PROGRESSIVE CHANGE IN ORGAN-FORMING CAPACITY
OF TOBACCO CALLUS DURING SINGLE
SUBCULTURE PERIOD

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The progressive loss of the capacity to form organized structures from plant callus tissues grown in vitro has been reported in several cases. Torrey (1967) tested a number of strains of callus tissue derived from root tips of the garden pea for their capacity to initiate organ formation over a period of 8 years. He found that during this prolonged period of subculturing there was a progressive loss of organ-forming capacity in all strains. Murashige and Nakano (1965) also found that prolonged culture had led to loss of capacity to form organs in tobacco. Syono (1965) observed that the gradual loss of organ-forming capacity during repeated subculturing occurred in many strains of carrot root callus and no organ was formed in calli subcultured over 38 months under any conditions attempted. The tendency towards loss of organ-forming ability has also been demonstrated in single isolated cells. Vasil and Hildebrandt (1965a) reported that single isolated cells of hybrid tobacco pith tissue cultured for 8 years divided and grew to form small colonies of over 50 cells in microcultures, but failed to differentiate roots and shoots. However, single isolated cells of fresh callus of hybrid tobacco pith tissue differentiated and developed into normal plants (Vasil and Hildebrandt 1965b).

On the other hand, several workers have obtained newly formed organs from calli cultured for a long time (Fox 1963; Melchers 1965; Vasil et al. 1966a, 1966b; Matsu shima et al. 1969). Steward (1967) reported that cells which had lost their ability to organize on a basal medium containing coconut milk regained it and formed roots and some shoots on the same medium after an intervening period of culture on the basal medium plus naphthalene acetic acid.

This paper shows that as a single callus grows and enters a prolonged period of stationary growth, there is a progressive increase in organ-forming capacity by the cells. The increase is probably paralleled by a tendency towards euploidy among the stationary growth phase cells.

MATERIALS AND METHODS

Strain XD-6 of tobacco callus was obtained from Dr. Hayashi of The College of General Education, University of Tokyo in 1968. This strain was originally isolated by
Filner (1965) in 1961, from the stem of *Nicotiana tabacum* var. Xanthi. The callus was subcultured monthly on the agar medium D, which was like that of Murashige and Skoog (1962) except that indole-3-acetic acid (IAA), kinetin and edamine were replaced with 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were maintained at 25°C in the dark.

The agar medium MS was employed for the organ-forming test and was like medium D except that the 2,4-D was replaced with 2 ppm IAA plus 1 ppm kinetin. The pH of both media was adjusted to 5.6-5.8 with 0.5 M NaOH before autoclaving. Forty ml of medium was pipetted into each 100 ml Erlenmyer flask. The flasks were covered with aluminium foil and then autoclaved at 120°C for 15 minutes.

When a mass of callus (ca. 5 mg dry weight) was transferred into fresh medium D, it proliferated and remained alive for a period of about 110 days. Three stages of callus grown on the medium D are shown in Fig. 1.

For cytological observation, cells were fixed and stained with 1% (w/v) orcein in 45% (v/v) acetic acid.

**RESULTS**

In the first experiment, samples of calli were transplanted into the medium MS at various stages after subculture to fresh medium D to see if organ formation would occur. The results are given in Table 1. Bud formation was never observed on the calli which were transplanted to medium MS during the first 30 days. But transfers made after 60 days showed an increasing tendency towards organ-forming capacity and greater longevity as well. A few rudimentary buds appeared on the 70 day or older transfers but failed to grow into shoots. Many buds were produced on the transfers which were made at 70 to 90 days. All these buds appeared at basal portion of the calli formed on medium MS. When the calli with the rudimentary buds were placed in the light, very abnormal shoots having semi-transparent and dark green leaves were
observed. Although these abnormal shoots did not develop further in culture, normal buds developed into small shoots with several leaves when transferred to the fresh medium MS. These shoots, however, ceased to grow and never developed further on medium MS, even though various concentrations of IAA (0.0-2.0 ppm) and kinetin (1 or 2 ppm) were tried. When these shoots were transferred to medium MS containing adenine (30-40 ppm), they began to develop again, roots being formed at the basal portion of the shoots. Figure 2 shows a plant obtained from such a 70 day transfer. This is a 14 month old plant formed from a small shoot which had remained for 5 months on the medium MS.

To look for the possible cause of the growth stage-dependent difference in the organ-forming capacity, cytological observations of the calli in various stages of growth were observed. Although these abnormal shoots did not develop further in culture, normal buds developed into small shoots with several leaves when transferred to the fresh medium MS. These shoots, however, ceased to grow and never developed further on medium MS, even though various concentrations of IAA (0.0-2.0 ppm) and kinetin (1 or 2 ppm) were tried. When these shoots were transferred to medium MS containing adenine (30-40 ppm), they began to develop again, roots being formed at the basal portion of the shoots. Figure 2 shows a plant obtained from such a 70 day transfer. This is a 14 month old plant formed from a small shoot which had remained for 5 months on the medium MS.

Table 1. Bud-forming capacity of the strain XD-6 of *N. tabacum* 

<table>
<thead>
<tr>
<th>Age of calli**</th>
<th>No. of calli tested</th>
<th>% of calli with buds</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>25</td>
<td>84</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td>90</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>105</td>
<td>26</td>
<td>16</td>
</tr>
</tbody>
</table>

* Bud-forming capacity was tested transferring samples into the medium MS.
** Days kept on the same medium after subculture to medium D.

Fig. 2. A plant, 14 month old, obtained from the callus kept on the same medium for 70 days after subculture to medium D. It remained as a small shoot for 5 months and then was developed on the adenine-contained medium. ×1/6
Fig. 3. 10 day old callus growing on medium D showing small protoplasma-rich cells and highly vacuolated cells. ×44
Fig. 4. Cells of 20 day old callus having prominent nuclei. ×44
Fig. 5. Cells of 55 day old callus. ×44
Fig. 6. Cells of 85 day old callus. ×44
Fig. 7. A cell without nucleus. ×100
Fig. 8. Cells inside other cells. ×100
Fig. 9. A cell with 2 nuclei. ×444
Fig. 10. A cell with 4 nuclei. ×444
Fig. 11. Anaphase bridge of chromosomes. ×444
made. Cells of the strain XD-6 exhibited a wide range of different sizes and shapes. In the early period on medium D, highly vacuolated cells, small cells with dense protoplasm and giant cells appeared (Fig. 3). The cells with dense protoplasm and prominent nuclei increased in number (Fig. 4) at a later stage of growth. Still later, however, the cells with dense protoplasm were not observed and highly vacuolated long filamentous cells were seen (Figs. 5 and 6). In the earlier period, cells with anaphase bridges (Fig. 11), and with 2, 3 and 4 nuclei (Figs. 9 and 10) were observed. In the several cases, cells without nuclei were observed (Fig. 7). Many cells which appeared to be inside other cells were seen in the early and middle stages (Fig. 8).

The somatic number of chromosomes is 48 in \textit{N. tabacum}. Although completely accurate chromosome counts could not be made, higher chromosome numbers than usual were observed in the early and middle stages of callus growth. The diameter of the circular interphase nuclei was measured at various stages of callus growth. The measurement was made with 100 nuclei in each stage. The nuclear size became smaller with the time of growth on medium D (Table 2). The size and shape of nuclei were relatively uniform in the later stage. Since cells with large prominent nuclei were predominant in 20 day old callus, the diameter of nucleus in that stage was not measured. Two nuclei sometimes fused and tended to form a single spherical nucleus.

### Table 2. Frequency of nuclei with various diameters in different ages of the strain XD-6 of \textit{N. tabacum}

<table>
<thead>
<tr>
<th>Age of calli*</th>
<th>No. of cells counted</th>
<th>Diameter of nuclei ($\mu$)</th>
<th>Mean diameter ($\mu$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8.6-15.0</td>
<td>15.0-20.0</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>100</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
<td>71</td>
<td>25</td>
</tr>
<tr>
<td>85</td>
<td>100</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>90</td>
<td>9</td>
</tr>
</tbody>
</table>

* Days kept on the same medium after subculture to medium D.

### Table 3. Frequency of cells having excess nuclei in various age of the strain XD-6 of \textit{N. tabacum}

<table>
<thead>
<tr>
<th>Age of calli*</th>
<th>No. of cells counted</th>
<th>No. of cell with more than 2 nuclei</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1640</td>
<td>205</td>
<td>12.5</td>
</tr>
<tr>
<td>20</td>
<td>1427</td>
<td>115</td>
<td>8.1</td>
</tr>
<tr>
<td>55</td>
<td>1692</td>
<td>189</td>
<td>11.2</td>
</tr>
<tr>
<td>70</td>
<td>1725</td>
<td>129</td>
<td>7.5</td>
</tr>
<tr>
<td>85</td>
<td>1093</td>
<td>55</td>
<td>5.0</td>
</tr>
<tr>
<td>100</td>
<td>761</td>
<td>26</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Days kept on the same medium after subculture to medium D.
which was larger than those in the 2 daughter cells of normal mitosis. Thus, an increase in nuclear size is probably associated with polyploidy. Some polyploidy probably also occurs by mitotic failure after chromosome replication. The appearance of many chromosome bridges suggests that a great deal of chromosome breakage also happens.

The number of multinucleated cells were also counted in each stage. With the time after subculture, the multinucleated cells decreased in number (Table 3). From these results, it is probable that in the strain XD-6 a progressive change from higher ploidy to lower ploidy occurs during the progressive stage after subculture on medium D. It is interesting that the decrease in ploidy is paralleled with the increase in the capacity of organ formation.

DISCUSSION

In plant callus tissues grown in vitro for a long period, a progressive loss of the capacity to form organized structures has been often reported. However, organ-forming capacity of the callus cultured for a long period without transfer to fresh medium has never been reported. Matsushima et al. (1969) reported that small shoot formation occurred in the tobacco cultured cell, clone XD-6 S, which has the same origin as XD-6 strain. But detailed data of the shoot formations were not reported. In the present paper, using a strain of tobacco callus subcultured for 8 years, the bud-forming capacity was tested in transfers made from callus allowed to age well past the stationary phase of growth. It was found that the shoot-forming capacity of the callus increased greatly during this period.

The occurrence of polyploidy and other aneuploid conditions in plant tissue cultured in vitro has been reported by many workers (Demoise et al. 1969; Torrey 1967; Blakely et al. 1964; Cooper et al. 1964; Fox 1963; Partanen 1963; Mitra et al. 1960, 1961; Das et al. 1956; Partanen et al. 1955; Naylor et al. 1954). Some of these studies also correlated chromosomal changes with organ-forming capacity (Partanen et al. 1955; Fox 1963; Torrey 1967). Torrey observed a progressive loss of organ-forming capacity in callus strains of the garden pea, and chromosomal analysis correlated with these tests showed that there was a progressive nuclear change in the cell population during the several years of repeated subculturing, from cells with simple diploid nuclei to a complex, heterogeneous population of cells showing aberrant nuclear features. Thus he suggested that the loss in organ-forming capacity is caused by the increase in abnormality of chromosomal constitution.

In this experiment, the nuclear features of callus cells sampled at various stages of growth from a single subculture were observed. Here polyploid and probably other aneuploid conditions were observed during early growth of a subculture. This observation seems to correspond to the many reports of increasing abnormality with prolonged subculturing (e.g., Torrey 1967). In this experiment, however, it was found that the tendency towards aneuploidy is reversed during the later (or stationary) phase of growth in subculture. The tendency towards euploidy during this stage is paralleled with an increase in organ-forming capacity. However, further study is necessary to validate this conclusion.
SUMMARY

Bud-forming capacity of XD-6, a strain of tobacco callus subcultured for 8 years, was tested at various periods after transferring to new culture medium. It was found that bud-forming capacity increased with the age of such a single culture. No bud-formation was seen in the earlier period but many buds were formed in the later period. Nuclear features of the callus were also observed at various periods. The cells exhibited a wide range of size and shape. The nuclei were exceptionally large and there were sometimes more than two per cell in the earlier period. In the later period, however, the size and shape of cells and nuclei were relatively uniform. Possible relevance of the nuclear features to the bud-forming capacity is discussed.

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LITERATURE CITED


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