*CELL DIVISION AND THE DIFFERENTIATION OF WOUND-VESSEL MEMBERS IN CULTURED STEM SEGMENTS OF COLEUS**

By D. E. Fosket

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS

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One of the questions frequently asked in developmental studies is whether cells must go through division in order to initiate differentiation. Recent studies on animal cell specialization indicate that the relationship between these two processes is complex. Stockdale and Topper[1] have shown that the mammary-gland epithelial cells of the mouse must undergo mitosis in order to initiate hormone-dependent differentiation. They conclude "that cell division may make a cell especially susceptible to environmental factors capable of eliciting changes in cell function." It is often observed that cell division is antagonistic to differentiation, in the sense that division leads to a loss of specialized cellular activity.[2] However, Cahn and Lasher[3] have found that undifferentiated chondrocytes in culture undergo differentiation and continue to produce a characteristic cell product while actively dividing.

In the intact plant, xylem differentiation is normally preceded, if not accompanied, by cell division. The procambial cells of higher plants ordinarily continue to divide after mitotic activity has ceased in the ground meristem and vascular elements begin to differentiate among the dividing procambial cells. In the shoot the procambial cells undergo several longitudinal divisions during primary vascular-tissue differentiation.[4] Although the root tip is often described as a compartmentalized system in which mitosis occurs in one region, while elongation and differentiation occur in other tissue regions, Jensen and Kavaljian[5] have observed mitotic activity throughout the provascular region of the onion root tip in the portion of the root they studied (the first 1850 μ).

Of course, these considerations do not suggest that there is a necessary or causal relationship between cell division and xylem differentiation, but only that these processes are fairly closely linked in time in the normal plant. In fact, some studies on vascular differentiation in roots would indicate that cell division and certain aspects of xylem differentiation can be experimentally separated. Torrey[6] found that pea root elongation could be blocked by exogenous auxin application at a concentration that accelerated the rate of primary xylem-element maturation. In the same paper he observed that iodoacetate treatment of pea roots, which blocked both cell elongation and division, also accelerated the rate of xylem element maturation. Torrey's work suggests that, once a cell has been induced to differentiate, the biochemical events that occur in the maturation of that cell are not dependent upon mitotic activity.

Unfortunately, it is not possible with the techniques now available to determine the developmental state from which a cell initiates xylem differentiation in the intact plant. The formation of xylem elements in response to wounding would appear to offer a more favorable system for the study of the relationship

between cell division and differentiation. It has been shown that a transverse incision through a vascular bundle of a higher plant will induce parenchyma cells in the vicinity of the wound to differentiate as xylem elements.[7, 8] We know approximately when these cells are induced to differentiate, the general time course of differentiation,[9] and the hormonal substance responsible for the initiation of this differentiation phenomenon.[10-12] By using the technique of Fosket and Roberts,[13] in which internodal stem segments of *Coleus* are cultivated *in vitro*, it is possible to obtain a quantitative estimate of the numbers of xylem elements formed in response to the wound produced in excising the segment.

In several studies of xylem regeneration in response to wounding, attempts have been made to obtain anatomical evidence that cells either do or do not divide prior to xylogenesis. Sinnott and Bloch[14] observed that many of the parenchyma cells divided once before differentiating in wounded *Coleus* stems. Freundlich[15] stated that at least some of the parenchyma cells became more highly specialized without dividing. Since it is difficult to determine conclusively that a large vacuolate parenchyma cell has or has not divided before differentiating, the question must remain open.

Materials and Methods.—Transverse internodal stem segments 2 mm in thickness were excised aseptically from the second internodes of uniaxial plants of *Coleus blumei* (Benth.), Princeton clone, using the technique previously described.[13] Plants were grown in soil in the Princeton University greenhouse and were between 5 and 7 weeks old when the segments were taken. Usually, four isolated segments were placed with their morphological apical ends up on the surface of sterile media in each 10-cm Petri plate. The tissue segments were cultured in a growth chamber at 25 ± 2°C with continuous fluorescent and incandescent light. After 7 days, the segments were weighed individually and cleared by a NaOH-chloral hydrate method.[13] The cleared segments were squashed onto glass slides in glycerin jelly to facilitate the counting of differentiated wound-vessel members. The basic culture medium contained 2% sucrose and 1% agar. Frequently this medium was supplemented with various substances, which were sterilized by filtration and added in the appropriate concentrations to the autoclaved sucrose-agar medium after it had cooled. In experiments to test the effect of 5-fluorodeoxyuridine (FUdR) on xylem differentiation, this compound (a gift of Hoffman-La Roche, Nutley, N.J.) was added to medium also containing uridine at 5×10^{-5} *M* to minimize any possible effects of 5-fluorodeoxyuridine on RNA synthesis. DNA was extracted from cultured stem segments by means of a modification of the Schmidt-Thannhauser technique suggested by Smillie and Krotkov[16] for higher plants. The DNA content of the perchloric acid extracts was estimated by means of the diphenylamine reaction for deoxyribose, using deoxyadenosine as a standard.[17] The numbers of cells present in the cultured stem segments were estimated by means of a maceration technique. Each stem segment was placed in 1 ml of a solution containing 5% HCl and 5% chromic acid for 24 hr. The fluid was then drawn repeatedly into a hypodermic syringe equipped with a no. 18 needle. A measured drop of this homogenate was placed on a glass slide and covered with a 22-mm-square cover slip. Four such slides were prepared and counted for each segment. Numerical data were then analyzed for significance by means of the *t* test.

Results.—Figure 1 illustrates the change in the amount of DNA/mg of fresh weight of the cultured stem slices. A progressive increase in DNA content of the tissues was observed through the first four days of the culture period. There was no further increase in the quantity of DNA/mg of fresh weight after the fourth day of culture. The numbers of cells present in the explants at isolation and after seven days of culture on sucrose-agar medium were determined. At

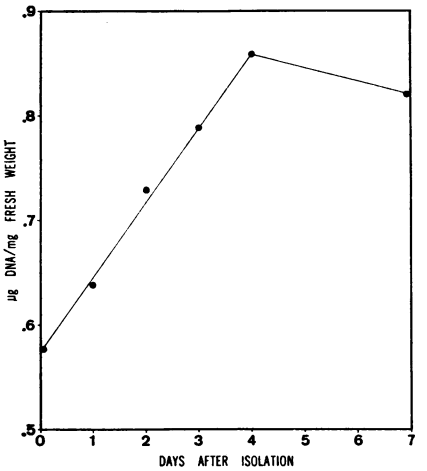

FIG. 1.—The change in DNA content of *Coleus* stem segments cultured for 7 days of 2% sucrose-1% agar medium. DNA was extracted and measured as described in *Materials and Methods*. Each point on the graph represents the DNA extracted from 24 stem segments, which were cultured for the indicated times.

isolation, an average of 439 ± 58 × 10^3 cells was found. After seven days in culture, this number had increased to 578 ± 61 × 10^3 cells. The average fresh weight of the tissue slices at isolation was 21.3 mg, which increased to 35.3 by the end of the seven-day culture period. From these data, the amount of DNA per cell can be calculated. At isolation each cell contained approximately 26 × 10^{-12} gm DNA. After seven days in culture, this figure was 50 × 10^{-12} gm DNA/cell. It is clear that cell division has occurred during the culture period; the data further suggest that DNA synthesis occurred in other cells which remained mitotically quiescent.

The wound-vessel members formed during the experimental period could be distinguished from the primary xylem formed prior to the excision of the segment by the pattern of secondary wall deposition. The primary xylem of the second internode of the *Coleus* stem consists of vessels with annular or helical thickenings. The wound-vessel members tend to be isodiametric with a reticulate pattern of secondary wall deposition.

Coleus internodal stem segments when cultured in the light for seven days on medium containing 2 per cent sucrose and 1 per cent agar (SA) formed approximately 400 wound-vessel members (Table 1). The addition to the SA medium of FUdR at a concentration of 10^{-5} *M* or 10^{-6} *M* almost completely blocked xylem differentiation during the experimental period. The addition of thymidine (10^{-4} *M*) to the medium containing FUdR (10^{-5} *M*) prevented the FUdR

TABLE 1. *The effect of 5-fluorodeoxyuridine on wound-vessel-member differentiation in cultured stem segments of Coleus.*

Treatment	Fresh weight (mg)	Number of WVM
2% sucrose-1% agar (SA)	33.7 ± 0.7*	426 ± 12.2*
SA + 10^{-5} *M* FUdR	33.9 ± 0.7	3 ± 0.4
SA + 10^{-6} *M* FUdR	30.2 ± 1.2	12 ± 1.1
SA + 10^{-5} *M* FUdR and + 10^{-4} *M* thymidine	33.6 ± 0.6	363 ± 24.0

* Each figure represents the mean from 24 stem segments cultured for 7 days on the medium indicated. The mean number of wound-vessel members (WVM) and the mean of the final fresh weights are given together with the standard error.

inhibition of wound-vessel-member differentiation. FUdR had no effect on the fresh-weight increase that occurred during the culture period, although it did prevent the increase in cell number.

Mitomycin C was also found to strongly inhibit xylem differentiation when it was incorporated into the SA medium (Table 2). Although mitomycin C did

TABLE 2. *The effect of mitomycin c on wound-vessel-member differentiation in cultured stem segments of Coleus.*

Treatment	Fresh weight (mg)	Number of WVM
Sucrose-agar (SA), light grown	33.1 ± 0.7*	484 ± 17*
Sucrose-agar (SA), dark grown	31.2 ± 0.6	204 ± 11.2
SA + 10/μg/ml mitomycin C	23.1 ± 0.4	0
SA + 1.0/μg/ml mitomycin C	30.0 ± 0.5	39 ± 3.9
SA + 0.5 μg/ml mitomycin C	30.3 ± 0.6	81 ± 5.1

* Each figure represents the mean from 24 stem segments cultured for 7 days on the medium and under the conditions indicated. The mean number of wound-vessel members (WVM) and the mean of the final fresh weights are given together with their standard errors.

not prevent segment growth at low concentrations (0.5 or 1.0 μg/ml), at 10 μg/ml it completely blocked both growth and differentiation. Since mitomycin C is light-sensitive, it was necessary to culture the segments in complete darkness to test the effect of this substance on xylem differentiation. A highly significant difference in the numbers of wound-vessel members formed in segments cultured in the light and in darkness was observed. Light stimulated wound-xylem differentiation, with approximately twice as many wound-vessel members being formed in the light as in the dark. Still, the addition of mitomycin C to the SA medium resulted in a substantial reduction in the number of wound-vessel members formed, as compared to either light- or dark-grown controls.

When tissue slices were incubated in the light for seven days on SA medium containing 0.04 per cent colchicine, normal wound-xylem formation was completely blocked (Table 3). However, a small number of highly abnormal wound-vessel members was observed after colchicine treatment. These abnormal wound-vessel members were characterized by a lack of pattern in the deposition of the secondary wall. Segments were also cultured on SA medium for three days and then transferred to SA medium containing 0.04 per cent colchicine. This treatment led to the production of relatively large numbers of abnormal wound-vessel members, as well as a few normal wound-xylem elements (Table 3). An estimation of the average number of cells in stem segments cultured for

TABLE 3. *The effect of colchicine on wound-vessel-member differentiation in cultured stem segments of Coleus.*

Treatment	Fresh weight (mg)	Number of normal WVM	Number of abnormal WVM
Sucrose-agar (SA), 7 days	36.2 ± 0.7	348 ± 18.5*	0
SA + 0.04% colchicine, 7 days	36.5 ± 0.7	0	43 ± 4.0*
SA for 3 days then SA + 0.04% colchicine, 4 days	35.4 ± 0.8	22 ± 3.1	216 ± 11.5

* The means presented for each treatment were obtained from 24 stem segments cultured under the indicated conditions. The mean number of wound-vessel members (WVM) and the mean of the final fresh weight are given together with their standard errors.

seven days on the colchicine-containing medium indicated that cell division was blocked by this substance.

Discussion.—The work of Haber and Foard is frequently cited as evidence that xylem differentiation can occur in the absence of cell division. Foard and Haber[18] reported the appearance of normal proto- and metaxylem elements in the roots of wheat seedlings growing without cell division after massive irradiation (800 kr) of the dry seed. However, Haber and Foard[19] have carefully pointed out that the development of xylem elements in these seedlings is a maturation process, rather than one in which differentiation is initiated. To quote these authors, ". . . at any cross-sectional level 'initiation' is apparently absent, but 'maturation' appears to continue normally. . . ." One might conclude that the xylem elements that were observed to mature during gamma seedling growth were already present in an immature form in the ungerminated seed.

The data of Figure 1 clearly show that DNA synthesis occurs in the stem segments of *Coleus* during the seven-day culture period. The average number of cells per explant was also found to increase during the culture period, but the magnitude of the increase was not sufficient to account for all the DNA produced by the explants. This observation suggests that some of the cells may have attained a higher level of ploidy without dividing. Since it has been shown that differentiating xylem elements frequently attain much higher levels of ploidy than surrounding tissues,[20, 21] either the higher levels of DNA per cell, or cell division, or neither of these could be involved in xylem differentiation by the cultured, internodal stem segments. The inhibition of wound-xylem formation by FUdR and mitomycin C suggests that DNA synthesis, or cell division, is a prerequisite for xylem differentiation.

FUdR has been shown to be a specific inhibitor of thymidylate synthetase in microorganisms,[22] and it has been found to inhibit the action of this enzyme in higher plants as well.[23] Since FUdR acts through its effect upon thymidine biosynthesis, exogenous thymidine will reverse the FUdR-inhibition of DNA synthesis. Matthysse and Torrey[24] observed the complete inhibition of diploid and polyploid mitoses in pea root segments cultured in a medium containing FUdR at 4×10^{-6} *M*. Nitsan and Lang[25, 26] have reported the inhibition of both DNA synthesis and gibberellic acid-induced elongation in FUdR-treated lettuce hypocotyls and lentil epicotyls. Matthysse and Torrey[24] and Nitsan and Lang[25] found that these FUdR effects were fully reversible with added thymidine.

Unfortunately, the biochemical effects of mitomycin C in higher plants are not as well studied. Mitomycin C has been found to inhibit the synthesis of bacterial DNA.[27–29] It also inhibits DNA synthesis in higher animal tissues.[30, 31] Iyer and Szybalski[32] have suggested that DNA synthesis is blocked by mitomycin C through the formation of cross-links between the complementary strands of DNA.

While the action of these two inhibitors on wound-xylem formation is suggestive of an involvement of DNA synthesis in the process of differentiation, apparently neither inhibitor is completely specific in its biochemical effects.

Schwartz, Sternberg, and Philips[31] found that RNA synthesis by the intestinal tissues of the rat declined when mitomycin C was given for longer than six hours. In *Escherichia coli*, RNA synthesis was initially stimulated by mitomycin C treatment, but again, longer incubation periods led to a decrease in the rate of RNA synthesis.[29]

Apparently FUdR also can affect RNA synthesis, although the effect appears to be more subtle. Flamm and Birnstiel[23] found that RNA synthesis continued at the same rate in FUdR-treated tobacco suspension cultures. The studies of Nitsan and Lang[26] indicated that a 48-hour incubation with FUdR did not significantly alter the amount of RNA in lentil epicotyls, as compared to untreated controls. However, FUdR did prevent the gibberellic acid-induced increase in the amount of RNA in these same tissues.

It would not be surprising to find that RNA synthesis was necessary for xylem differentiation. While there is little direct evidence to indicate that differentiating xylem elements pass through a stage in which they are dependent upon RNA synthesis, Fosket and Miksche[33] have found that protein synthesis is necessary for wound xylem formation. Also, preliminary work by the author suggests that a relatively large increase in the rate of C^{14}-uridine incorporation into RNA occurs during the first 24 hours after the isolation of *Coleus* stem segments.

Because of these considerations, the results of the studies with mitomycin C and FUdR should not be considered conclusive evidence that DNA synthesis, or cell division, must occur before cells can be induced to differentiate as xylem elements. The effects of colchicine are, however, more convincing. Colchicine does not inhibit DNA synthesis, but rather inhibits cytokinesis by blocking spindle formation. DNA synthesis and mitosis occur normally through metaphase in meristematic plant tissues treated with colchicine. Since the spindle fails to form, the chromosomes do not migrate to the poles and the ploidy level of the cell is doubled.[34] The fact that colchicine blocks wound-vessel-member formation when given continuously for seven days indicates that the increased ploidy level observed in differentiating xylem elements is not a sufficient condition for their differentiation. The inhibition of xylogenesis by colchicine suggests that cells must undergo cytokinesis before initiating differentiation.

Harris and Bajer[35] found the spindle fibers of dividing cells of *Haemanthus* endosperm to consist of microtubules, which were attached to the chromosome at the kinetochore. Microtubules have also been observed in the cytoplasm directly opposite the developing bands of secondary wall thickening of differentiating xylem elements.[36, 37] Pickett-Heaps[38] studied the ultrastructural effects of colchicine on dividing cells and differentiating xylem elements in coleoptiles and root tips of *Triticum vulgare*. The use of colchicine led to the disappearance of the microtubules in both types of cells. However, the subsequent effect of loss of the microtubules was different. Colchicine blocked cytokinesis, but it did not prevent secondary wall deposition in the differentiating xylem elements. Wall material continued to be deposited in the absence of the microtubules, but the normal pattern of deposition was lost.

Although the observation of Pickett-Heaps would suggest that xylem differentiation itself is not blocked by colchicine treatment, his observations ex-

tended over a relatively short time period (2–3 hr). It was possible that a longer exposure to colchicine, such as was employed in this investigation, would inhibit xylem differentiation directly through its effect on wall deposition. Therefore, an attempt was made to distinguish between these possible effects. The data of Figure 1 indicated that approximately 75 per cent of the DNA increase had occurred by the end of the third day of the culture period. Examination of segments at various time intervals after culturing has shown that the first partially differentiated xylem elements appear after three days of culture. The majority of the differentiating xylem elements are just beginning secondary wall synthesis at this time. If colchicine were to inhibit xylem differentiation by preventing secondary wall deposition, one would expect colchicine application on the third day to be as effective in preventing xylem differentiation as the continuous cultivation of stem segments on the colchicine-containing medium. Clearly this was not the case. Incubation of *Coleus* stem segments with colchicine for four days, beginning on the third day of the culture period, led to the production of relatively large numbers of aberrant wound-vessel members. This result would verify the observation of Pickett-Heaps that colchicine does not block secondary wall deposition. Furthermore, this finding substantiates the hypothesis that colchicine blocks xylem differentiation by preventing cytokinesis in *Coleus* segments cultured for seven days on medium containing colchicine. It would seem then that cells must divide before wound-xylem differentiation can occur in cultured *Coleus* stem segments.

Summary—Internodal stem segments of *Coleus* 2 mm in thickness were cultured *in vitro* for seven days on a medium containing 2 per cent sucrose and 1 per cent agar (SA), or on SA medium supplemented with inhibitors of DNA synthesis and mitosis. Mitomycin C (0.5–1.0 μg/ml) and 5-fluorodeoxyuridine (FUdR, at 10^{-5} or 10^{-6} M) both inhibited wound-xylem differentiation, as compared to the SA controls. The addition of thymidine to the FUdR-containing medium restored xylem formation. When segments were cultured on a medium containing colchicine (0.04%), normal xylem differentiation was completely blocked, although a few abnormal xylem elements were observed. Segments cultured on SA medium for three days and then transferred to SA + 0.04 per cent colchicine formed relatively large numbers of abnormal wound-vessel members and a few normal xylem elements. The DNA content of the tissues grown on SA medium was determined by extraction procedures at daily intervals after isolation. The amount of DNA/mg fresh weight of tissue increased through the fourth day of the culture period. No further increase was observed after the fourth day. Estimation of cell numbers from macerated tissue segments indicated that cell division occurred during the culture period. It was concluded that cells must divide in order to initiate wound-vessel-member differentiation.

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