Acta Bot. Neerl. 42(2), June 1993, p. 237–244

Determination of cell-wall porosity by microscopy: walls of cultured cells and pollen tubes

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SUMMARY

In previous studies the porosity of the plant cell wall has been estimated to lie between 3·5 and 6 nm, depending on the cell type investigated and the method used. *Morinda citrifolia* cells from cultures at various stages of growth were incubated for 60 min with a colloidal gold sol (CGS) of size range 1·9–7·9 nm. Penetration of the cell wall by these gold particles was limited to the log phase of the culture growth cycle, with maximum uptake occurring in early log phase. The size of particles observed within the early log phase cells ranged from 2·5 to 5·7 nm. Since the cell wall was not, as in other methods, disrupted or altered, we conclude that the wall pores are up to 5·7 nm mean diameter at this early stage, but decrease rapidly as the culture ages. These results were confirmed with observations on the uptake of FITC-labelled dextrans. FD 20S dextrans (*c.* 6·6 nm diameter) failed to penetrate the wall, while FD 10S (*c.* 4·5 nm diameter) was taken up across the wall and into the protoplast. Further work using this approach demonstrated that pollen tubes of *Nicotiana* sp. were less permeable than those of *Tradescantia* sp. With the former, FD 20S only penetrated to the cytoplasm very slowly (18 h to become detectable), while with the latter, uptake was more rapid (1–2 h to become detectable).

Key-words: cell-wall porosity, colloidal gold sol, FITC-dextrans, *Morinda citrifolia*, *Nicotiana* sp., pollen tubes, *Tradescantia* sp.

INTRODUCTION

Cell-wall porosity determines the size of molecules that can gain access to the plasma membrane surfaces of plant cells. Generally solutes can pass freely across cell walls, but movement of macromolecules is severely restricted. Cell-wall porosity to such macromolecules may change during cell growth and differentiation, and may vary from tissue to tissue.

Existing methods for estimating cell-wall porosity (e.g. Carpita *et al.* 1979; Tepfer & Taylor 1981) are laborious and involve disrupting cells or exposing them to unusual conditions. The absence of a rapid method for examining cells in their natural state has inhibited studies on this topic, so that little is known of wall porosity, and its variation, in normal or diseased plant material.

In this present study the cell-wall porosity of suspension cultured cells of *Morinda citrifolia* was measured using both electron opaque (colloidal gold) and fluorescent (FITC-dextran) markers. By preserving the integrity of the cell wall and examining the cells at various periods throughout the culture growth cycle, accurate measurements on the level of cell-wall porosity were obtained. The results established the efficiency of the FITC–dextran method, which was then applied to the study of pollen tube wall porosity with a view to establishing limits to the sizes of molecules able to interact with pollen-tube plasma membranes *in vivo*.

MATERIALS AND METHODS

Cell culture

Morinda citrifolia *suspension cells.* Suspensions of *M. citrifolia* cells were cultured in conical flasks in NAX culture medium, a modified Gambourg B-15 medium (Zenk *et al.* 1975). The flasks were kept on a rotating platform (99 rpm) at a constant temperature of 25°C. After 21 days the nutrients in the medium became depleted at which time the maximum biomass yield had been reached. The suspension culture was maintained by subculturing into fresh medium every 21 days.

Pollen culture. Pollen of the two species was sown on culture media as follows:

Tradescantia virginiana medium: 10% sucrose, 0·01% boric acid, 10^{-2}M $CaCl_2$, 5×10^{-3}M KH_2PO_4, pH 6·8.

Nicotiana tabacum medium: 5% sucrose, 0·03% Amicase (Sigma), 12·5% polyethylene glycol 4000 (BDH special), 15·0 mM MES/KOH pH 5·9, 1·0 mM $CaCl_2$, 1·0 mM KCl, 0·8 mM $MgSO_4$, 1·6 mM H_3BO_3, 30 μM $CuSO_4$, 10 μg ml^{-1} Rifamycin.

Colloidal gold sol uptake experiments

Morinda citrifolia cells from suspension cultures at various stages of growth were centrifuged in 40 ml aliquots ($1·5 \times 10^6$ cells ml^{-1}) for 5 min at 1000 rpm. The supernatant was discarded and the cells resuspended in a treatment solution containing 5 nm colloidal gold sol (Biocell Research Lab) diluted in a 1:1 mixture with double strength NAX, to make a final volume of 40 ml. This mixture was then transferred to a 100-ml conical flask and replaced on the rotating platform.

After 1 h in the gold sol, the cells were spun down and rinsed twice in fresh medium followed by primary fixation for 2 h in 2·5% glutaraldehyde made up in fresh medium. Cells were then resuspended in 3% technical agar (OXOID agar No. 3). When solid the agar was cut into 1 mm^3 cubes and postfixed for 5 min in 2% osmium tetroxide in 0.08 M pipes buffer, pH 6·5. The fixed material was stained *en bloc* in 0·05% uranyl acetate for 1 h. Cells were then dehydrated using an acetone series and embedded in Spurr's resin (Agar Scientific Ltd.). Ultrathin sections were examined with a JEOL 2000EX TEMSCAN without post-staining

FD experimental work

Cells from 7-day old cultures were centrifuged for 3 min at 100 ***g***. The medium supernatant was discarded and the pellet of cells was resuspended in fresh medium containing 1 mM fluorescent dextrans (Sigma) (FD-20S, FD-10S or FD-4, see Table 1). Cells were incubated for time periods ranging from 3 to 18 h. After incubation, the cells were centrifuged as before and resuspended in fresh medium. This step was repeated twice. Samples were

Table 1. Sizes of fluorescent dextrans used in this study

Name	Mol. wt.	Diameter
FD-20S	17 200	6·6 nm
FD-10S	9400	4·5 nm
FD-4	4400	3.5 nm

(Granath 1958: Peters 1986: Terry & Robards 1987: Owen & Thompson 1991.)

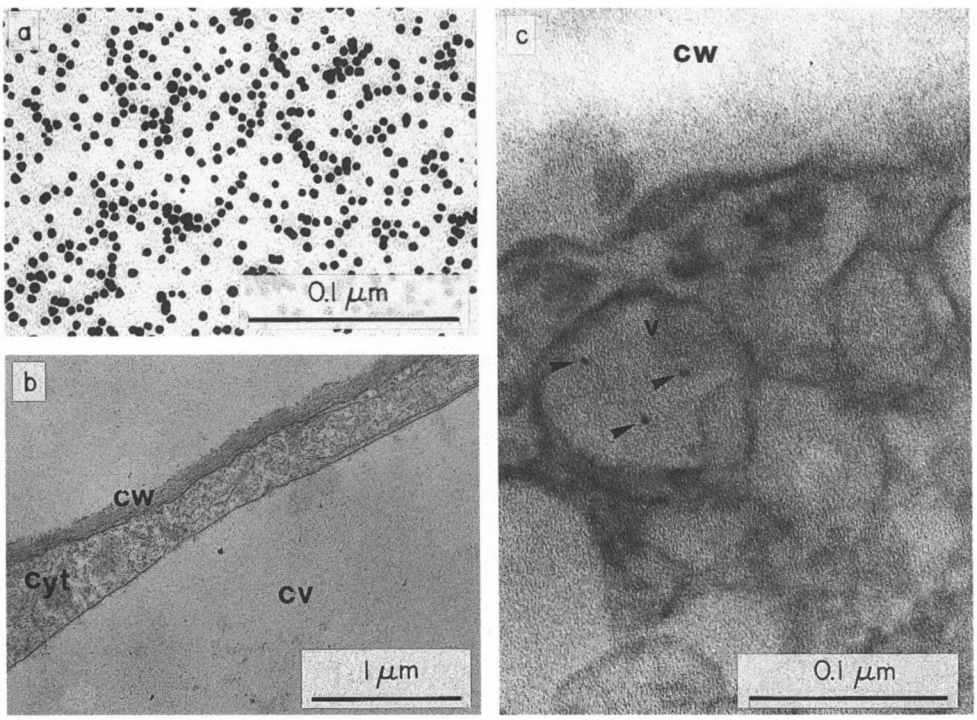

Fig. 1. (a) Colloidal gold sol (Biocell Research Lab) with an average particle diameter of 5 nm. (b) Section of *Morinda citrifolia* cell showing a large central vacuole (cv), which occupies 80–85% of cell total volume, surrounded by a thin layer of cytoplasm (cyt) and cell wall (cw). Cells are 100–150 μm in length depending on age and 30 μm in diameter. (c) Section of *M. citrifolia* cell from a culture in early log phase, incubated in a NAX medium containing 5 nm colloidal gold sol, for 60 min. Particles were located at various sites within the cell including terminal end vesicles (v) of individual cisternae.

examined in an epifluorescence microscope system. Fluorescent dextrans (Sigma) were added to the pollen tube growth medium to a final concentration of 1% either before sowing the pollen, or after pollen germination and a period of tube growth.

Thin-layer chromatography

Tests were carried out for the presence of free FITC and low molecular weight dextrans in the fluorescent dextran preparations, culture media, media recovered from growing pollen

Table 2. Uptake of colloidal gold particles at different phases of the culture cycle

Phase	Day	Particles h^{-1} $cell^{-1}$
Lag	3	100
Early log	7	1800
Late log	12	810
Stationary	16	100

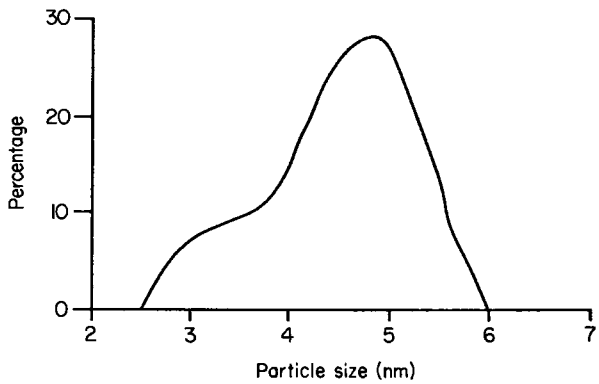

Fig. 2. Size distribution of gold particles observed in section profiles of *Morinda citrifolia* cells treated with colloidal gold sol.

tubes and extracts of tubes grown in the presence of fluorescent dextrans. Silica gel TLCs were run with a solvent system of 3:1 chloroform:ethanol to detect free FITC. Dried plates were viewed in UV-light.

RESULTS

Colloidal gold sol (CGS) experiments

Colloidal gold particles supplied to the cells were found to have a size range of 1·9–7·9 nm diameter when viewed on formvar grids. The spherical, electron opaque particles exhibited very little clumping (Fig. 1a).

The cells, when examined in the electron microscope, were seen to consist of a large central vacuole (80–85% of cell total volume) surrounded by a thin layer of cytoplasm and a primary cell wall (Fig. 1b).

In sections of *M. citrifolia* cells exposed to the gold sol for 1 h, gold particles were found in the cell wall and plasma membrane and in the cell protoplast (Fig. 1c). CGS penetration was maximum during early log phase growth (Table 2). The size of the particles which penetrated the wall during this phase ranged from 2·5 to 5·7 nm (Fig. 2). The frequency and size of particles entering the cells decreased during the later stages of log growth.

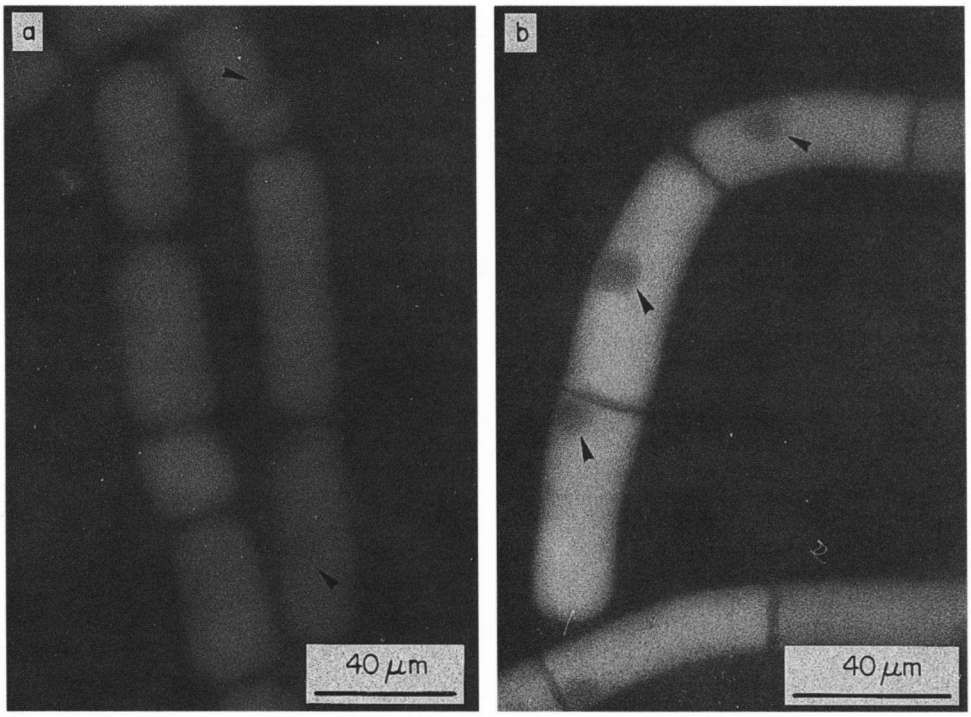

Fig. 3. Light micrographs of *Morinda citrifolia* cells under blue light conditions. The cells are seen to have taken up FITC-dextran (10S) after 4 h (a) and 18 h (b). The nuclear region, which does not contain the fluorochrome conjugate, can be seen as a dark region (arrow) against the brighter background of the stained vacuole.

FITC dextran (FD) experiments

Morinda citrifolia *experiments.* No uptake of FD-20S was observed after 18 h of incubation. After 4 h of incubation with FD-10S, fluorescence of the cells was observed (Fig. 3a). After 18 h the cells were clearly seen to fluoresce strongly (Fig. 3b). The thinness of the cytoplasmic region around the central vacuole made it difficult to discern whether or not the FD was restricted to the vacuole, however the nuclear region was seen as a dark area (Fig. 3b). FD-4 was not taken up into the protoplast, though plasmolysed cells whose plasma membrane was damaged showed heavy staining of the cytoplasm.

Pollen tube experiments. Tubes were grown in culture and then exposed to 1% FD-4 in growth medium for varying periods of time before washing in fresh medium and examination in a fluorescence microscope. After a short time period, 15–30 min, fluorescence was found in the tube at the tip end in both species. Longer exposure to FD-4 (1–3 h) resulted in fluorescence occurring in the main tube back to the pollen grain except where the presence of mature callose plugs prevented access of the fluorochrome to the older parts of the tube (Fig. 4b).

Larger fluorescent dextran molecules entered the tubes more slowly. FD-10S entry occurred over a similar time period to that seen with FD-4 in *T. virginiana*, but the uptake was much slower in *N. tabacum*. Tubes of *N. tabacum* examined after 30 min incubation in FD-10S appeared dark against the light background caused by residual fluorescence material in the medium (Fig. 4c). After 1–3 h most tubes of this species showed

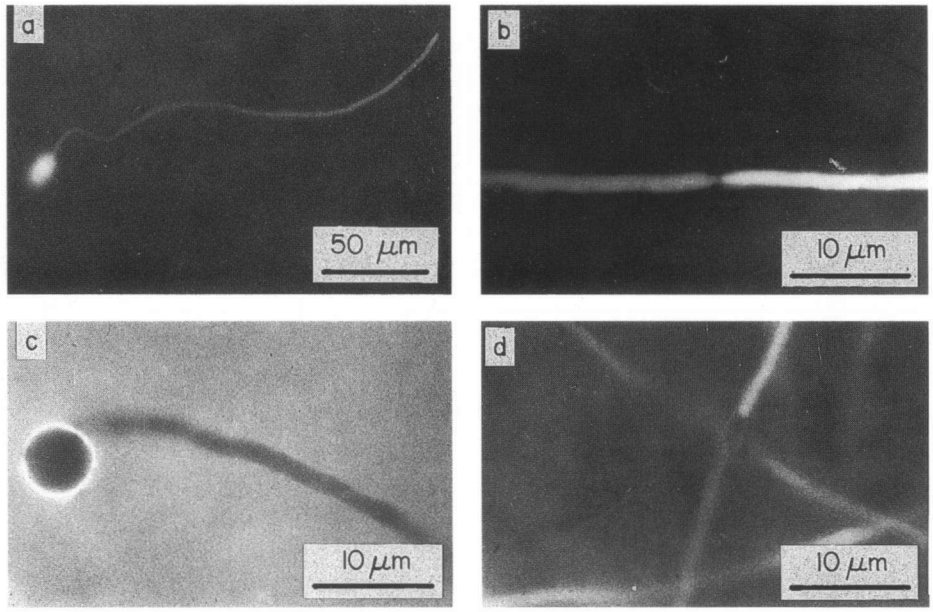

Fig. 4. (a)–(d) Photomicrographs of living pollen tubes taken using an epifluorescence illumination system after rinsing in fresh medium, without fluorescent dextran. (a) *Tradescantia virginiana* pollen tube after a 90 min incubation in FD-20S. Fluorescent material has accumulated in the grain (left) after moving in from the tip region (× 300). (b) *Nicotiana tabacum* pollen tube after 1 h in FD-4. The tube tip is to the right of the figure, a forming callose plug has restricted entry of the fluorochrome to the older part of the tube (× 1500). (c) *N. tabacum* pollen grain and tube after 30 min in FD-10S. The unstained grain and tube are seen against the residual background medium fluorescence (× 1500). (d) *N. tabacum* pollen tubes after 3 h in FD-10S. The dextran has penetrated the pollen tube walls. The brightly fluorescent contents of the tube in the upper part of the figure stop abruptly at a callose plug (× 1500).

fluorescence in the mature parts of the tube, but some tubes were still unlabelled (Fig. 4d). FD-20S crossed the wall slowly into *T. virginiana* pollen tubes, with uptake only observed after 1–2 h (Fig. 4a). However, 18 h incubation was required before FD-20S could be detected in tubes of *N. tabacum*.

Purity of dextrans and identity of intracellular fluorescence. Thin-layer chromatography was used to test the commercially-supplied dextrans and to determine whether these were degraded by growing pollen tubes. Free FITC could not be detected in ethanol extracts of the commercial dextrans (results not shown), or in aqueous solutions of FD-4 in media conditioned by growing pollen tubes, or in aqueous extracts of washed pollen tubes from these media. These extracts left a fluorescent spot at the origin, as did the FD-4 solutions. The aqueous TLC chromatograms also showed that none of the above samples contained low molecular weight fluorescent dextrans. Further, separate growth experiments not detailed here have shown that FD-4 had no effect on growth rates of tubes over a period of many hours.

DISCUSSION

Cleland (1968) stated 'Wall loosening cannot be separated from wall extension.' He proposed that wall extension may occur by a form of 'chemical creep', i.e. bonds may break and reform in new configurations, leading to a loosened wall. The enhanced permeability found during log phase growth of *M. citrifolia* cultures corresponds to the

early period of growth and cell-wall extension. The absence of gold sol uptake in the lag and stationary phases of the growth cycle does not imply that the wall is impervious to all the marker but rather that a decrease in wall porosity has reduced the level of particle penetration into the wall structure. As can be seen in Fig. 1a, very few particles are in the 2·5 nm size range, less than 4% of the marker population is below 2 nm, while 40% is below 3 nm, therefore even a slight reduction in wall porosity would have a dramatic effect on the amount of gold sol which may penetrate the wall due to the fixed size of the colloidal gold particles.

The estimated maximum pore size of 5·7 nm is considered to be realistic. The disadvantages of this method are that the number of cells which can be examined at any one time is limited, since work is carried out at a magnification of approx. 40 000 times and the protocol used is time-consuming.

Fluorescent-dextrans (FD) of various sizes were therefore also used as size markers. Though the shape and size of the FD molecule is not as clearly defined as the CGS particles, it does enable the rapid observation of marker uptake by a large population of cells. The results of the FD-20S and FD-10S experiments agreed well with the CGS marker findings. FD-20S has an approx. diameter of 6·6 nm, which is greater than the estimated maximum pore size of 5·7 nm, and failed to enter the cell. The FD-10S has an approx. diameter of 4·5 nm and was taken into the cell. With respect to wall porosity, the FD-4 (approx. diameter 3·5 nm) results also agree with the colloidal gold data. Though it failed to be taken up by living protoplasts, the staining of moribund cells indicates that the FD-4 can penetrate the cell wall.

Though much work in recent years has concentrated on the various aspects of cell-wall structure, debate still exists as to which wall constituents most directly affect wall porosity. The issue is a complex one since the wall is composed of polysaccharides, lectins, lignins, waxes and structural proteins. The popular model of the cell wall is that of a layered lattice structure of cellulose polymers which are associated with hemicellulose strands and interwoven with a pectin polymer network. The proportions of various constituents may vary from cell to cell or even within regions of the same cell wall (McCann *et al.* 1990; Roberts 1992). The cellulose structure forms the cell infrastructure and therefore would appear to have an important determining effect on wall porosity. Delmer *et al.* (1992), however, found that when suspension-cultured tomato cells were grown in the presence of a cellulose inhibitor, 2,6-dichlorobenzonitrile (DCB), wall porosity was not altered even though the cellulose–xyloglucan network was greatly depleted. These results suggest that the pectic network, and not the cellulose and xyloglucan, plays a major role in determining wall porosity.

The results of the experiments with pollen tubes grown *in vitro* show that walls are able to support the transport of macromolecules up to about 10 000 Da in size, but are only slowly permeable to larger molecules. Tests were undertaken to confirm that the fluorescence was emanating from labelled, intact dextran molecules. No evidence was found for any contamination of the high molecular weight dextrans with either free FITC or FITC linked to low molecular weight dextrans. In addition the pollen tube cultures did not appear to degrade the dextrans to such molecules, either in the medium or intracellularly. Extracts of pollen tubes containing fluorescent material run on TLC plates gave only a single fluorescent spot at the origin, as did FD-4. Pollen tube growth is inhibited by free FITC (Steer & Steer 1989), but FD-4, at the concentration used in the uptake experiments, had no effect on tube growth over a prolonged time period, in contrast to the rapid appearance of fluorescence in the tubes.

These observations support the conclusion that fluorescence in the tubes is due to the entry of high molecular weight dextrans, which must have traversed the cell wall. The

permeability of the walls is not uniform along the length of the tube, as judged by the labelling pattern. This is consistent with restriction of entry to the growing tube tip region. Subsequent movement within the tube transports the dextran to mature regions.

Permeability in the tip region is limited to molecules in the 5–7 nm range, with *N. tabacum* permeability being at the bottom of this range and *T. virginiana* at the top. These conclusions follow from the limited uptake of FD-10S and FD-20S dextrans.

In *Nicotiana alata* glycoproteins from the style (Jahnen *et al.* 1989a) have been detected in pollen tubes, which may be involved in incompatibility reactions (Gray *et al.* 1991). These glycoproteins are 28 000–34 000 Da (Jahnen *et al.* 1989a), estimated by gel electrophoresis against standards. A possible consequence of the present results is that such incompatibility factors could only gain entry when the growing tube tip passes through the tissue; they cannot enter mature tubes. This may also provide an explanation for the very high concentrations of these glycoproteins in the stylar tissue (Cornish *et al.* 1987).

By examining wall porosity in different cells under varying conditions much can be learnt about the determining factors affecting wall porosity. The present study has shown that the fluorescent dextran size marker method is accurate, simple and fast, making it ideally suited for large-scale examination of cell-wall porosity.

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