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SHOOT HISTOGENESIS IN TOBACCO CALLUS CULTURES

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SUMMARY

The earliest histological event observed in light-grown shoot-forming tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) callus was the deposition of safranin-stainable substances (probably suberin) on cut, exposed cell surfaces. This was followed by the initiation of cell files and the appearance of starch granules. Nodules with lignified tracheary elements also were observed in the upper part of the callus. Pronounced starch accumulation occurred in the lower part of the callus in which protrusions of tissue into the medium occurred. Meristemoids were found in these protrusions as well as elsewhere. In between meristemoids, parenchyma cells with starch granules of varying sizes were observed. Cell strands that connected with the meristemoids also were observed. These strands often terminated at the surface of the protrusions at which point shoot apices originated. The earliest shoots were formed in these protrusions. With time, additional shoots were formed in other parts of the bottom of the callus and finally in the top part of the callus on prolonged culture. The determination of the loci at which shoot primordia were formed sequentially was interpreted in relation to the physiological gradient concept.

Key words: shoot histogenesis; tobacco callus; meristemoids; lignified nodules.

INTRODUCTION

For over 30 years callus cultures of plants have been used to study factors regulating organ formation and cell differentiation. These studies have shown inter alia that a basic regulatory mechanism underlying organ formation in plants involves a balance between auxin and cytokinin (1), a relatively high cytokinin-to-auxin ratio favoring shoot formation. In tobacco callus, it also has been shown that concentration, diffusion or physiological gradients of metabolites and/or growth substances may be important in determining the loci at which shoot primordia are formed (2). In addition, the accumulation of starch and its utilization have been shown to be associated with organ formation in tobacco (3–5) as well as rice callus (6). In both tissues the gibberellin-repression of shoot formation has been correlated with a reduction in starch content. However, no detailed study of the histogenic events involved in shoot formation in tobacco callus seems to have been made although some histochemical and ultrastructural studies have been made during organogenesis (3–5).

Details of the histology of shoot-forming tobacco callus are presented here, particularly in relation to (a) the role of physiological gradients in determining the morphology of the upper and lower parts of the callus, and (b) the distribution of the deposition and disappearance of starch granules.

MATERIALS AND METHODS

In this experiment, tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) callus, initially isolated from stem segments and subcultured at least four times at 5- to 6-week intervals, was used. Subcultured tobacco callus was transferred to the shoot-forming medium which consisted of Murashige and Skoog's mineral salts (7) supplemented with 10^{-5} M indole-3-acetic acid (IAA), 10^{-5} M kinetin, 90 mg per l adenine sulfate, 50 mg per l tyrosine,

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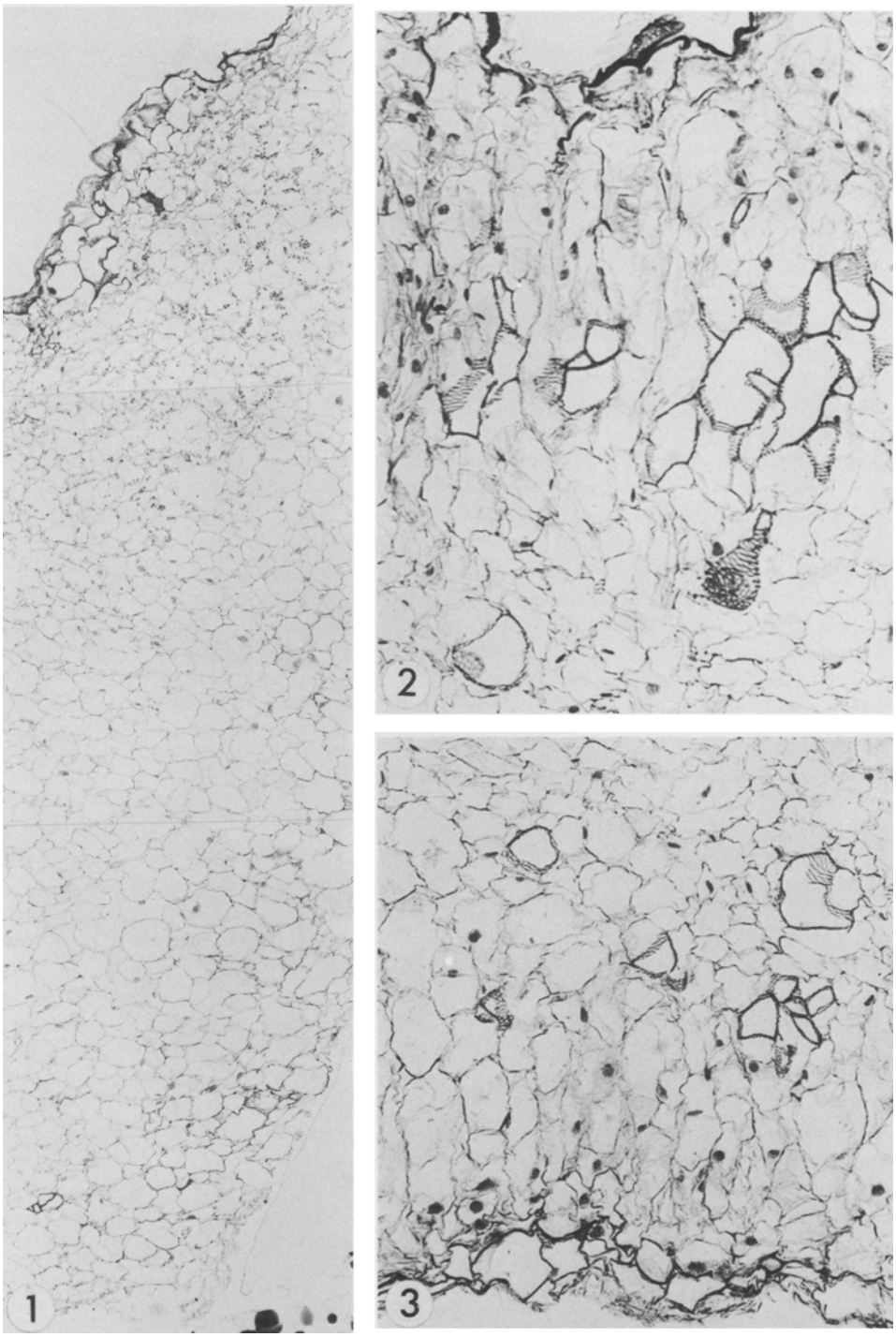

FIG. 1. A section of tobacco callus, at 7 days in culture, on a shoot-forming medium showing portions of the lower part at the bottom, middle and top part of the tissue. Dark round figures are Dowex granules indicating the lower callus surface in contact with medium. ×60.

FIG. 2. Cell files in the upper part of shoot-forming tobacco callus at 10 days in culture. ×100.

FIG. 3. Cell files in the lower part of shoot-forming tobacco callus at 10 days in culture. ×100.

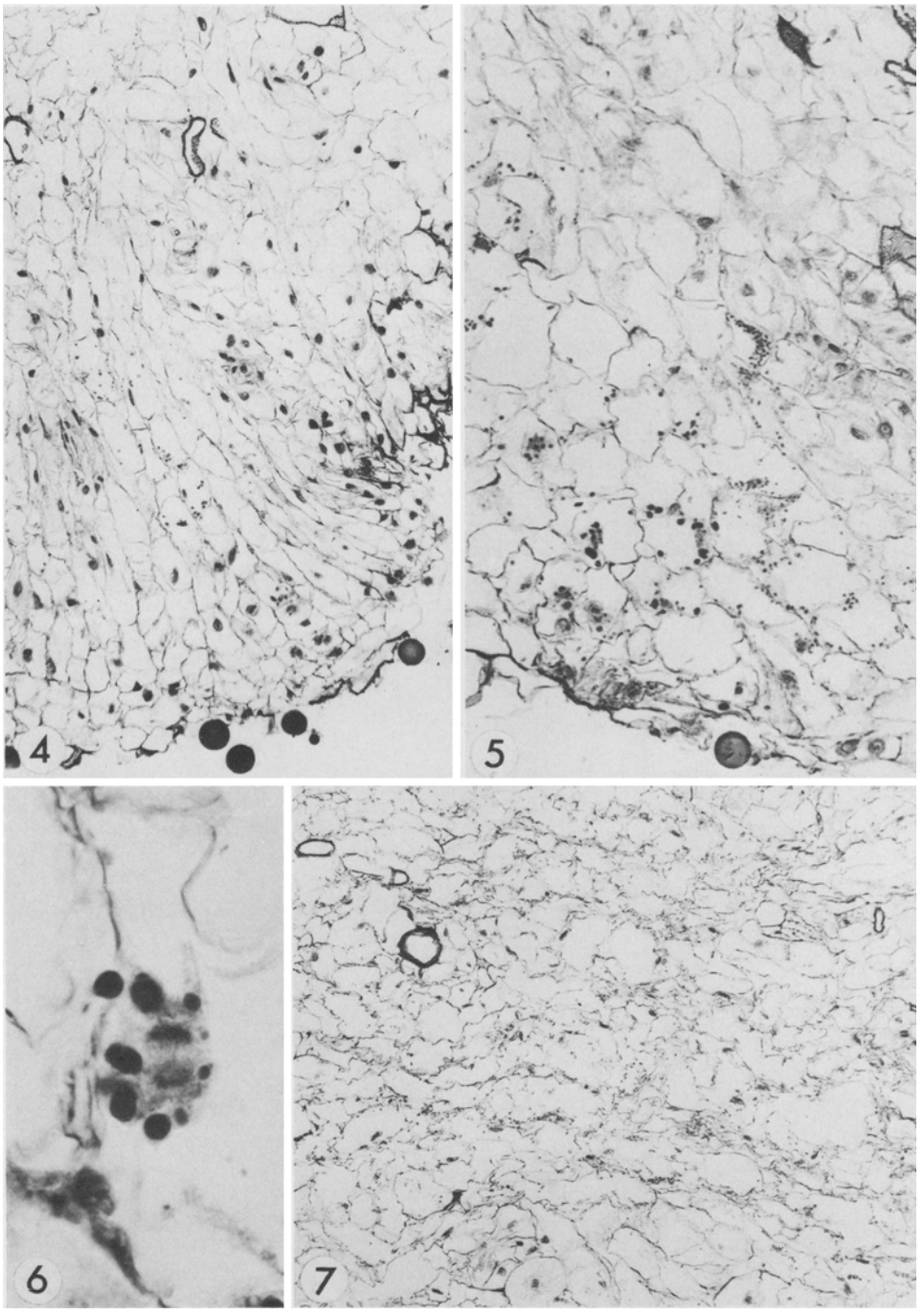

FIG. 4. Shoot-forming tobacco callus showing broad cell files of the protrusion tissue at 10 days in culture. ×90.

FIG. 5. Deposition of starch grains in the parenchymatous cells in a protrusion at 10 days in culture in shoot-forming tobacco callus. ×150.

FIG. 6. Telophase chromosomes in the cells of a protrusion at 10 days in culture in shoot-forming tobacco callus. ×600.

FIG. 7. Central parts of shoot-forming tobacco callus at 10 days in culture. ×90.

170 mg per l NaH_2PO_4, 0.4 mg per l thiamine-HCl, 100 mg per l inositol, 30 g per l sucrose, and 9 g per l agar (5). Two pieces of tobacco callus (each approximately 80 mg) served as inoculum in 50-ml Erlenmeyer flasks containing 25 ml of the medium. Cultures were maintained in continuous light at 27° ± 1° C or darkness.

Seven to 14 days after inoculation, the callus pieces were fixed whole in FPA (Formalin, propionic acid, 100% ethanol and water 2:1:10:7). Before fixation, the basal parts of some pieces were embedded in 2 g per 100 ml of agar, or a few granules of Dowex resin were placed on the agar surface in order to record the proper orientation of the cultured tissue. The pieces alone or with the agar were embedded in Paraplast after dehydration through a tertiary butanol series. Microtome sections were cut approximately 10 μm in thickness. Sections were stained in tannic acid-ferric chloride and safranin (8) and observed with the light microscope (Leitz Orthoplan). Starch grains were stained red with safranin and also were identified with the IKI reaction (8).

Results

An examination of the external morphology of the light-grown shoot-forming tobacco callus revealed that at 7 days, some of the inoculum had changed to a light-green color but there were no discernible changes in the external morphology. By 10 days, the greening process had continued. New callus appeared on parts of the inoculum that were in contact with the medium, and parts of the inoculum had changed to a brown color. By 14 days, protuberances could be observed on the lower parts of the green-colored tissue; these protuberances were most often inside the medium. With further time in culture leafy vegetative shoots emerged from these protuberances. These were dark green in color, and shoot apices could be found among these leaves. Dark-grown cultures remained colorless with the tendency for the tissue to become firmer and light brown in color. The same morphological patterns could be observed as in the light-grown cultures. In prolonged periods in culture (beyond 35 days), vegetative shoots were found on the upper parts of the callus. Finally, the development of these vegetative shoots on emergence from the callus was slower in the light-grown cultures.

Since earlier studies (3-5) had indicated that the key histogenic events leading to shoot formation occurred in 7- to 14-day-old cultures, we concentrated our histological study during that period. In 7-day-old shoot-forming callus, in which the tissue histology was essentially similar to the original inoculum, starch grains were observed in some parenchyma cells located in the center of the tissue. Most of these were probably carried over with the inoculum. Deposition of starch was occasionally seen in the upper parts (Fig. 1). At this stage, starch distribution did not have any relation to meristemoid formation because no meristemoids were as yet observed in the tissue. Wall materials in the crushed cells of the upper surface often were stained deep red with safranin (Fig. 1). Such cells had characteristics similar to those observed during wound repair on the surface of explants. In the lower portion, no changes were observed even in the cells in contact with the medium surface (Fig. 1).

In 10-day-old callus, extended cell files, including cells that often had folded walls or were crushed, surrounded the inoculum (Figs. 2, 3). Nuclei were prominent in these cell files. Such cell files were more developed in the upper part of the tissue (Fig. 2). Xylem elements and tracheids often were observed at the border between the inoculum and the cell files. Meristemoids began to arise from the areas of preferential cell division near xylem elements and tracheids. The xylem elements, tracheids and meristemoids were found in greater abundance in the upper parts than in the lower parts of the callus, but they soon became well developed in the lower part of the light-grown tissue. In the upper parts of the callus, starch grains were not seen in the cell files, and thickened cell walls stainable with safranin were noted in the outer surface in the peripheral cells (Fig. 2); such cell walls were seen as well on the lower surface (Fig. 3).

It was characteristic of the lower part that broad files of cells protruded from parts of the callus surface that were in contact with the medium (Figs. 4, 5). Large and small starch grains were deposited in the enlarged or elongated cells in the protrusion. It was clear from the observation of the large starch grains surrounding telophase chromosomes (Fig. 6) that these enlarged cells containing starch grains divided further to give rise to the protrusion. Starch grains also were observed in the cells of the inoculum situated in the central portion of the callus (Fig. 7).

In 14-day-old shoot-forming callus, meristemoids were observed at discrete distances from the lower surface layer of callus. They generally

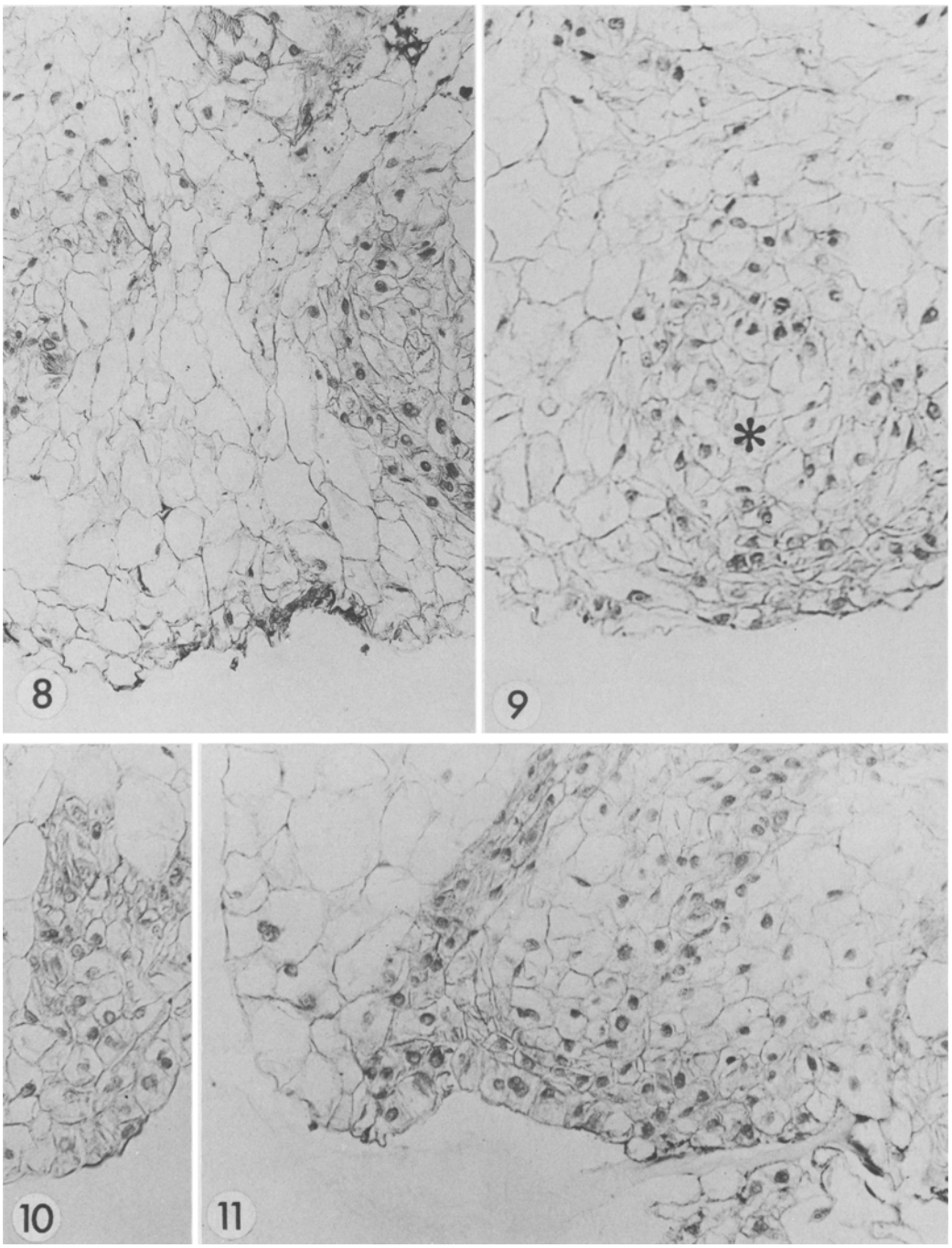

FIG. 8. Parenchymatous tissue from the lower part of shoot-forming tobacco callus at 14 days in culture. Note also part of a meristemoid. ×100.

FIG. 9. A meristemoid (*) in the lower part of shoot-forming tobacco callus at 14 days in culture. ×150.

FIG. 10. Cells having densely stainable cytoplasm in the lower part of shoot-forming tobacco callus at 14 days in culture. ×100.

FIG. 11. A shoot apex-like structure in tobacco callus at 14 days in culture. ×150.

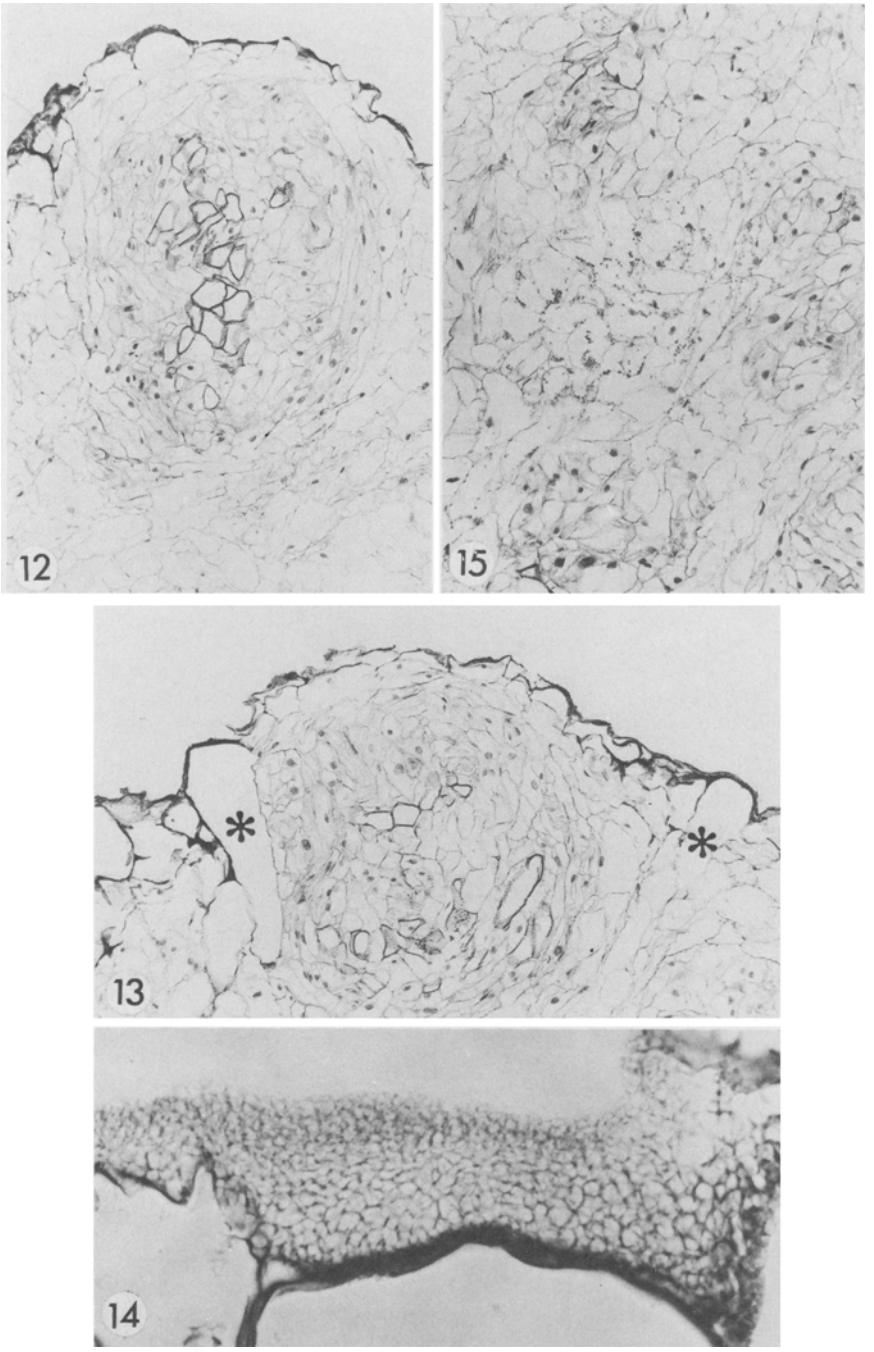

FIG. 12. A nodule with lignified tracheary elements in the upper surface of shoot-forming tobacco callus at 14 days in culture. ×90.

FIG. 13. A lignified nodule and hypertrophied cells (*) in the upper part of the tobacco callus at 14 days in culture. ×90.

FIG. 14. Mosaic of granular deposits in the surface of exposed, cut cells in tobacco callus at 14 days in culture. ×150.

FIG. 15. Deposition of starch grains in intermeristemoidal area in the lower part of shoot-forming tobacco callus at 14 days in culture. ×90.

were located more frequently in the lower or central parts than in the upper parts; especially well developed ones were seen in the central parts. Often parenchymatous tissue was found between the meristemoids and the lower surface of the callus (Fig. 8), but a few meristemoids were in contact with the lower surface (Fig. 9). Periclinal divisions occurred in the cells in the parenchymatous protrusion and caused newly formed cells to protrude further outward.

Nuclei having binucleoli were sometimes observed in the surface or subsurface cells of the parts where the parenchymatous protrusions were in contact with the medium surface. The presence of cells having such nuclei probably implies a high activity of cells in these parts. In fact, some cells having densely stainable cytoplasm were observed in and near the lower surface of the parenchymatous protrusions (Fig. 10). Cell strands connected with meristemoids were observed in the protrusion. A shoot apex-like structure arose from loci where these cell strands connected with the surface cell layer which was in contact with the medium (Fig. 11). Such cell strands and the surrounding cells did not contain starch grains. The features mentioned above also were observed in the lower parts of dark-grown cultures.

The above situation was never observed in the upper parts of the shoot-forming callus. In this case, meristematic nodules were observed near the upper surface, but these always included lignified vascular elements (Figs. 12, 13). Hypertrophied cells in the surface layer occasionally were found near these nodules (Fig. 13) although unbroken nuclei could be observed in these cells. These cells may have been torn by the growth of meristematic nodules and the division and enlargement of the surrounding cells.

On the face view of the surface cell layer, which was obliquely cut, a thick mosaic of granular deposits, about 7 μ in diameter, was observed (Fig. 14). This indicates the secretion of safranin-stainable substances and was suggestive of the regeneration of epidermal-like cells.

At this stage parenchymatous cells in general had few starch grains; any starch grains present in the cells were found at particular loci. Starch was deposited in the enlarged cells surrounding meristemoids but not in isodiametric cells situated in the center of the meristemoids (Fig. 15). Also they often were seen in the elongated cells having lens-shaped nuclei that were located in intermeristemoidal areas. Fig. 16 shows the deposition of starch grains in the cells surrounding a meristemoid that has a cell strand extending toward the lower region of the callus. In most cases starch grains were found on the lower walls of the cells (Figs. 17, 18) or around the nuclei (Fig. 19). Starch deposition was more frequent in the center and lower parts than in the upper parts of the tissue. This feature could be correlated with the increased appearance of meristemoids in these parts as well as with the presence of the protrusions in which meristemoids also were observed.

Discussion

The first histological event observed in shoot-forming tobacco callus was wound-healing which took place on the exposed surface. This was recognizable through the accumulation of safranin-stainable substances in the cell walls, particularly on the upper parts of the callus; this material was probably suberin (9).

The formation of relatively uniform cell files followed. These files were formed as a result of periclinal division and elongation of the cells. Such cell files also have been observed in rice callus (10). Tracheary elements also were seen close to the cut inoculum surface. Soon afterwards, active meristematic centers could be observed in close proximity to these elements. Starch grains first appeared in the inoculum and only subsequently in the newer-formed tissues. Most of this activity initially took place in the upper half of the callus. Such activity in the upper parts of the callus also was seen during the process of callus growth by Caplin (11) who examined tissue in which no organ formation was taking place. Thus these processes can be assumed to be unrelated to the shoot-forming process.

The earliest histological event that could be related to shoot formation was the appearance of regions of active cell division in the lower part of the callus as reported earlier (5). This was followed by the formation of broad protrusions, composed of elongated parenchymatous cells, which were found on the callus surface in contact with the medium. Starch grains of various sizes were found in these regions and the grains decreased in size as the protrusions enlarged. Cell strands could be observed in these protrusions; they appeared to be connected with isodiametric cells at the center of the meristemoids. Surrounding the meristemoids were parenchymatous cells which still contained starch grains. With further development the cell strands extended toward the surface from which a shoot apex arose.

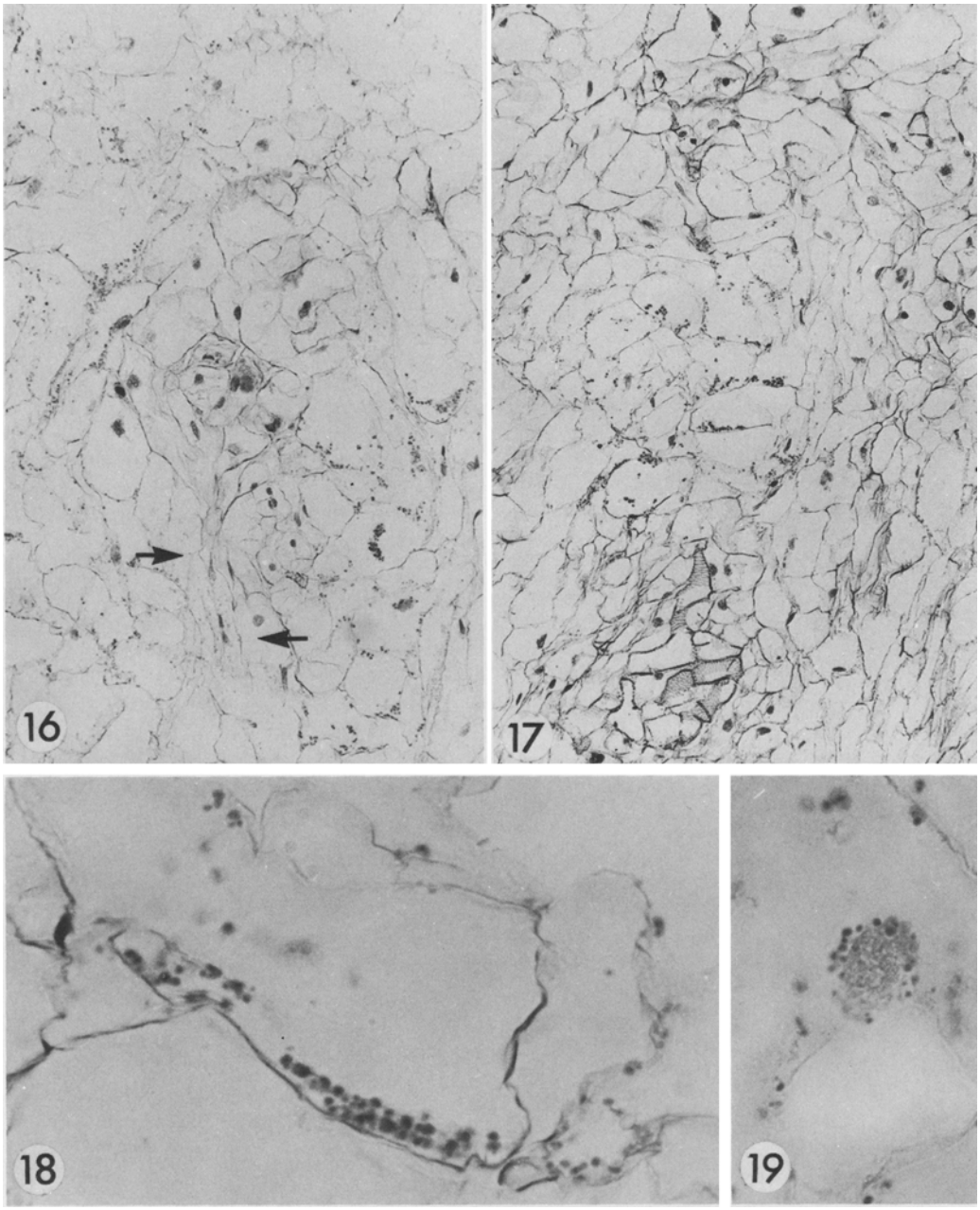

FIG. 16. Connection between a cell strand (*arrows*) and a meristemoid with surrounding cells containing starch grains in callus at 14 days in culture. ×100.

FIG. 17. Starch grains on the lower walls of cells from intermeristemoidal regions in shoot-forming tobacco callus at 14 days in culture. ×100.

FIG. 18. Starch grains on the lower walls of cells from shoot-forming tobacco callus at 14 days in culture. ×600.

FIG. 19. Starch grains surrounding a nucleus from a cell in the intermeristemoidal region of shoot-forming tobacco callus. ×600.

The earliest shoots that appeared were from the protrusions in the lower part of the tobacco callus. It was postulated earlier that physiological gradients of materials into the tissue from the medium were operative in determining the loci at which meristemoids were initiated (2). In that study, the position in the tissue from which shoots emerged (and thus presumably indicative of regions in which the primordia were formed) could be altered by inverting the shoot-forming callus at different times during the culture period. Our histological findings are consistent with this hypothesis since the earliest-formed histogenic centers were in the protrusions closest to the tissue-medium interface and thus in tissue regions closest to the morphogenic substances in the medium. The operation of such gradients also could lead to the formation of meristemoids and finally shoots in the upper parts of the callus; indeed, we were able to observe such shoots on prolonged culture. However, we have not examined the origin or development of these later-formed organs.

The formation of shoots in vitro was first reported to occur in submerged tissue (12). This was thought to reflect possibly a lower oxygen tension. Indeed, this view has recently been supported (13). In this later study, a reduction in the dissolved oxygen concentration in cell suspensions of carrot tissue led to the onset of adventive embryogeny. Thus the formation of shoots in the partially submerged protrusions reported here also could be a consequence of such a reduced oxygen tension. This reduction in oxygen tension has been correlated with an increase in the endogenous ATP level, and we have previously shown that shoot initiation is a high-energy-requiring process (2, 14, 15).

The accumulated starch and the free sugars from the medium have been shown to be utilized during meristemoid and shoot formation (3–5, 16). In this study, we found that these carbohydrates also were utilized in the development of the intermeristemoidal regions and in forming the cell strands as the size of the starch grains decreased in these regions.

Nodules containing lignified elements were observed in both the top and bottom portions of the tissue. However, most of these nodules were formed in the upper part of the callus, early in culture, and did not give rise to shoots. We have no evidence that those in the lower part of the callus formed shoots although it has been suggested that such nodules could form organs (17). In any case, the majority of the nodules found in the lower part of the callus was identical to the meristemoids, i.e. without lignified elements, and gave rise to shoot primordia as previously reported (3–5).

In conclusion, this paper offers a histogenic model for shoot formation in tobacco callus. The development sequence begins with (a) the formation of cell files around the inoculum; (b) the appearance of tracheary elements in the areas between the cell files and inoculum; (c) the occurrence of meristematic centers near the tracheary elements; (d) the formation of broad protrusions on the callus surface in contact with the medium; and, finally, (e) the induction of a shoot apex from the surface of the protrusion.

In this study we were able to confirm some of our earlier studies, and also to determine that the histogenic process of shoot formation in light-grown callus was essentially similar to that of dark-grown tissue. Furthermore, we were able to obtain a greater insight into the developmental processes leading to shoot formation. Since much of our work is concerned with the physiology of the organ-forming process, the observations made in this study give additional important background information for a proper interpretation of the physiology of the process.

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