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# KINETIN INDUCED CHANGES IN CELL WALL COMPOSITION OF TOBACCO CALLUS

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**Key Word Index**—*Nicotiana tabacum*; Solanaceae; callus tissue; kinetin; friability; cell walls; hemicellulose; extensin.

**Abstract**—Culture of tobacco callus on high or low kinetin in light or darkness leads to changed tissue texture and associated changes in cell wall composition. In particular, friable callus (low kinetin, darkness) cell walls have a greater extensin content and an altered composition of arabinose and xylose containing hemicelluloses compared with cell walls of compact callus (high kinetin, darkness). The possible importance of these differences in determining callus friability is discussed.

## INTRODUCTION

In plant tissue culture the degree of tissue coherence, as well as growth rate and the ability to differentiate, is related in a complex manner to the supply and balance of nutritional factors in the culture medium. In particular, suitable levels of auxins, cytokinins and gibberellins have been used to induce friability in solid [1, 2] and suspension [3–5] callus tissue and in cultured explants [6]. Since exposure of tissues to treatments which affect cell wall integrity, such as chelating agents [7–9] and wall degrading enzymes [10], facilitate the fragmentation of tissues, it has been recognized that friability is most probably related to an altered cell wall metabolism.

A study of Paul's Scarlet rose suspension culture has shown that the induction of friability is associated with an increase in the activity of endogenous wall degrading enzymes [11]. Other studies have sought to explain friability in callus on the basis of some difference in cell wall composition or structure [12, 13]. Since slight structural modifications are known to change greatly the properties of polysaccharides, the differences involved could be small ones [14]. We decided to re-examine this proposition by using a solid tobacco callus culture in which other aspects of carbohydrate metabolism have previously been studied [15]. When cultured in darkness on media containing high ($2.5 \times 10^{-6}$ M) or low ($5 \times 10^{-8}$ M) kinetin levels a firm compact or a loose friable tissue form can be reliably induced: on the other hand, in light the same culture media each produce a compact form of callus [2]. We report here that induction of a friable callus is correlated with a significant alteration in the composition of the cell wall hemicelluloses and in the content of extensin.

## RESULTS

The appearance of the tissue grown on the two levels of kinetin, in light and in darkness, was as previously described [2]. The firm compact callus (dark, high kinetin) and in particular the loose friable callus (dark, low kinetin) contained a variable degree of brown pigmentation from batch to batch. Friability might be associated with polysaccharides that are loosely attached to the wall and such components could be lost by tissue extraction in aqueous buffer. We therefore chose to homogenise tissue in 80% EtOH (13, see Experimental) and this procedure yielded cell wall preparations with 32% (±3%) carbohydrate. The preparations contained small amounts of lignified cell wall fragments, derived from xylem cells which were occasionally differentiated in the otherwise parenchymatous callus cell mass under all treatments. It was considered that these small amounts of secondarily-thickened cell walls did not present a serious problem in terms of affecting the analyses of the major primary cell wall material. Any starch in the cell wall preparation would be solubilised by exhaustive hot water extraction. Assuming that all the glucose extracted represents starch, the total starch contamination in the wall preparation can be estimated to be no more than 6% for the dark grown callus (Tables 2, 3), and 8% for the light (data not presented). Because of the significant differences discovered, most attention has been given in this work to the cell walls of dark-grown friable (F) and compact (C) callus. In these cases cell walls were prepared from several separately cultured batches of tissue, and the analyses are presented as an average composition of several separately cultured batches (Tables 1–5).

The monosaccharide composition of the cell walls of the four callus treatment conditions, and of tobacco pith parenchyma from which the callus was originally derived, is presented in Table 1. The major components were glucose, galactose, arabinose, xylose and uronic acids: mannose and rhamnose were present in small amounts, and trace amounts of fucose were occasionally found (the presence of small amounts of ribose, probably representing cytoplasmic contamination, is omitted in presenting analyses). The levels of arabinose and xylose, and their relative ratios, were clearly and reproducibly different in the cell walls of dark-grown friable and compact tissue, but otherwise the cell wall compositions were very similar. Tobacco pith cell walls and light-grown compact

Table 1. Effect of high and low kinetin on cell wall monosaccharide composition (%) and hydroxyproline content of tobacco callus cultured in darkness and light

| Sugar | Darkness* | | Light | | Tobacco pith parenchyma |
|---|---|---|---|---|---|
| | Low kinetin (F) | High kinetin (C) | Low kinetin | High kinetin | |
| Rha | 1.7 (0.2) | 2.0 (0.1) | 1.1 | 1.4 | 1.5 |
| Ara | 14.7 (0.8) | 8.6 (0.7) | 11.6 | 11.2 | 4.9 |
| Xyl | 4.0 (0.3) | 8.7 (1.1) | 3.5 | 5.1 | 7.3 |
| Man | 1.9 (0.6) | 2.1 (0.1) | 1.3 | 1.7 | 4.0 |
| Gal | 19.7 (2.5) | 16.8 (2.1) | 13.8 | 16.5 | 11.1 |
| Glc | 46.5 (3.4) | 47.9 (3.6) | 61.3 | 54.7 | 65.3 |
| Uronic acids | 11.6 (1.3) | 13.4 (1.7) | 7.5 | 9.5 | 6.0 |
| Ara/Xyl ratio | 3.8 (0.2) | 1.1 (0.03) | 3.3 | 2.2 | 0.7 |
| Hyp (% total wall carbohydrate) | 5.9 (0.2) | 3.6 (0.5) | 5.5 | 5.0 | 0.8 |

* Mean (s.e.m.) for 3 batches.

Table 2. Effect of high and low kinetin upon amounts of warm water extractable polysaccharides, pectin, hemicelluloses and cellulose in dark cultured tobacco callus cell walls

| | Amount carbohydrate recovered in fraction/ total carbohydrate in cell wall (%). Mean (s.e.m.) for 4 batches. | |
|---|---|---|
| | Low kinetin (F) | High kinetin (C) |
| Warm water | 15.0 (4.2) | 18.4 (5.3) |
| Pectin | 46.9 (3.1) | 53.0 (8.9) |
| Hemicellulose | | |
| KOH | 20.9 (1.5) | 25.0 (5.0) |
| $NaOH–HBO_3$ | 8.2 (3.4) | 5.3 (1.3) |
| total | 34.0 (2.9) | 29.5 (6.1) |
| Cellulose | 17.6 (4.5) | 19.5 (7.2) |

callus cell walls had a higher glucose content and showed a low or intermediate value for the arabinose:xylose ratio compared to the dark grown tissues. The hydroxyproline content of dark-grown friable callus cell walls was somewhat greater than those of light-grown compact tissues, and considerably greater than that of the dark-grown compact callus. Tobacco pith parenchyma had a hydroxyproline content much lower than that of any callus.

When cell walls from the outer ("new tissue") and inner parts (corresponding to the original compact inoculum) of the callus were examined there was no major difference in their composition; both parts showed the same high or low arabinose:xylose ratio as in the F and C whole callus (data not presented). Culture on low kinetin therefore apparently changes the wall composition of all cells in the same way.

The F and C cell walls were fractionated by exhaustive extraction with hot water, alkali and alkali–borate. The amounts of material extracted are shown in Table 2.

The monosaccharide composition of the hot water extractable pectins is shown in Table 3 and there was no significant difference in the composition of F and C pectins. The pectins had low levels of arabinose and xylose; the majority of the wall content of these sugars remained in the residue, which still displayed the already noted difference in their relative ratios. However there may be differences in the structure of the pectins, or in their arrangement in the cell wall, because extraction of F and C cell walls with warm water solubilised polysaccharides in similar amounts but of differing composition, particularly in the relative amounts of galactose and uronic acids (Tables 2, 4).

A large part of the glucose, and all of the other sugars remaining in the residue, were removed by alkali plus alkali-borate extraction (Table 5). The alkali-extracted

Table 3. Effect of high and low kinetin upon monosaccharide composition (%) of pectins extracted from dark cultured tobacco callus cell walls. Mean (s.e.m.) for 3 batches

| Sugar | Hot water extraction | | | |
|---|---|---|---|---|
| | Extract | | Residue | |
| | Low kinetin (F) | High kinetin (C) | Low kinetin (F) | High kinetin (C) |
| Rha | 1.8 (0.3) | 2.6 (0.2) | 1.4 (0.4) | 0.7 (0.2) |
| Ara | 6.2 (0.6) | 5.4 (1.3) | 19.9 (0.6) | 8.8 (1.3) |
| Xyl | 2.0 (0.3) | 2.6 (0.1) | 5.8 (0.2) | 12.4 (0.1) |
| Man | 0.2 (0.2) | 0.2 (0.2) | 2.0 (1.4) | 3.0 (0.3) |
| Gal | 24.2 (3.2) | 23.2 (4.8) | 11.1 (1.2) | 9.0 (0.9) |
| Glc | 12.2 (2.8) | 11.2 (5.0) | 58.6 (1.6) | 65.2 (1.3) |
| Uronic acids | 53.6 (2.5) | 55.5 (8.4) | 1.3 (0.6) | 1.1 (0.4) |
| Ara/Xyl ratio | 3.2 (0.5) | 2.1 (0.6) | 3.4 (0.1) | 0.7 (0.1) |

Table 4. Effect of high and low kinetin on monosaccharide composition (%) of warm-water soluble polysaccharides from dark cultured tobacco callus cell walls. Mean (s.e.m.) for 3 batches

| Sugar | Low kinetin (F) | High kinetin (C) |
|---|---|---|
| Rha | 2.6 (1.7) | 1.7 (0.4) |
| Ara | 9.8 (2.7) | 5.3 (1.0) |
| Xyl | 3.6 (0.7) | 3.7 (0.6) |
| Man | 0.9 (1.1) | 0.1 (0.1) |
| Gal | 56.3 (4.0) | 39.3 (8.5) |
| Glc | 5.8 (5.8) | 4.6 (4.6) |
| Uronic acids | 19.5 (6.8) | 42.2 (7.7) |
| Gal/Uronic acids ratio | 3.5 (1.3) | 1.0 (0.4) |

hemicelluloses were richer in both arabinose and xylose than those subsequently extracted by alkali-borate, had a somewhat greater arabinose:xylose ratio, and tended to have a lower galactose and uronic acid content (data not presented). Apparently substantial amounts of arabinose were lost by this extraction because the hemicellulose arabinose:xylose ratio was much lower than that in the residue (Table 3). The loss was accountable for in material remaining in the 80% ethanolic supernatant after hemicellulose precipitation, which was *ca* 7% of the total wall carbohydrate and contained 68% (±10%) arabinose and 24% (±5%) glucose.

## DISCUSSION

Because of the high growth rate of tissue under all treatment conditions the callus cell wall composition after 4 weeks growth essentially reflects the pattern of wall deposition during culture. In darkness, the cell walls of friable and compact tobacco callus contain large amounts of pectins and hemicelluloses, and small amounts of cellulose (Table 2). It has been suggested that friability may be associated with a change in the relative amounts of pectins, hemicelluloses and cellulose [12]. Table 2 shows that in tobacco callus there is no basis for such an argument when the data for several preparations is examined.

There is a clear difference between dark grown friable and compact callus in the amounts of cell wall arabinose and xylose (Table 1), sugars mostly found in the hemicelluloses (Tables 2, 3, 5). There is no difference between the gross pectin sugar compositions of the two tissue forms: however, a mild extraction technique extracted polysaccharides that differed in their relative amounts of galactose and uronic acids (Table 4), suggesting that there may be a difference in pectin structure or wall location in friable and compact callus cell walls. Similarly, a weakly-acidic galactan with differing composition between friable and compact strains of *Rosa glauca* callus has recently been identified [16].

The faster growing friable callus tissue has a greater wall hydroxyproline content than the slower growing compact tissue (Table 1), and therefore does not show the inverse correlation between growth rate and extensin synthesis usually observed in intact plant tissues. It is not easy to reconcile this finding with the idea that increased deposition of extensin leads to a cessation of cell expansion by cross-linking elements in the wall [17]. The hydroxyproline content of friable and compact callus cell walls is closely correlated with the content of arabinose (hyp:ara 0.49, 0.42 respectively, Table 1). Hydroxyproline-containing cell wall protein ("extensin"), which has been shown to contain arabinose- and galactose–oligosaccharide side chains, is generally understood to play mainly a structural role in the cell wall [18].

Thus a compact callus cultured in darkness on a low-kinetin instead of a high-kinetin medium develops a friable texture associated with changes in the hemicellulose and extensin compositions (and possibly pectin structure). When cultured in light on either medium the cell wall composition changes in a different way (Table 1) but the tissue remains compact in form. Clearly then a changed cell wall composition is not always linked to a change in callus tissue texture.

Friability of a tissue is characterised by a low degree of intercellular adhesion so that a moderate mechanical shear stress along the intercellular boundary causes the tissue to dissociate. Suspension cultures, in which the cell population is dispersed as single cells or small clusters of cells, may be viewed as an extreme case of friability in which cells separate after cytokinesis, or at some subsequent time [5], due to the continuous agitation of the medium [4]. The structural properties of the plant cell wall are a function of the degree of linkage, by covalent and noncovalent bonds, between the constituent matrix polysaccharides (pectins and hemicelluloses), and between them and the cellulose microfibrils [14]. Small changes in the structure of polysaccharides alter the physiochemical properties of the polysaccharides, and could thereby profoundly change the mechanical properties of the cell wall [14]. We have shown that in solid tobacco callus, induction of friability in darkness is associated in part with a large increase in the amount of arabinose, and a large decrease in the amount of xylose, in cell wall hemicelluloses. We suggest that these changes decrease the strength of intermolecular bonding in the cell wall matrix, which results in tissue friability. The changes in the amount of cell wall extensin which we have observed could also alter the bonding in the matrix.

The alteration in cell wall composition as a compact callus inoculum develops into friable tissue means that a lowering of the level of exogenously applied kinetin leads to an increase in the synthesis of extensin, and also to a change in the relative activity of the arabinosyl and xylosyl glycosyl–transferases.

Table 5. Effect of high and low kinetin upon monosaccharide composition (%) of total (alkali plus alkali-borate) extractable hemicelluloses from dark cultured tobacco callus. Mean (s.e.m.) for 3 batches

| Sugar | Low kinetin (F) | High kinetin (C) |
|---|---|---|
| Rha | 3.1 (1.1) | 3.1 (0.4) |
| Ara | 16.7 (2.5) | 8.2 (1.0) |
| Xyl | 12.1 (1.9) | 27.7 (4.5) |
| Man | 2.9 (2.2) | 3.0 (1.9) |
| Gal | 18.5 (3.5) | 17.7 (3.4) |
| Glc | 38.2 (7.8) | 29.4 (3.6) |
| Uronic acids | 8.4 (2.0) | 11.0 (4.7) |
| Ara/Xyl ratio | 1.4 (0.2) | 0.30 (0.04) |

## EXPERIMENTAL

*Tissue culture.* Tobacco (*Nicotiana tabacum* cv Wisconsin 38) callus was isolated in early 1974 from stem pith segments and maintained in stock culture on a Murashige–Skoog medium as previously described [2, 15]. For experimentation the kinetin concn in the culture medium was either $2.5 \times 10^{-6}$ M (high kinetin) or $5 \times 10^{-8}$ M (low kinetin). Four inocula were planted in each flask.

*Cell wall preparation.* After 4 weeks growth, callus (25–50 g fr. wt) was harvested, removing any adhering agar, and immediately macerated (2 × 2 min) at top speed in an homogeniser in 80% EtOH (taking into account the tissue $H_2O$ content). The macerated tissue was collected by filtration, and then re-suspended in 80% EtOH and passed through a French pressure cell. The macerate was left to settle overnight, and the supernatant, which contained mostly starch granules and small cell wall particles, was carefully removed. The sedimented material was refluxed (3 × 1 hr) in 80% EtOH to remove low MW material, especially sucrose, and then dried by solvent exchange (EtOH–$Me_2CO$). This cell wall preparation (3–5% of original fr. wt) was stored in tightly capped bottles and used for analysis and fractionation. Microscopical examination of the material revealed some residual starch granule contamination; it was assumed that all the starch was solubilised by hot $H_2O$ extraction (see Results). Tobacco pith parenchyma cell walls were obtained from greenhouse grown plants in a similar way.

*Fractionation of cell walls.* Cell wall material (500 mg) was exhaustively extracted in 2% sodium hexametaphosphate, pH 3.7 at 100° (30 ml, 5 × 3 hr) [19]. The extract was collected by centrifugation, filtered, reduced in vol by rotary evaporation at 40°, dialysed against dist $H_2O$ (4 × 2 hr), precipitated by addition of 4 vols EtOH, re-dissolved in $H_2O$ and stored deep frozen (Pectin fraction). A portion of the residue was washed with $H_2O$ and dried for analysis. The depectinized residue was further extracted with 24% KOH–1% $NaBH_4$ (30 ml, 40°, 24 hr) followed by 17.5% NaOH–5% $HBO_3$–0.1% $NaBH_4$ (30 ml, 40°, 3 × 24 hr). The two extracts (either separately, or pooled at this stage) were neutralised with conc HCl, and polysaccharide material recovered as described above (Hemicellulose fractions). The residue (Cellulose fraction) was exhaustively washed with $H_2O$ (until *ca* pH 7) and dried. In addition, a portion of the cell wall material was extracted with $H_2O$ (40°, 24 hr) and polysaccharides recovered as described above (warm $H_2O$ extractable polysaccharides). In all cases, the EtOH supernatant after polysaccharide precipitation was retained for analysis. The whole fractionation scheme was carried out twice.

*Analysis of cell wall and fractions.* Insoluble material (about 2 mg carbohydrate) was hydrolysed by dissolving in 0.05 ml 72% w/w $H_2SO_4$ (3 hr at room temp), and then after addition of 1.4 ml $H_2O$ (dilution to 4% w/v) at 120° for 1 hr in a Teflon-capped tube. Fractions already dissolved in $H_2O$ were hydrolysed directly after addition of the appropriate amount of $H_2SO_4$. The $Ba_2CO_3$ neutralised hydrolysate was assayed for neutral and acidic sugars, and analysed by GLC of the alditol acetates as previously described [20]. Individual analyses, initially replicated, were found to be reproducible, and were therefore in later work only performed once on each sample. The uronic acid content of the cell wall was not sufficient to account for that found in subsequent fractions (Tables 1, 2, 3, 5). This may be due to loss of uronic acids as $Ba^{2+}$ salts during neutralization [21]. Hydroxyproline was assayed [22] in hydrolysates (6N HCl, 110°, 24 hr under $N_2$), which had been clarified by resin filtration [23].

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