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Structural, Physiological, and Biochemical Gradients in Tobacco Pith Tissue

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Abstract. **Explants of tobacco pith taken at various distances from the apex of a mature stem show a sharp gradient in growth potential *in vitro;* growth is highest in the extreme apical and basal explants, and is minimal in explants removed *ca.* 75 cm from the apex. Calluses produced by the vigorously growing basal explants are harder and more compact than those produced from more apical explants. The gradient in growth potential is directly correlated with gradients in RNA, protein of cell sap and soluble N per unit fresh weight, but is inversely correlated with peroxidase activity. Cell size increases from apex to base of plants.**

The peroxidase activity of pith explants is electrophoretically resolvable into 2 isoperoxidases, moving anodically at pH 9.0. During *in vitro* culture, this activity rises, due to the formation of several new isozymes moving toward the cathode. The appearance of these isozymes occurs most rapidly in apical and extreme basal explants.

Tobacco pith tissue has been traditionally regarded as uniform in structure and behavior (6, 17, 18). Thus, when explants are removed for tissue culture, no particular precautions are stipulated regarding the region in the plant from which the tissue is taken. The variability noted in the results of some bioassays based on pith has usually been attributed to variations in technical procedures and occasionally to polyploidy and asynchronous cultures (2, 18). However, such explanations cannot be universally applied, since variability has been reported not only for the growth of tissue explants *in vitro* but also for chemical analyses of total DNA and RNA in freshly excised pith (5, 17) made with the perchlorate method (13). In previous work (9) it was shown that such variability could be significantly reduced if the pith were consistently taken from the same site on the plant. This suggested that metabolic gradients might account for some of the chemical and biological variability. A recent report by Kerstetter and Keitt on indoleacetic acid decarboxylation (7) supported this notion. To explore this matter further, we undertook a study of some chemical properties and growth potential of pith tissue from various sites along the mature tobacco stem.

Materials and Methods

Plant Material. Greenhouse grown mature tobacco plants (Cv. Wisconsin-38) with about 35 leaves were excised just before flowering. The plants were defoliated and divided into stem segments 12 cm long starting 20 cm below the growing point and ending 15 cm above soil level. The pith of the upper 4 cm of each segment was extracted by means of a sterile No. 2 cork-borer (diam. 5 mm). The resulting cores were used either for analysis or, after division into 3 mm long pieces by a standard cutting tool, for aseptic culture (6). These pieces were very uniform along the plant, in no instance varying by more than 5 %.

Anatomical Measurement. Free hand sections of the pith were made and cell size determined with a Leitz-Panphot microscope equipped with phase-contrast optics. The cells were photographed and measured at 80X magnification.

Tissue Culture. Twenty ml of 2I-MW medium (18) containing 0.2 mg kinetin (Kin) and 2.0 mg indole-3-acetic acid (IAA) per liter were placed in 50 ml flasks; sometimes 10 ml medium in 25 mm diameter culture tubes was substituted. The mineral solution containing organic additives was adjusted to pH 6.3 before the addition of agar. Pith cylinders (2 per flask and 1 per tube) with an initial fresh weight of 55 ± 2 mg were implanted on the solidified medium and were grown at 25° under weak illumination (*ca.* 100 ft-c) for 30 days. During that period samples were removed for analysis. Each determination was based on 4 or 5 repetitions, each involving 2 tissues.

Chemical Analysis. Soluble nitrogen was extracted for 18 hr at 4° with a sufficient volume of 80 % ethanol as to allow a final concentration of 70 %. After evaporation of the alcohol, nitrogen was determined in a semimicro Kjeldahl apparatus. Insoluble nitrogen in the residue was determined in

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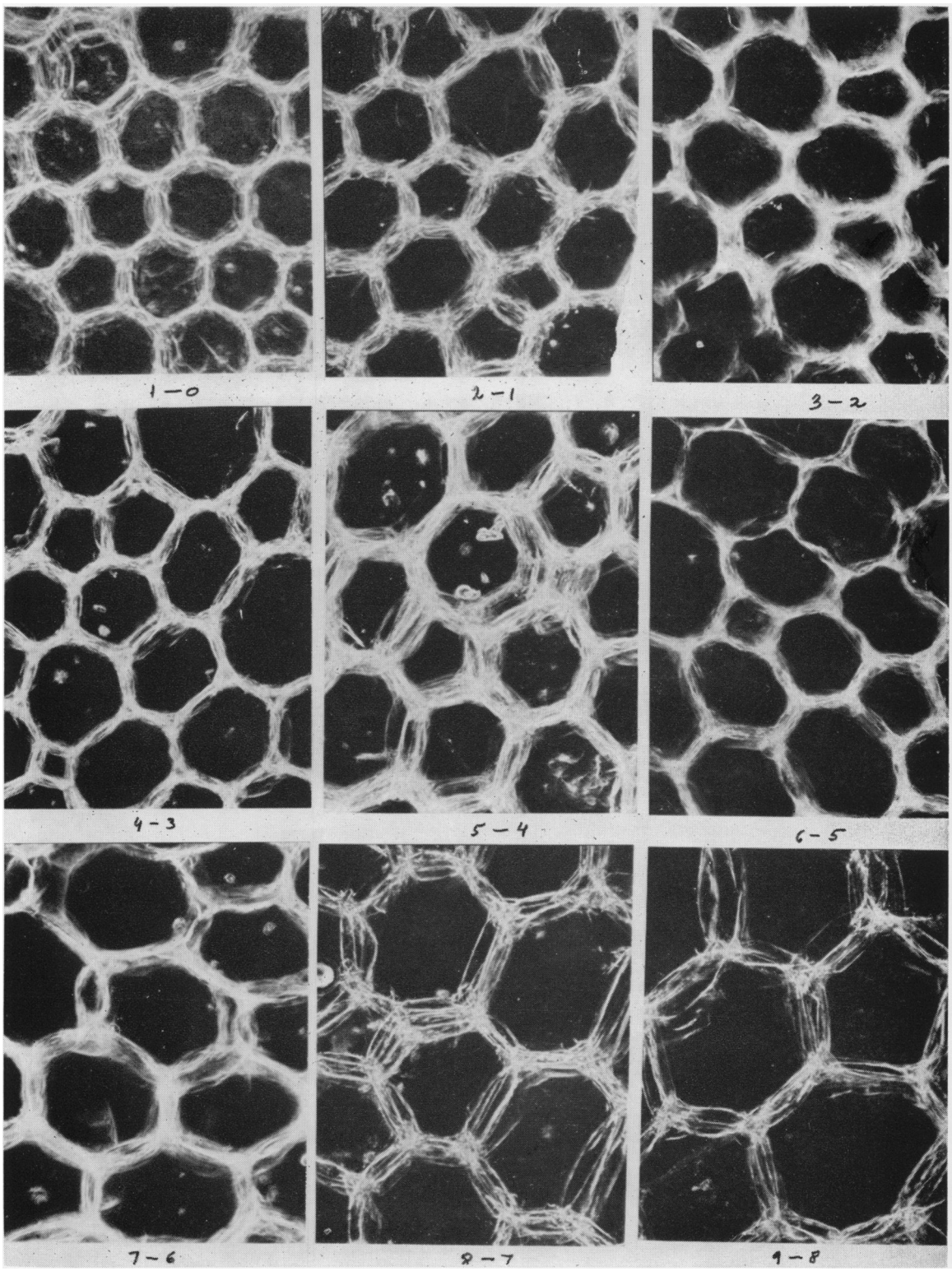

FIG. 1. Transverse sections of tobacco pith at various distances from the apex. Segment 0 is taken 5 cm below apex; thereafter all sections were cut at intervals of 12 cm. Phase contrast microscopy; magnification 80×.

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the same way. A direct determination of protein in the cell sap could be made by squashing the tissue onto a porcelain plate, absorbing 6 μl of the sap onto 3 MM filter paper and analyzing by the Lowry method (11). RNA and DNA were determined by a modification (9) of the Ogur-Rosen perchlorate method (13).

Enzymatic Determination. Total peroxidase activity was determined in 6 μl sap using an equimolar guaiacol-H_2O_2 system at pH 5.8 (12). Readings were taken with a Bausch and Lomb Spectronic 20 colorimeter at 470 nm at intervals of 15 sec (15). Results are expressed as △OD units per 15 sec. The isozymic composition of the peroxidase was determined by means of electrophoresis on starch gel at pH 9.0 with a borate buffer mobile phase at pH 8.3 (12). A potential of 10 v/cm was applied for 75 min. The zymograms were developed with the guaiacol-H_2O_2 reagent after transverse slicing of the gel and were recorded by Polaroid photography.

Results

Cells of pith increase in size from the apex to the base of the plant (table I). This increase in size is gradual from just below the apex down to the sixth segment. Between this and the next segment relatively larger increases in cell size were observed. The pith cells in all segments were rather uniform and isodiametric, so that the size differences were easily demonstrable (fig 1). Despite the marked difference in cell size along the plant the percentage dry weight was remarkably constant, ranging from 5.63 to 6.90 %. There seemed to be a slight tendency toward an increase towards the base of the plant.

The concentration of certain classes of metabolites in the pith changes considerably as one progresses down the plant. The soluble N fraction decreased markedly from the apex to the lower third of the plant; thereafter the concentration increased slightly again (fig 2). The insoluble N fraction was generally low and rather constant except for slightly higher levels near the apex. The soluble protein of the expressed sap, determined in a different set of experiments by the Lowry method, revealed a gradient of protein concentration from apex to segment 4, then a plateau, followed by a slight rise at the very base (fig 3).

Table I. *Cell Size of Tobacco Pith Tissue at Various Locations Along the Stem*

Cell size index = $\frac{\text{cell length} \times \text{cell width}}{100}$. Each value represents the mean of 100 measurements.

Segment No.	Distance from apex	Cell width	Cell length	Index of cell size
	cm	*μ*	*μ*	
0	5	40.5	40.2	16
1	20	42.4	41.3	18
2	32	44.4	44.8	20
3	44	50 4	50.6	25
4	56	50.2	51.8	26
5	68	52.6	53.6	28
6	80	55.0	56.7	31
7	82	73.9	77.8	57
8	94	89.4	88.8	79

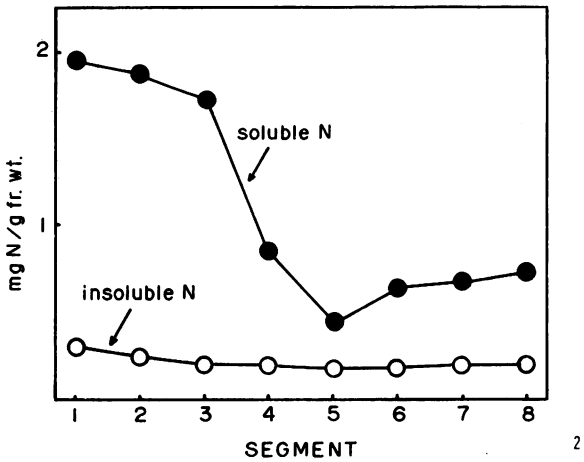

FIG. 2. Content of insoluble and soluble nitrogen in tobacco pith sections taken at various distances from the apex. Segment numbers as in figure 1. Mean data from 3 experiments of 3 plants each. Maximum variability within segments was 3.1 %.

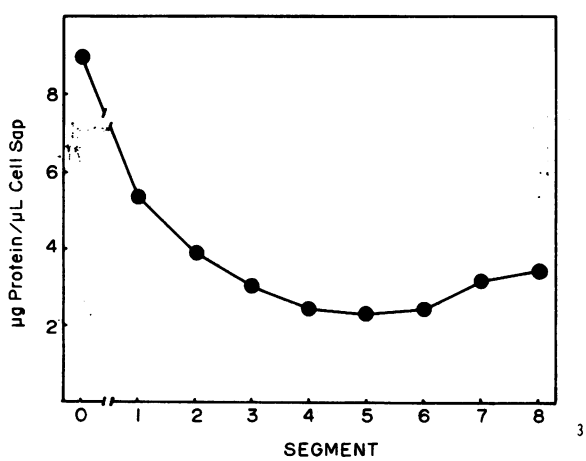

FIG. 3. Protein content of tobacco pith cell sap by method of Lowry *et al.* Segment numbers as in figure 1.

RNA distribution resembled that for soluble N (fig 4). A gradual reduction in RNA content along the plant was apparent down to segment 6, with a slight increase thereafter. DNA showed a similar pattern of basipetal reduction, but nearly no increase at the bottom of the plant. The reduction in DNA could be expected in view of the increase in cell size down the plant; since each cell has 1 nucleus, and since the bulk of cellular DNA is in the nucleus,

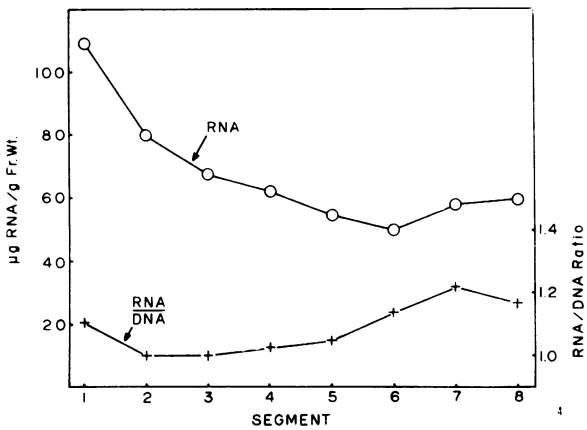

FIG. 4. RNA concentration and RNA:DNA ratio of tobacco pith tissue. Experiment as in figure 2. Maximum variability within segments was 2.6 %.

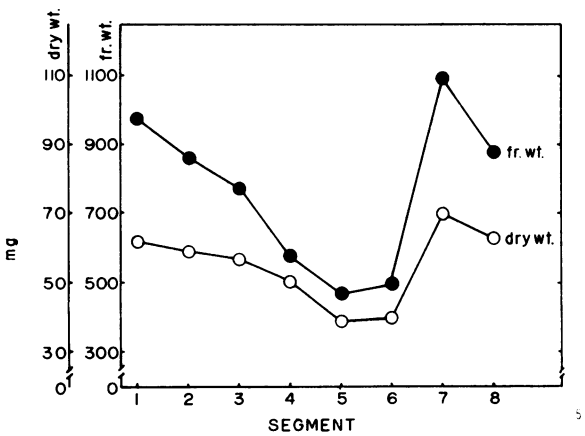

FIG. 5. Weight of tobacco pith callus formed from various sections after 30 days of culture on 2I-MW medium with 2.0 ml/l IAA, 0.2 mg/l Kin. Segment numbers as in table II.

the lower the concentration of nuclei, the lower will be the DNA content. This situation is well described in the RNA:DNA ratio, which increases somewhat toward the base of the plant.

The changes in the RNA, soluble N and sap protein of the pith along the plant were generally correlated with the growth potential of an explant of that tissue *in vitro*. The fresh and dry weights of the callus tissue produced after 4 weeks of culture of pith tissue originating from the different segments is shown in figure 5.

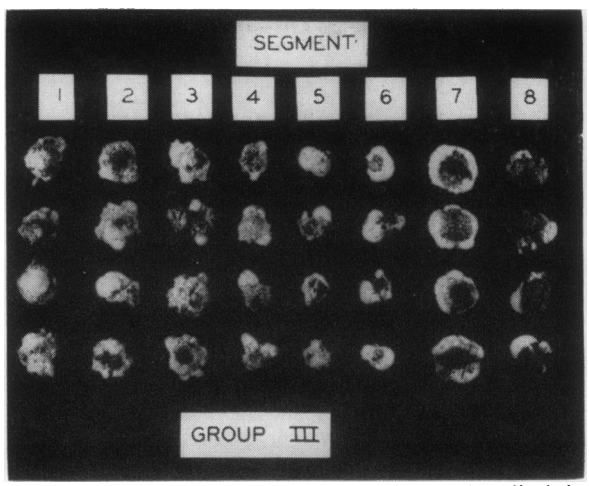

FIG. 6. The appearance of the calluses described in figure 5.

The nature of the callus produced from the various explants was different. While the tissues produced from segments 1 to 6 was soft and loose, that of the last 2 with relatively vigorous growth was hard and compact (fig 6). Compact tissue with small cells was also detectable in localized regions of the tissues originating from segment 6.

Table II. *The Growth Potential of Pith Tissue Excised From Various Locations Along the Stem in Plants at Various Stages of Maturity*

Each value is the mean of 24 tissue weights. There were 3 experiments, each involving 4 flasks containing 2 explants each, cultured for 30 days.

Segment	Cm from apex[1]	Stage I Young Fr wt	Stage I Young Dry wt	Stage II Growing Fr wt	Stage II Growing Dry wt	Stage III Mature Fr wt	Stage III Mature Dry wt	Stage IV Flowering[2] Fr wt	Stage IV Flowering[2] Dry wt
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
1	20	1509	83	1087	63	978	62	1306	73
2	32	547	46	783	56	863	59	1337	67
3	44	669	48	677	53	785	57	835	57
4	56	794	53	504	43	579	50	866	56
5	68			837	54	473	39	775	55
6	80			820	54	501	40	528	45
7	92					1098	70	1279	82
8	104					878	63	902	64

[1] Last segment about 15 cm above the soil line.
[2] Length does not include the inflorescence.

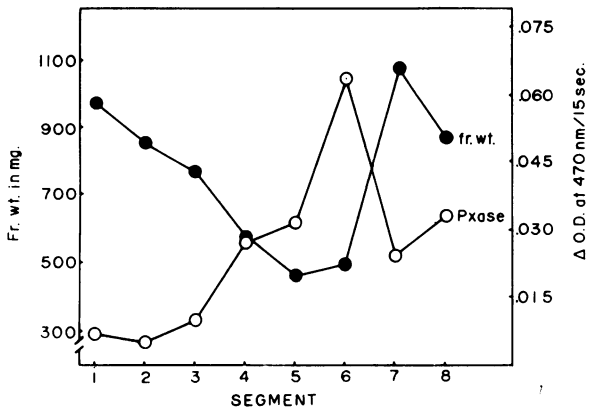

FIG. 7. A comparison of growth potential and total peroxidase activity of tobacco pith tissue. Segment numbers as in figure 1. Fresh weight after 30 days in culture; peroxidase activity in original pith (mean from duplicate samples of 3 plants).

The gradient of growth potential of the pith along the tobacco stem can be noted in young plants as well as in old, fully flowering plants (table II). The increased growth potential of the pith in the lower part of the plant, although present in the young plants, increases markedly with time.

Since IAA must be added to the medium to induce growth of the pith *in vitro,* it was deemed advisable to observe possible gradients in IAA-destroying capacity. A recent report (7) describes an increasing decarboxylation of ^{14}COOH IAA from progressively more basal pith explants. Since IAA destruction is mediated by peroxidase, we set about to measure total peroxidase activity in the various segments. A comparison between the growth potential of the tissue expressed as fresh weight after 4 weeks in culture and the peroxidase activity of the original explant is shown in figure 7. A striking inverse correlation between these 2 quantities is evident. Although the level of peroxidase activity varied somewhat from plant to plant, the trend along any one stem was always the same.

When tissue is cultured *in vitro,* its peroxidase activity may be significantly altered, generally toward increased activity. If one plots the reciprocal of culture time (in hr) needed for a given tissue to develop a peroxidase activity with a final OD/min value of 0.600, the curve in figure 8 is obtained. Now, there is clearly a positive correlation between growth potential and the rate of development of peroxidase activity. The detailed kinetics of the increase of peroxidase activity in cultures derived from different locations is shown in figure 9. Clearly, both the youngest and oldest parts of the pith are most active in synthesizing peroxidase, at least up to 100 hr in culture.

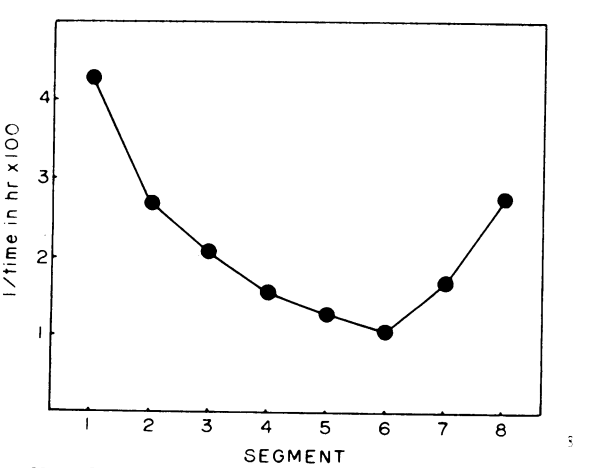

FIG. 8. Peroxidase activity in pith calluses from various segments. (numbers as in figure 1). Activity expressed as the reciprocal of hr in culture required to attain a constant (Final OD = 0.600) peroxidase activity (experiment as in figure 7).

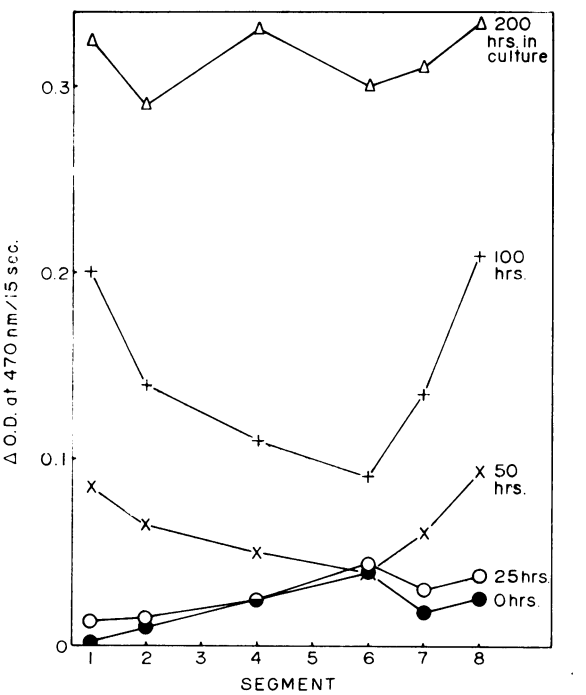

FIG. 9. The effect of time in culture on peroxidase activity in various tobacco pith segments. (Numbers as in figure 1). (medium 2I-MW, and 2 mg/l IAA and 0.2 mg/l Kin).

The nature of the increase in peroxidase activity was studied by means of starch gel electrophoresis. The isozymal composition and relative activity of various segments harvested after 0, 50, 100, and 200 hr in culture, after reaction with guaiacol-H_2O_2, is shown in figure 10.

It is clear that while the peroxidase activity of the original pith all along the plant is dependent on 2 anodic isozymes, the additional activity is due to the appearance of several new cathodic isozymes. The new isoperoxidases appeared earliest in the pith

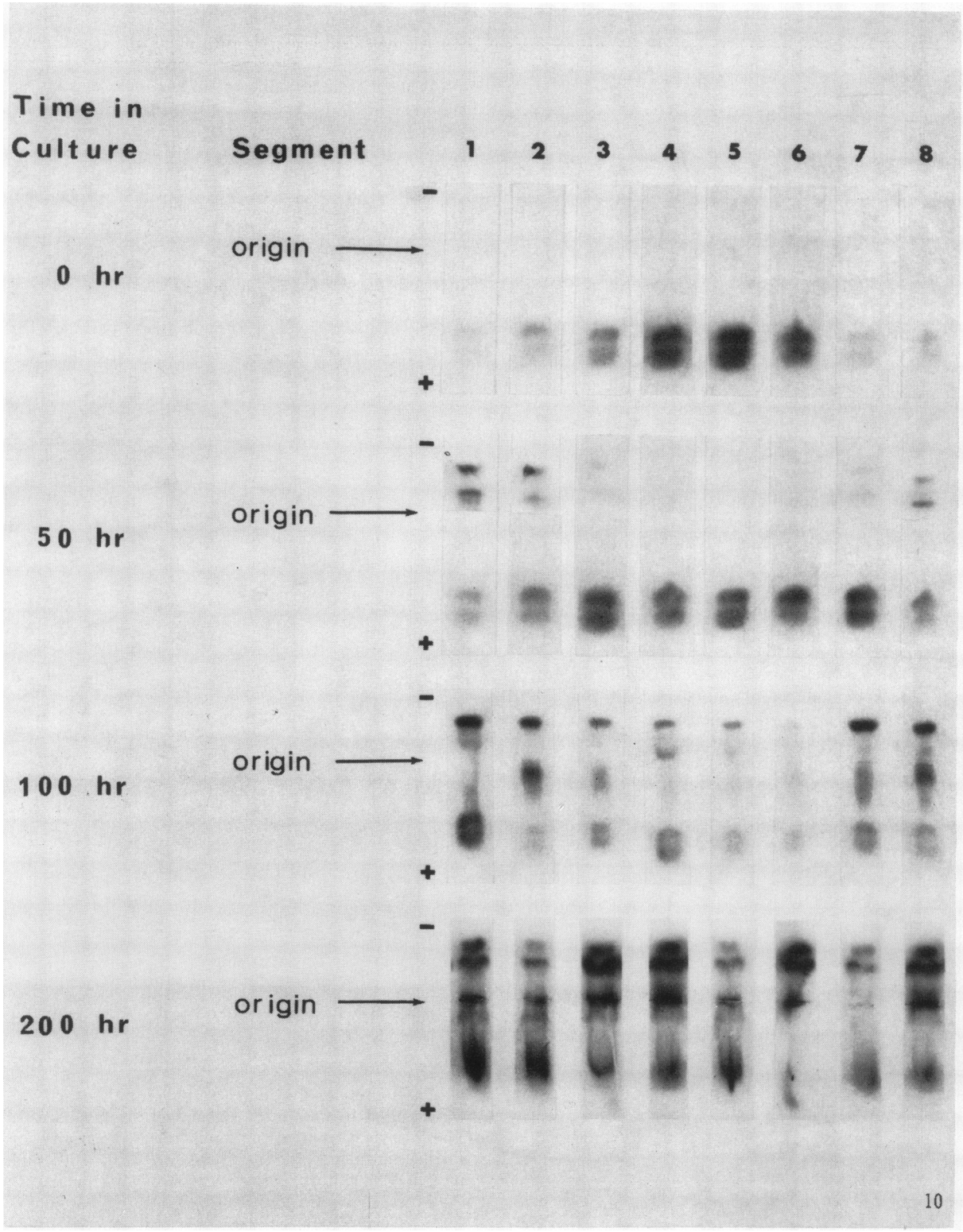

FIG. 10. The effect of time in culture on peroxidase isozyme composition and content in tobacco pith from various segments. (Numbers as in figure 1).

tissue from the youngest and oldest parts of the stem, and gradually became dominant over the original ones, which did not change appreciably in the first 50 hr in culture. After 200 hr, when the development of an additional cathodic isoperoxidase was apparent and the original anodic isoperoxidases had largely disappeared, no significant difference in isozyme composition or intensity could be found between the various tissues. The total activity at this stage of development was very high, resulting in some diffusion of the dark colored oxidation product of guaiacol. This caused a smearing of the patterns by the time of the photographic recording.

Discussion

The increase in pith cell size along the tobacco stem from apex to base is, by itself, convincing evidence of the existence of gradients in pith tissue. The chemical and physiological data presented here serve only to further document this conclusion. This demonstrated inhomogeneity, especially in growth response, suggests that when pith explants are used to assay for cytokinins, either similar tissue must be excised from comparable stems or explants must be suitably randomized prior to inoculation into different media. This caveat is probably well known to workers in the field, and instinctively obeyed by many researchers. Yet, the magnitude of the observed differences indicates that the inhomogeneity ought to be spelled out for all to appreciate.

The basipetal gradient in peroxidase activity in the structurally homogeneous pith cells parallels that originally reported for IAA oxidase in etiolated pea epicotyls (3) and since then in many other systems, including *Lens* roots (14) and *Pelargonium* pith (10). Recently, a similar gradient in the ability of tobacco pith to decarboxylate ^{14}C-carboxyl labeled IAA has been reported (7).

In both tobacco (4) and *Pelargonium* pith (10), the youngest pith cells are devoid of peroxidase, and develop peroxidase activity within 24 hr after excision and implantation on aseptic media, to an extent determined largely by the medium. These facts speak against the view (1, 22) that peroxidase is a "constitutive" rather than an "adaptive" enzyme. In both systems, IAA has been shown first to repress and later to induce specific isoperoxidases; in tobacco pith, the repressed and induced isozymes are different, while in *Pelargonium* pith, they are the same. The repression by IAA of the *de novo* formation of a specific isoperoxidase has also been reported in excised green pea stem sections (12). The demonstration (16) that the heme group of peroxidase is not essential for IAA oxidase activity, together with the presence in the tissue of other heme enzymes such as the cytochromes makes it likely that the control of peroxidase synthesis, both by excision and auxin, is attributable to control of synthesis of the apoprotein moiety of the enzyme.

It is intriguing to note that those tissues initially lowest in peroxidase activity are precisely those which form peroxidase most rapidly after excision and culture (fig 9; compare time 0 with 100 hr). This clearly indicates that the initial low level of the enzyme in tissues with high growth potential is the result of a repression type control mechanism; clearly these cells *can* make the enzyme, but are prevented from doing so by some metabolic control. It also demonstrates the different nature of the systems regulating the formation of anodic and cathodic isoperoxidases. In this context, it should be noted that in tobacco pith (4), *Pelargonium* pith (10) and pea stem tissue (12), the isoperoxidases induced or repressed in response to excision or auxin treatment are always cathodic at pH 9.0. Those isoperoxidases which develop normally in tobacco pith are anodic (4) and in pea stem both anodic and cathodic (12). *Pelargonium* pith does not normally form peroxidase at all *in situ* (10).

While the physiological meaning of the gradients and rapid changes in peroxidase activity is still unclear, the correlation with growth patterns continues to suggest an important connection between the two.

Acknowledgment

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