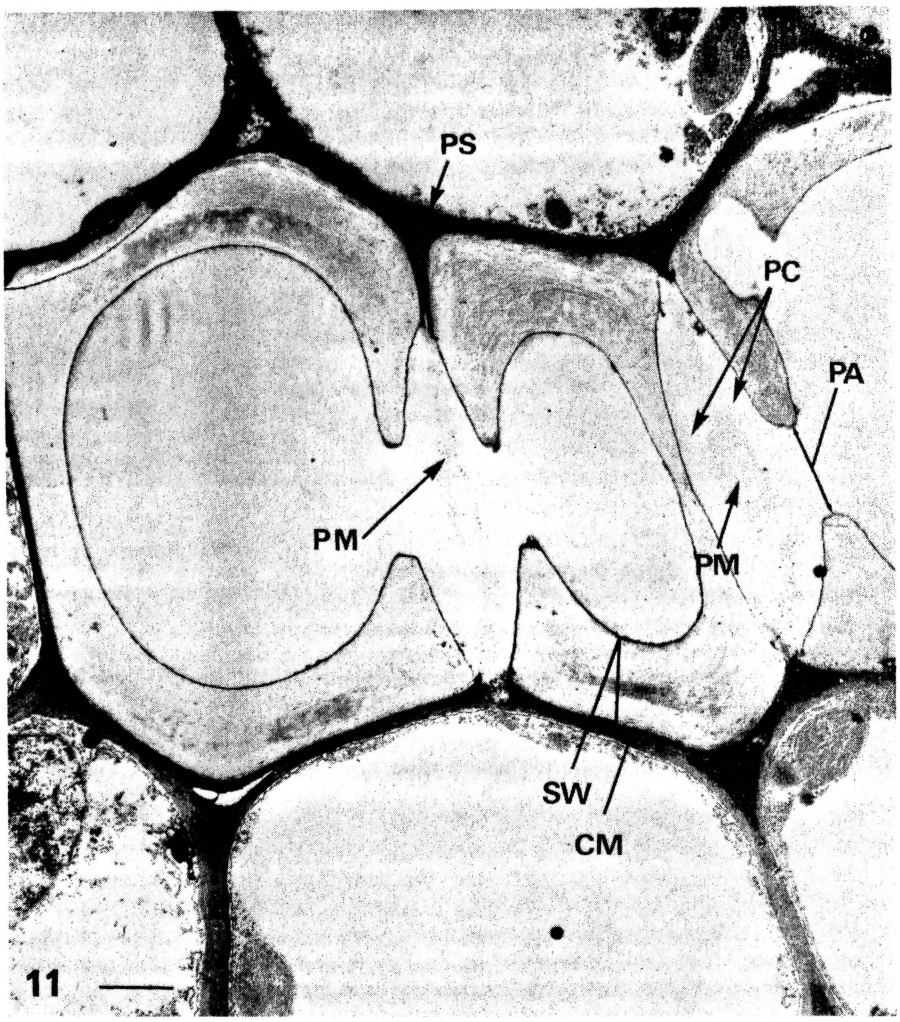

Fig. 11.

TS through transfusion tracheids at most advanced stage of differentiation. STAIN: Same as in Fig. 8. These cells have no cytoplasmic contents, neither are there any pectic substances in the pit membranes (PM). Darkly stained pectic substances (PS) are present in compound middle lamellae (CM) when adjacent to mesophyll cells. PA, pit aperture; PC, pit cavity; SW, secondary wall.

formvar-coated grids. No staining can be seen in the control (Fig. 1) whereas the pectin particles and fibres in the full treatments (Figs. 2 and 3) appear an intense black. There is apparently no difference in intensity of staining between the longest and shortest treatments.

Pectin particles incorporated into the formvar coating are shown in Figs. 4 and 5. Here, too, the control pectin is unstained while the fully stained pectin, at the periphery of a hole in the formvar membrane, is an intense black.

In Fig. 3 (pectin) and Fig. 6 (pectic acid) both groups of pectic substance have been dusted onto formvar and stained with the shortest treatment. In pectic acid, there is very little, if any, methylation of the carboxyl groups (Salisbury and Ross, 1969), and so the amount of stain complex formed would be expected to be proportionally less than the amount formed with pectin, which has a much greater degree of esterification of carboxyl groups (Bonner and Varner, 1965). However, it is difficult here to see any difference in intensity of staining between the two groups of pectic substances. This could be as a result of a certain degree of methylation having taken place in the pectic acid sample.

In all methods and staining sequences used on the extracted pectic substances, the same rather inconclusive results were obtained. The fundamental problem proved to be the unreliability in composition of the pectic preparations themselves.

The alkaline hydroxylamine-ferric chloride treatment of the leaf sections resulted in good differential staining of the compound middle lamellar pectic substances, even though an impression of the effect of different staining times on intensity of staining could not be gained because of the rapid disintegration of the copper grids. In *Welwitschia* leaf tissue, the distribution of pectic substances in the walls of transfusion tracheids, especially in the pit regions, during some stages in their differentiation is illustrated in Figs. 7–11. Transfusion tracheids form a specialized transfusion tissue when in association with parenchyma cells and so far have been found only in the leaf tissue of some gymnosperms and in leaf, root and stem tissue of *Welwitschia* (Gilliland *et al.*, 1971).

FIGS. 7–11.
Transfusion tracheids in leaf of one-year-old *Welwitschia* seedling. Bars represent 1 μm.

Figure 7, the control, shows a young transfusion tracheid which has formed bordered pit pairs where it lies adjacent to other transfusion tracheids, and a blind pit where adjacent to a mesophyll cell. There is no great distinction between the intensity of staining of the compound middle lamella and that of the cytoplasmic cell contents.

Figures 8, 9, 10 and 11 are fully stained sections of transfusion tissue showing stages of increasing maturity of transfusion tracheids. The pectic substances of the compound middle lamellae have become much more intensely stained

than the cytoplasmic contents (if still present) and the control wall pectic substances of Fig. 7. Pectic compounds were present in large quantities in the compound middle lamellae and were densely accumulated in the intercellular regions at the junctions of cells. They were also present, but very sparsely distributed, in the secondary walls of transfusion tracheids at the earlier stages of differentiation.

During maturation of a transfusion tracheid, breakdown of pectic substances (presumably by hydrolytic enzymes) in a pit region of the compound middle lamella begins from the side immediately adjacent to the cell lumen and gradually progresses outwards (Figs. 8, 10, 11). The compound middle lamella broadens noticeably in the pit region (Fig. 8). If the pit forms part of a pit-pair between two transfusion tracheids, breakdown commences from the side of the older wall and probably is completed as the younger cell ages (Figs. 8, 10). Eventually all pectic compounds in the pit membrane between the two cells disappear (Fig. 11). On the other hand, if the pit is a blind pit, e.g. in a transfusion tracheid wall opposite a mesophyll cell as in Fig. 9, only partial breakdown in the wall between the two cells takes place and the primary wall of the mesophyll cell, in which the pectic substances are less dense, remains intact, seemingly unaffected by the agent of pectic substance breakdown.

If this agent is a type of enzyme (as is suggested by the pattern of breakdown), its synthesis in a transfusion tracheid would be possible only as long as the cytoplasmic contents remain functional. In a situation as is present in Fig. 9, therefore, it is difficult to imagine that the older transfusion tracheid is capable of synthesizing further the enzymes which will continue the breakdown process in the compound middle lamella. It seems feasible that, at this stage, there may either be a delay in the process until breakdown commences from a younger transfusion tracheid on the other side of the cell wall, or, if the other cell is a mesophyll cell, there will be no further breakdown of pectic substances at all.

Conclusion

The usefulness of the hydroxylamine-ferric chloride stain in histochemical localisation of pectic substances at the subcellular level is obvious. However, this technique must still be greatly refined before it can be used as a reliable method for the quantitative histochemical determination of these compounds in plant material.

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