Cell Enlargement and Cell Division in Excised Tobacco Pith Tissue

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Introduction

In recent years tissue culture techniques have been employed to advantage in studies of hormone or growth factor requirements in plants as well as in studies of certain aspects of the functions and interrelationships of such factors which cannot be readily determined in intact plants. However, the experience with excised organs and fragments consisting of complex tissues and also with callus cultures as experimental material often has been that various cell types or regions of the tissue respond differently to different substances and to different concentration of a single substance. Difficulties have arisen therefore in interpreting the effects of the applied substances, particularly in relating the effects of treatments to specific changes in growth and tissue composition.

It has been found that when tobacco stem segments, consisting of cortical, vascular, and pith tissues, are cultured on White's medium with various growth substances added, the pith cells undergo some proliferation as well as enlargement on media containing indoleacetic acid (IAA), naphthaleneacetic acid, or other substances with auxin activity (Skoog and Tsui, 8).

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CELL ENLARGEMENT AND CELL DIVISION

In contrast with this behavior, pith parenchyma sections freed of vascular elements fail to grow appreciably on the same control medium in the presence of suitable concentrations of IAA the pith cells respond with an enormous cell enlargement entirely unaccompanied by cell division.

In view of this differential response of the pith cells in the presence or absence of vascular elements and the unusual magnitude of the cell enlargement in response to IAA, a detailed study of the growth of tobacco tissue in vitro has been carried out, the results of which are reported in this paper.

Materials and Methods

Young rapidly growing internodes of 2—3 ft. tall tobacco plants (Nicotiana tabacum, var. Wls. no. 38), grown in seven inch pots in the greenhouse, were used as stock material for the preparation of stem segment and pith section cultures. Culture media and methods of preparing sterile cultures were essentially those described by Skoog, Tsui (8). The basic nutrient medium contained the following subs (mg/l): Ca(NO₃)₂ 100, KNO₃ 80, MgSO₄ 35, KCl 65, KI 0.5, MnSO₄ 25, Fe₃(SO₄)₂ 3.5, ZnSO₄ 1.5, H₃BO₃ 1.6, KH₂PO₄ 37.5; glycine 2, thiamine hydrochloride 0.1, nicotinic acid 0.5, and pyridoxine hydrochloride well as 20 g/l sucrose and 10 g/l agar. The pH of the solution was adjusted initially to about 5.0 with 0.1 N HCl or NaOH. The vitamins, purine, and purified glycine and indole-3-acetic acid (Eastman Kodak Co.) were recrystallized before use.

Pith tissue sections free of vascular tissue and of uniform dimension were prepared as indicated in Figure 1. Stem segments were cut with an edge cutting tool into one centimeter long cylinders. The cylinders were longitudinally bisected with a second cutting tool, into blocks which were then placed in a sealed Petri dish of pith parenchyma sections of dimension 10×1×1 mm. These pith sections were cultured on the surface of 50 ml nutrient agar medium in 125 ml Erlenmeyer flasks. Two to four sets were placed in each flask in individual experiments, and the cultures were grown in an environment of the building where conditions were maintained at approximately 25° C. and continuous, weak diffuse illumination was provided. Growth was measured in terms of changes in weight and dry weight of pooled samples of six to twenty sections each treatment. The fresh weight determinations were carried out by blotting off the surfaces and weighing the samples in glass stop.
weighing bottles. The dry weights were determined after keeping the sections for 48 hours in a forced air oven at 65°C. In general, measurements of fresh and dry weights were reproducible within 10 per cent with samples of ten sections each, but only results based on much greater differences than this between treatments will be considered in this report.

Results

Cell Enlargement

Results of an experiment with serial concentrations, from 0 to 20 mg/l., of IAA added to the medium are presented in Figure 2. During the first two to three days in culture, all sections generally remain visibly unchanged, but some growth occurs as indicated by increases in weights of the sections and occasionally in size of individual surface cells. The sections with 0.02—2.0 mg/l. IAA treatments then begin rapid expansion, the exact starting time, duration, and final extent depending on the concentration of IAA supplied, and apparently also on the growth vigor or other seasonal variations in the plant material. The optimal concentration varies slightly from one experiment to the next but generally is 2—3 mg/l. Higher concentrations of IAA, from 4 to 20 mg/l. result in progressively less increase and finally complete inhibition of growth. The progress of cell expansion for optimal treatments with IAA is shown in Figure 3. The enlargement of the individual cells involves marked elongation followed by further increase in volume to produce practically isodiametric cells. The cells in the upper surface layers expand first, and as they round up into spheres are peeled away from the tissue underneath. The process continues for 10—14 days, i.e., until all or nearly all the tissue has been transformed into a loose mass of practically separate giant cells. Many cells undergo about a tenfold increase in but the optimal average increase for all cells is ca. 6 times.

It may be seen from comparison of Figures 2 a and b, that there is a three to fourfold increase in dry weight, the increase in fresh (water uptake) is proportionally still greater. These general feature IAA effect have been reproduced consistently in numerous experiments a two year period.

A complete mineral medium was used above. However, excim Newcomb (unpublished) have shown that the rapid cell enlargement place also on media with all nutrients removed except the sugar a

Figure 3. Photomicrographs of pith fragments showing stages in IAA induced cell enlargement (A) after 6 days; (B) after 10 days on a culture medium with 2.0 mg/l. IAA
Table 1. Effect of IAA (2.0 mg/l) on dry weight, total sugar, and reducing sugar content of tobacco pith sections. Expl. started Oct. 7, 1959 and harvested Oct. 30, 1959.

<table>
<thead>
<tr>
<th>Treatment and age of culture</th>
<th>Average dry wt. mg/segment</th>
<th>% dry weight of fresh wt.</th>
<th>% total sugars of dry wt.</th>
<th>% reducing sugars of fresh wt.</th>
<th>% reducing sugars of dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.71</td>
<td>4.95</td>
<td>69.9</td>
<td>1.55</td>
<td>28.7</td>
</tr>
<tr>
<td>(0 days)</td>
<td>3.82</td>
<td>3.93</td>
<td>67.8</td>
<td>1.39</td>
<td>24.9</td>
</tr>
<tr>
<td>Control</td>
<td>5.84</td>
<td>5.56</td>
<td>47.6</td>
<td>1.78</td>
<td>32.0</td>
</tr>
<tr>
<td>(23 days)</td>
<td>5.28</td>
<td>4.97</td>
<td>49.3</td>
<td>1.72</td>
<td>31.7</td>
</tr>
<tr>
<td>IAA</td>
<td>10.7</td>
<td>4.25</td>
<td>54.2</td>
<td>1.40</td>
<td>31.4</td>
</tr>
<tr>
<td>(25 days)</td>
<td>17.4</td>
<td>4.49</td>
<td>62.8</td>
<td>1.49</td>
<td>33.0</td>
</tr>
</tbody>
</table>

1 Data by Robinson (1956).
2 Values obtained from a composite sample of pith sections.

In fact, although the sugar is often required, occasionally only the IAA is essential. The expansion process, therefore, cannot be osmotically controlled through salt uptake. Nor is the effect of sugar purely osmotic, for analyses carried out by Robinson (7) shows that in spite of increases in total dry weight and sugar content, there is no increase in percentage sugar content during the early stages but a gradual drop from an initial 69 to 48 per cent of the dry weight in the controls and to ca. 59 per cent in the IAA treated sections at the end of the growth period (Table 1). Possibly the water is taken up by an active non-osmotic mechanism, as has been proposed for potato tuber tissue (Hackett and Thimmann, 4; Hackett, 3), but as increases in cell wall materials, particularly pectic substances occur prior to and concomitant with cell expansion of the pith tissue (Wilson and Skoog, 9) and pending conclusive evidence to the contrary, the water uptake may be a secondary phenomenon of growth accompanying the increase in cell surface and regulated by existing osmotic properties of the tissue.

Extensive anatomical examination of sectioned material has revealed no evidence of cell division in control or rapidly enlarging, IAA treated pith sections. However, Sterling and Mallott (unpublished) found marked enlargement of the nuclei in expanding cells treated with IAA. A detailed cytological study of the pith sections with and without IAA treatments for optimal cell enlargement is reported by Naylor et al. (6).

**Proliferation and Organ Differentiation**

In attempts to obtain cell division in pith sections free from vascular tissues, pure substances which are known to stimulate plant growth such as adenine and various vitamins and amino acids, were tested in several concentrations, negative results. Similarly, traumatic acid, which promotes cell division, was found inactive when applied to tobacco pith sections in concentrations of 1, 10, and 100 μg/l.

Next attempts were made to obtain cell division in pure pith sections by placing them in physical contact with vascular tissue of stem segments. After placing the cut pith sections in contact with their corresponding vascular tissue failed to induce cell division in the pith, because the pith tissue tended to curve and thus to lose contact with the pith. However, the pith tissues were planted on the agar surface and short vascular segments were placed on their upper surfaces, cell division was observed at the basal end of a few pith sections in each experiment (Fig. 4), and with the pith sections on the agar surface, the water and nutrient flow to the pith was passing through the segments, induction of cell division was obtained in 95—100 per cent of pith sections in all experiments (4 b), whereas control sections placed on the medium failed to divide (4 a).

When pith sections were planted in the center of the agar surface, they were surrounded by, but not in contact with, a large number of cells. Cell divisions were also observed at the basal end of pith sections. The minimum effective ratio of stem segments to pith sections was ca. 8:1 in these experiments. These results demonstrate that the material from the vascular tissue is diffusible through agar, and show that the induction of cell division in pith sections on top of vascular tissues was not due merely to the arrangement whereby the ends of the sections were projecting, free from contact with the medium or other factors. This fact was also demonstrated by placing pith sections on one on
another or by mounting them on porous gypsum blocks which were placed on the agar surface. In the latter case, cell division did not occur. In the former case, occasionally one of the two sections, generally the one on top, exhibited some cell division at the basipetal surface, but invariably much less so than pith sections mounted on vascular tissue in the same experiment. As might be expected, the pith sections themselves seem to contain small amounts of the active material but generally less than required under the conditions of these experiments to produce visible callus growth.

As a preliminary step toward isolating the active material, Seitzfiltered aqueous extracts were prepared from stem internodes, with epidermis, cortex, and most of the pith removed. About 0.1 ml of extract was applied to each pith section by puncturing its surface with a hypodermic syringe. After a nine day growth period, callus formation was observed at the basipetal ends of the pith sections so treated. However, this effect of tissue extracts was not obtained consistently in subsequent experiments.

Turning to other sources, it was found that when a coconut-meat concentrate (Mauney et al., 5) corresponding to 15 per cent by weight coconut meat was included in the culture medium, very active cell division occurred in the pith sections particularly at their basipetal ends but also over their entire upper surfaces (Figure 5 a). In fact, on this medium the tissue can be maintained indefinitely in subcultures, and is capable of differentiation to produce vascular strands and organs such as buds. A malt extract (10 per cent malt per 100 ml medium (Figure 5 b)) could be substituted for the coconut extract (cf. Blakeslee and Satina, 1).

Discussion

The above results show that tobacco pith tissue may be cultured under conditions which permit either enormous enlargement of the cells in response to IAA without the occurrence of cell division or rapid proliferation of the cells in response to an additional growth factor. However, in nuclear material are also associated with cell enlargement.

The nature of the material which induces division of the pith cells is not identical with a coconut factor which stimuluses the growth of carrot tissues, because more highly purified fractions of this factor, which are active on carrot tissue, were inactive on tobacco pith. Nor is it traumatic acid, the so-called wound hormone, which stimulates cell division in bean pod parenchyma. It should be noted that the substance evidently exerts its effect primarily at the point of application whereas active material for pith cell division is transported through tissue and exerts its effect first at the basipetal end of the sections. The data, however, need not necessarily indicate that the substance is per se transported, because the distribution of growth may be determined by the distribution of auxin. Low concentrations of IAA enhance the effect of active material and were included in the medium in most experiments. Results of attempts to characterize the active material by solvent fractionation and chromatographic techniques are as yet inconclusive.

The marked and differential growth responses of the pith tissue culture as described above make it an excellent material for studies of changes in respiration, enzyme activities, and tissue composition associated with enlargement and/or proliferation of plant cells. The results of studies are reported elsewhere.

Summary

Tobacco pith tissue (Nicotiana tabacum, Variety Wisconsin no. 38) to grow on a modified White's nutrient agar medium, but undergoes nuclear enlargement resulting in a tenfold increase in size of cells when optimal concentrations (ca. 2-3 mg/l) of indoleacetic acid (IAA) are added to the medium. Higher and lower concentrations elicit less response, and 20 mg/l IAA are often completely inhibitory.

No cell divisions have been found in pure pith tissue either in the absence of added IAA or in connection with the cell enlargement in response to optimal concentrations of IAA even though there is an increase in nuclear material in the expanding cells. Cell divisions do occur, however, in tissues with attached vascular strands, or in severed pith tissue placed in contact with vascular tissues.

The material active in inducing cell division is obtained in water extracts from vascular tissue and various plant products such as coconut milk. It is diffusible through agar, but is not replaceable by traumatic
In its presence callus formation and organ differentiation as well as continued \textit{in vitro} growth of the pith tissue are obtained.

The marked differential growth responses of pith tissue to IAA and various plant extracts make it an excellent material for studies of metabolic and compositional changes associated specifically with the process of enlargement or proliferation of plant cells.

References