WALL REGENERATION BY PLASMOLYSED
CELLS FROM TISSUE SUSPENSION CULTURES
OF SPHAEROCARPUS DONNELLII

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SUMMARY

De novo synthesis of wall material by cells of Sphaerocarpus donnellii was followed using radioactive tracer techniques. Uniform uptake of [14C]glucose by plasmolysed suspension cultures was demonstrated autoradiographically. Liquid scintillation counts of acid- or alkali-soluble wall carbohydrates indicate that hemicellulose and cellulose make up the major wall fractions in control and plasmolysed tissues. The amount of label in walls of plasmolysed cells was significantly lower than in those of controls. Higher relative radioactivity in pectin and hemicellulose, and lower radioactivity in cellulose were found in plasmolysed tissue. These compositional changes may enhance wall stability by providing numerous sites for cross-linkage between cellulose microfibrils of regenerated walls.

INTRODUCTION

Deposition of wall material in plant cells is a continuous event accompanied by alteration and turnover of pre-existing wall material. Autoradiography of cell walls whose protoplasts have been fed with labelled sugars indicates that deposition occurs uniformly throughout the wall during growth, as opposed to being layered (Ray, 1967). Since the cell wall is always present around the protoplast, it has been thought that the old wall might serve some role in the deposition process.

The dependence of wall synthesis on pre-existing wall material can now be studied using naked plant protoplasts. Wall regeneration by isolated protoplasts has been investigated at the structural level in several plant species (Pojnar, Willison & Cocking, 1967; Horine & Ruesink, 1972; Burgess & Fleming, 1974; Fowke, Bech-Hansen & Gamborg, 1974; Burgess & Linstead, 1976). Use of protoplasts for biochemical analysis of the regeneration process has received less attention. Hanke & Northcote (1974) reported that pectic components were not assembled into the regenerated wall of isolated soybean callus protoplasts, but rather were secreted into the medium. They suggested that the absence of pectins could explain the occurrence of budding in protoplasts, and might also explain anomalous wall structure (Horine & Ruesink, 1972; Burgess & Fleming, 1974). The most detailed investigation of biochemical components of a regenerated cell wall (Takeuchi & Komamine, 1978), indicates that there may be similarities between the sugars of the extracellular polysaccharides

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which are released into the medium and the original wall, but that distinct differences exist between the composition of the regenerated and original cell wall.

A technique somewhat simpler than the use of enzymically isolated protoplasts has been described for biochemical analysis of *de novo* wall regeneration (Boffey & Northcote, 1975). This technique involves the use of plasmolysed tissue. Plasmolysis results in separation of protoplasts from adjacent cell walls. Large numbers of viable protoplasts can then be studied without resort to wall enzyme treatment. The newly regenerated wall can be distinguished from the old wall if the tissue is fed a radioactive carbon source. Under these conditions, only new wall material becomes radioactively labelled. Boffey & Northcote (1975) employed this technique with plasmolysed tobacco leaf disk cells. In this study, the technique of plasmolysis was used to investigate wall regeneration in liquid suspension cultures of the liverwort, *Sphaerocarpos donnellii*.

**MATERIALS AND METHODS**

**Plant material**

Female gametophytes of *Sphaerocarpos donnellii* Aust. were grown axenically in Prat's liquid medium (Doyle, 1967). Clonal subcultures were maintained at 20 °C under 12-h daily illumination with cool white fluorescent light (2500 lux outside the 250-ml Erlenmeyer flasks) for periods of up to 30 days prior to use.

**Microscopic histochemistry**

Freshly harvested material, in some cases plasmolysed for 3 days in media containing 0.4 M mannitol, was used to test microscopically for the presence of starch and wall components. The following standard reagents or techniques were used: iodine in potassium iodide for starch, iodine in potassium iodide and sulphuric acid as well as birefringence in polarized light for cellulose, ruthenium red and hydroxylamine-ferric chloride for pectins, chlorine sulphite for lignin, aniline blue for callose, Sudan IV for cutin, and mercuric-bromphenol blue for proteins (Jensen, 1962). Protein content per g dry wall weight was also determined colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Radioactive labelling**

About 0.4 g fresh weight of plant material was acclimated for 3 h in the dark in either carbon-free liquid Prat medium (control), or medium plus 0.4 M mannitol as osmoticum (experimental). After dark acclimation, tissues were transferred to 20 ml of their respective medium containing 10 μCi of D-[U-14C]glucose (sp. act. 313 mCi/mmol, New England Nuclear). Aliquots of the radioactive media were removed to determine initial radioactivities. Plant material was then incubated in the dark for 6 h at 20 °C. After this period, aliquots were again taken from each solution to determine final radioactivities. The tissues were removed and washed with 3 changes (20 ml, 30 min) of their respective non-radioactive media.

**Autoradiography**

Radioactively labelled tissue (control and experimental) was pressed between blotting paper and dried overnight at 56 °C. Autoradiographs were then made using Kodak Industrex M X-ray plates. The exposure period was 5 days.
Extraction procedure

Freshly harvested, radioactively labelled tissues were extracted twice, 10 min each with 95% boiling ethanol. Ethanol extracts were separated from the tissue and pooled by filtration through glass fibre filters. The extracted plant material was dried over P₂O₅ under vacuum at 50 °C for 8 h, and then weighed.

Desiccated tissues were treated for 2 h with 15 ml of 2% (w/v) sodium hexametaphosphate at pH 3.7, 99 °C (Stoddart, Barrett & Northcote, 1967). An aliquot was removed from each solution for determination of radioactive pectins and starch. Filtered extracts were cooled and then adjusted to pH 7 with 2 M NaOH. NaCl was added to give a final concentration of 0.05 M. Salivary amylase (10 ml) was added along with 3 drops of toluene, and samples were stored for 4 h at 37 °C to allow for the digestion of starch (Olmait & Northcote, 1962). After amylase treatment, salts and free sugars were removed by dialysis against 4 × 2-l. of water for a period of at least 12 h. Volumes of the solutions with remaining non-diffusible pectins were measured and retained. Hemicelluloses were removed from ethanol- and acid-insoluble material by treatment with 5% (w/v) KOH for 2 h, 20 °C, followed by 24% (w/v) KOH for a further 2 h. Each solution was filtered from the tissue with glass fibre filters and neutralized with acetic acid. Aliquots (1 ml) of all the above extracts were immediately assayed for radioactivity. The insoluble material remaining after alkaline extraction was digested with 1 ml of NCS™ Tissue Solubilizer (Amersham/Searle Corp.) for 48 h at 45 °C, and also assayed.

Loss of radioactively labelled macromolecules into the growth medium was investigated in both plasmolysed and unplasmolysed conditions by pooling the incubation and wash media and then dialysing against 4 × 4-l. of water for a 48-h period. Volumes of the dialysed solutions were measured and aliquots were taken for radioactive assay.

Radioactivity determinations

Samples prepared for assay were immediately placed in plastic scintillation vials to which 10 ml of a universal liquid scintillation cocktail (Aquasol, New England Nuclear) were added. The samples were counted in a Beckman Model LS-100C liquid scintillation counter, using a preset error of 0.2%, and a maximum counting time of 20 min. A chemoluminescence problem with NCS™-solubilized samples was minimized by dark incubation for 96 h. Counts per min (cpm) were converted to disintegrations per min (dpm) by subtracting the cpm of the non-radioactive blanks and correcting for efficiency by the internal calibration standard method. Samples were compared on the basis of cell wall dry weight (determined after ethanol extraction).

RESULTS

Histochemistry

Plasmolysed cells of Sphaerocarpos donnellii, and regeneration of their walls, may be readily detected at the light-microscope level (Fig. 1). Old and newly formed cell walls of this species respond to histochemical staining in a fashion similar to the primary walls of higher plant cells. These walls lack lignin, a characteristic shared by almost all liverworts (Siegel, 1962). Cellulose and pectin are the only positively stained wall components. The ruthenium red reaction for pectin results in uniform staining of the wall. Hydroxylamine-ferric chloride reaction for esterified pectins is negative. Callose (α(1→3) glucan typically deposited in response to wounding) is known to occur as a normal component in developing walls of Sphaerocarpos spores (Doyle, 1975), but could not be detected in the regenerated walls of plasmolysed tissue. Callose has previously been identified as a wall polymer secreted by isolated onion root and Vinca rosea protoplasts (Prat & Roland, 1971; Takeuchi & Komamine, 1975).
M. A. Grusak, R. J. Thomas and B. H. Marsh

1978), but is not a detectable wall component of soybean protoplasts (Horine & Ruesink, 1972).

While protein could not be detected histochemically in *Sphaerocarpos* walls, it was detected colorimetrically. Protein content of cleaned wall material (11.1% of total wall dry weight) is in the 10% wall protein range found in some higher plant tissues (Albersheim, 1976). The percentage of protein in regenerated walls was not determined colorimetrically due to the difficulty involved in separating old from new walls in the plasmolysed tissue.

![Fig. 1. Plasmolysed cells of *Sphaerocarpos donnellii* growing in liquid suspension culture (Doyle, 1967). (A) Light micrograph of cells after 1 h in osmoticum (0.4 M mannitol). (b) Birefringence of cellulose in old and regenerated walls as seen under polarizing optics. ×350.](image)

**Total radioactive uptake**

 Autoradiography of tissues incubated in d-[U-14C]glucose solutions demonstrates uniform uptake of label into *Sphaerocarpos* cells (Fig. 2). This is in contrast to the pattern of uptake observed in tobacco leaf disks (Boffey & Northcote, 1975) where the presence of a cuticle blocked uptake in all but a layer of cells adjacent to the cut margin.

As with tobacco leaf disks, the mean value for total radioactive uptake is lower in plasmolysed *Sphaerocarpos* tissue than in controls (Table 1). The difference in *Sphaerocarpos*, however, is not significant at the 95% confidence level. In plasmolysed and control tissues of both *Sphaerocarpos* and tobacco leaf disks, much of the absorbed label is apparently respired. The percentage of radioactive label incorporated into cells is significantly greater in controls than in plasmolysed tissues. The amount of labelled macromolecules lost to the medium (as determined after dialysis) was similar in both cases (Table 1).
Wall regeneration in *Sphaerocarpos*

Fig. 2. Autoradiograph of *Sphaerocarpos donnellii* female gametophytes treated with $[^{14}C]$glucose. (A) Dried plant mounted on blotting paper. (B) Corresponding X-ray plate developed after a 5-day exposure period. ×15.

### Table 1. Percent uptake of $[^{14}C]$glucose by plasmolysed suspension cultures of *Sphaerocarpos donnellii*

<table>
<thead>
<tr>
<th></th>
<th>Plasmolysed</th>
<th>Control</th>
<th>$t$-test, $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake</td>
<td>3.03 ± 0.52</td>
<td>7.25 ± 1.83</td>
<td>0.090</td>
</tr>
<tr>
<td>Retained in tissue</td>
<td>2.23 ± 0.25</td>
<td>5.32 ± 0.89</td>
<td>0.024</td>
</tr>
<tr>
<td>Macromolecules lost to medium</td>
<td>0.69 ± 0.09</td>
<td>0.60 ± 0.15</td>
<td>0.657</td>
</tr>
<tr>
<td>Remainder (mostly respired)</td>
<td>97.08 ± 0.29</td>
<td>93.88 ± 1.02</td>
<td>0.040</td>
</tr>
</tbody>
</table>

* Percentage of initial label per g dry weight of tissue after 6 h incubation in darkness; mean and standard error of 3 determinations.
† Percentage of the total radioactive uptake.

Radioactivity in extracted fractions

Analysis of extracted fractions from tissues incubated in $[^{14}C]$glucose indicates lower incorporation of label into wall fractions of plasmolysed than into control samples (Table 2). The ethanol-soluble extracts contain the highest percentage of label in both control and plasmolysed tissue. Starch, histochemically localized in chloroplasts by the iodine in potassium iodide staining procedure, is also labelled during the incubation period. The ratio of radioactivity in ethanol- and amylase-soluble fractions is 13.9:1 for plasmolysed cells, compared with 7.6:1 for controls.

Detailed analysis of the wall fractions indicates the presence of dilute acid-soluble (pectin), dilute alkali-soluble (hemicellulose-A), concentrated alkali-soluble (hemicellulose-B), and alkali-insoluble (cellulose) substances (Fig. 3). These substances have been identified previously in *Sphaerocarpos* (Bricker & Doyle, 1964). The pectic content of *Sphaerocarpos* is low compared to most higher plants, a characteristic common to hepatics (Schulz & Lehmann, 1970; Thomas, 1977).

Quantitative comparison between plasmolysed and control wall fractions of *Sphaerocarpos* (Fig. 3) shows statistically significant differences at the 95% confidence
level in all but the hemicellulose-A fraction \((P = 0.089)\). The pectin content of the regenerated wall is higher compared to controls. This result contradicts the observed decrease in pectins seen in plasmolysed cells of tobacco leaf mesophyll (Boffey & Northcote, 1975), and the total lack of pectins incorporated into regenerated walls of isolated soybean protoplasts (Hanke & Northcote, 1974). The percentage of hemicellulose-B in new Sphaerocarpos walls is higher than that incorporated in controls. The percentage of cellulose is lower. In tobacco leaf disks, total hemicelluloses make up a higher percentage in plasmolysed tissue compared to controls, whereas the percentage of cellulose remains the same. Regenerated walls of isolated Vinca rosea

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**Table 2. Percent of radioactivity in ethanol-soluble, starch, and total wall extracts from \[^{14}C\]glucose-treated cultures of Sphaerocarpos donnellii**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Plasmolysed</th>
<th>Control</th>
<th>(t)-test, (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol-soluble</td>
<td>76.10 ± 0.74*</td>
<td>48.92 ± 0.93</td>
<td>0.000</td>
</tr>
<tr>
<td>Amylase-soluble</td>
<td>5.47 ± 0.31</td>
<td>6.47 ± 0.18</td>
<td>0.032</td>
</tr>
<tr>
<td>Wall</td>
<td>18.43 ± 0.66</td>
<td>44.62 ± 1.58</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Mean and standard error of 4 determinations.

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![Graph](image-url)  

**Fig. 3.** Radioactivity in cell wall extracts from \[^{14}C\]glucose-treated cultures of Sphaerocarpos donnellii. Bars represent means of 4 determinations. Standard error is indicated by vertical line at the top of each bar. Student's t-tests indicate the following significance probabilities between corresponding extracts from experimental and control treatments: pectins, \(P = 0.008\); hemicellulose-A, \(P = 0.089\); hemicellulose-B, \(P = 0.004\); cellulose, \(P = 0.001\).
protoplasts are low in both hemicellulose and pectin, and lack cellulose completely (Takeuchi & Komamine, 1978).

DISCUSSION

The microscopic histochemical analysis of *Sphaerocarpos* gametophytes reveals a number of features that make this tissue useful for wall regeneration studies. Since the tissue is largely undifferentiated, composed entirely of cells with primary wall composition, quantitative analyses are greatly facilitated. ‘Leaves’ of this tissue are only one cell thick, so that plasmolysis is easily accomplished and easy to observe (Fig. 1). Radioactive uptake of exogenous label (Fig. 2) is presumably facilitated by the absence of a surface cuticle on cells grown in liquid suspension culture.

Increased pectin and hemicellulose content of regenerated *Sphaerocarpos donnellii* walls is a finding in direct contrast to decreased matrix sugar content of newly regenerated walls in higher plants. Northcote and co-workers consider the loss or absence of pectin material in regenerated walls of higher plant protoplasts to be a factor which significantly contributes to anomalous wall structure (Hanke & Northcote, 1974; Boffey & Northcote, 1975). Pectins and hemicelluloses are normally an important component of the matrix polysaccharides. Matrix polymers are chemically linked to cellulose microfibrils. Microfibrils in turn, usually occur in ordered, netlike arrays (Roelfsen, 1965), with matrix material filling the spaces within the net. In a turgid cell (i.e. with pressure against the wall), individual microfibrils slip on one another to relieve tension. The presence of matrix material dampens the slip, and therefore both minimizes displacement of microfibrils and results in uniform distribution of wall stress. Increased pectic and hemicellulose content of regenerated walls of *Sphaerocarpos* may therefore help stabilize the new wall. This hypothesis is supported by the observation that newly isolated protoplasts of *Sphaerocarpos* (Taylor, 1979) do not bud as frequently as those isolated from higher plants (Taylor, personal communication). Analysis at the ultrastructural level is needed to determine whether these regenerated walls show a less anomalous structure compared to those of other species (Pojnar et al. 1967; Burgess & Fleming, 1974; Fowke et al. 1974; Burgess & Linstead, 1976).

The synthesis and deposition of hemicelluloses and pectic polysaccharides by plant cells is associated with the Golgi apparatus (Harris & Northcote, 1971; Fowke & Pickett-Heaps, 1972). Golgi vesicles are believed to fuse with the plasma membrane, releasing wall material and possibly enzymes for incorporation into the wall. Golgi body number remains constant in both plasmolysed and unplasmolysed tissues of higher plants (Sitte, 1963; Prat, 1972; Robinson & Cummins, 1976). The deposition of cellulose microfibrils is vaguely understood, although this process is closely associated with the outside surface of the cell membrane (Wooding, 1968; Bowles & Northcote, 1972, 1974). Separation of the plasmalemma from the old cell wall during plasmolysis would disrupt membrane-associated cellulose synthesis. Continued deposition of matrix sugars by Golgi, accompanied by decreased cellulose synthesis due to plasmolysis, would result in the radioactive incorporation pattern observed in
Sphaerocarpos, i.e. higher relative matrix sugar content and lower cellulose content during regeneration (Fig. 3).

Cell wall terminology employed in this report is based on solubility criteria and histochemical staining rather than chemical composition. Preliminary colorimetric and chromatographic characterization of Sphaerocarpos wall hydrolysates (Bricker & Doyle, 1964; Marsh & Thomas, unpublished) indicates that dilute acid- and alkali-soluble wall fractions contain hexoses (glucose, galactose and mannose), pentoses (arabinose and xylose) and uronic acids (glucuronic and galacturonic acids). The percentage of these sugar moieties in soluble wall fractions from [14C]glucose-treated samples of Sphaerocarpos has not yet been determined. Changes in the relative proportions of wall polysaccharides due to plasmolysis, however, probably account for the observed differences in wall solubility. A correlation between wall solubility and differential incorporation of wall sugars has been established in tobacco mesophyll cells (Boffey & Northcote, 1975), where plasmolysis apparently has a differential effect on the mechanism for arabinan versus polygalacturonorhamnan synthesis.

The inhibitory effects of mannitol and other plasmolysing agents on wall incorporation are well documented (Bayley & Setterfield, 1958; Ordin, 1960; Cleland, 1967; Robinson & Cummins, 1976). This inhibition during plasmolysis may be due to osmotic stress, but has also been explained, at least in part, by the absence of wall primer material (i.e. the old wall from which the membrane has been separated) as a site for organized deposition of wall precursors. Thus, much of the newly synthesized wall polymer is lost to the culture medium. After incubation of Sphaerocarpos for 6 h in [14C]glucose, there is no significant difference between the amount of labelled extracellular macromolecules (based on percentage of uptake) detected in the plasmolysing or non-plasmolysing culture media (Table 1). This is probably a reflexion of how quickly Sphaerocarpos protoplasts begin to regenerate stable walls. Some trapping of ethanol-insoluble, non-wall-incorporated material between the membrane and the old wall of intact tissue may also be occurring. Further work with isolated protoplasts is required as a test for the latter possibility.

The contributions of M.A.G. and B.H.M. to this work represent portions of Senior theses at Bates College.

REFERENCES


Wall regeneration in Sphaerocarpos


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