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Computer-Assisted Image Analysis of Tissues of Ethrel-Treated *Pinus taeda* Seedlings[1]

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ABSTRACT

A microcomputer based video image analyzer is described and demonstrated in the analysis and comparison of tissue types of 6-month-old Ethrel-treated and control seedlings of loblolly pine (*Pinus taeda* L.). Two different programs were employed. 'Topographer I' sought, identified, and measured cell or resin canal lumen areas, and 'Area' measured tracheid length and silhouette areas. Ethrel treatment induced an increase in radial growth and a decrease in shoot elongation and needle growth. Bark production was stimulated by the same treatment. The tracheid lumens of Ethrel-treated plants were shorter and had a smaller cross-sectional area, whereas the tracheid cell walls increased in thickness. The treatment also increased resin canal formation in the xylem.

The extensive use of automatic image analysis and its associated new technology in the collection and processing of data of biological material has been hindered by the high cost of the instrumentation. In the past, the instrumentation has required either a minicomputer or a dedicated microprocessor packaged in a costly image analyzer (5, 6). We have developed a versatile low cost image analyzer (Fig. 1) which we have used here to quantify anatomical observations as described below.

Ethrel is a commonly used ethylene-releasing compound (9). When it is applied to plants, it is taken up by them and breaks down to liberate the phytohormone ethylene. The image analyzer was used to analyze different tissue types of control or Ethrel-treated seedlings of loblolly pine.

MATERIALS AND METHODS

Plant Material. Seedlings of loblolly pine (*Pinus taeda* L.) were grown in the greenhouse at 26 ± 2°C day, and 16 ± 2°C night, in 145-cc 'cone-tainers' (Ray Leach Cone-tainer Nursery, Cranby, OR), in a soil mixture consisting of 1 part perlite, 2 parts vermiculite, and 2 parts peat moss, with 18:6:12 osmocote 9-month time release fertilizer added at a rate of 2.48 kg/m^3 of mix (7). Thirty 3-month-old seedlings were treated with a 5% solution of Ethrel (83 mM Ethephon) (neutralized just prior to application) once every 2 d brushed on the side of the hypocotyls. This treatment was continued for 3 months. At the end of the treatment period, stem height and width and fresh weights were recorded and compared to controls which had been brushed with distilled H_2O. Ten samples of both treatment and control seedlings were fixed in FAA (Formalin:acetic acid [80%]:ethanol; 1:1:8), dehydrated, embedded in paraffin, and then sectioned for anatomical analysis. Sections were stained with safranin-fast green. Five samples of both treatment and control seedlings were prepared for tissue maceration by acid hydrolysis in order to determine tracheid length. Macerated tracheids were stained with safranin in order to enhance contrast for image analysis.

Image Analysis. The DARWIN system (Digital Analyser of Resolvable Whole-pictures by Image Numeration) uses an Apple II+ microcomputer with 48K memory, a Colorado Video 270A video digitizer (Colorado Video, Inc., Boulder, CO), and an analytical quality video camera (Dage vidicon model VC-65-S), with external remote control gain, target, gamma and black level controls. We have developed the digitizer-to-computer interface board and associated software packages. The system is set up according to the block diagram in Figure 2. The analog video signal from the camera is converted into a digital signal by the digitizer. The digital signal represents the grey scale value (from 0 = black to 255 = white) for the points in the scan field of the camera. The computer can request the grey scale value for any given point by sending two 16-bit control words to the digitzer via the interface board. The control words include matrix coordinates to access any one of 245,760 points in a 512 × 480 array (for faster scanning, a 256 × 240 array containing 61,440 points may be used). The grey scale value for the selected point is then returned to the computer via the interface board. Once in the memory of the computer, analysis of the image takes place according to the software which has been loaded. The system can be used for analysis of both microscopic and macroscopic material, photographs, negatives, or video tape signals. With appropriate software, the computer is instructed to recognize and measure the topographical features of interest in the image. Thus, the system does not use light pens or graphics tablets, but requires the instructed computer itself to recognize and identify the features of interest and then to make whatever measurements are included in the program.

In this work, two programs were employed. The first, Topographer I, can count, measure the area of, and report the density of up to 100 topographically similar features. If desired, it will also produce a low resolution, graphic reconstruction of the image scanned, which can be compared to the original image to insure proper analysis. Before the computer can carry out these measurements, the operator must instruct the instrument to distinguish the object from the background. This procedure is essential to all programs. The operator can input to the computer light and dark gray level limits which would allow for recognition of an object based on its gray level. When the scan is performed, the image is scanned from the top to the bottom of a column and left to right column by column. When a pixel possessing a density within the limits ascribed to the images to be analyzed is identified, its

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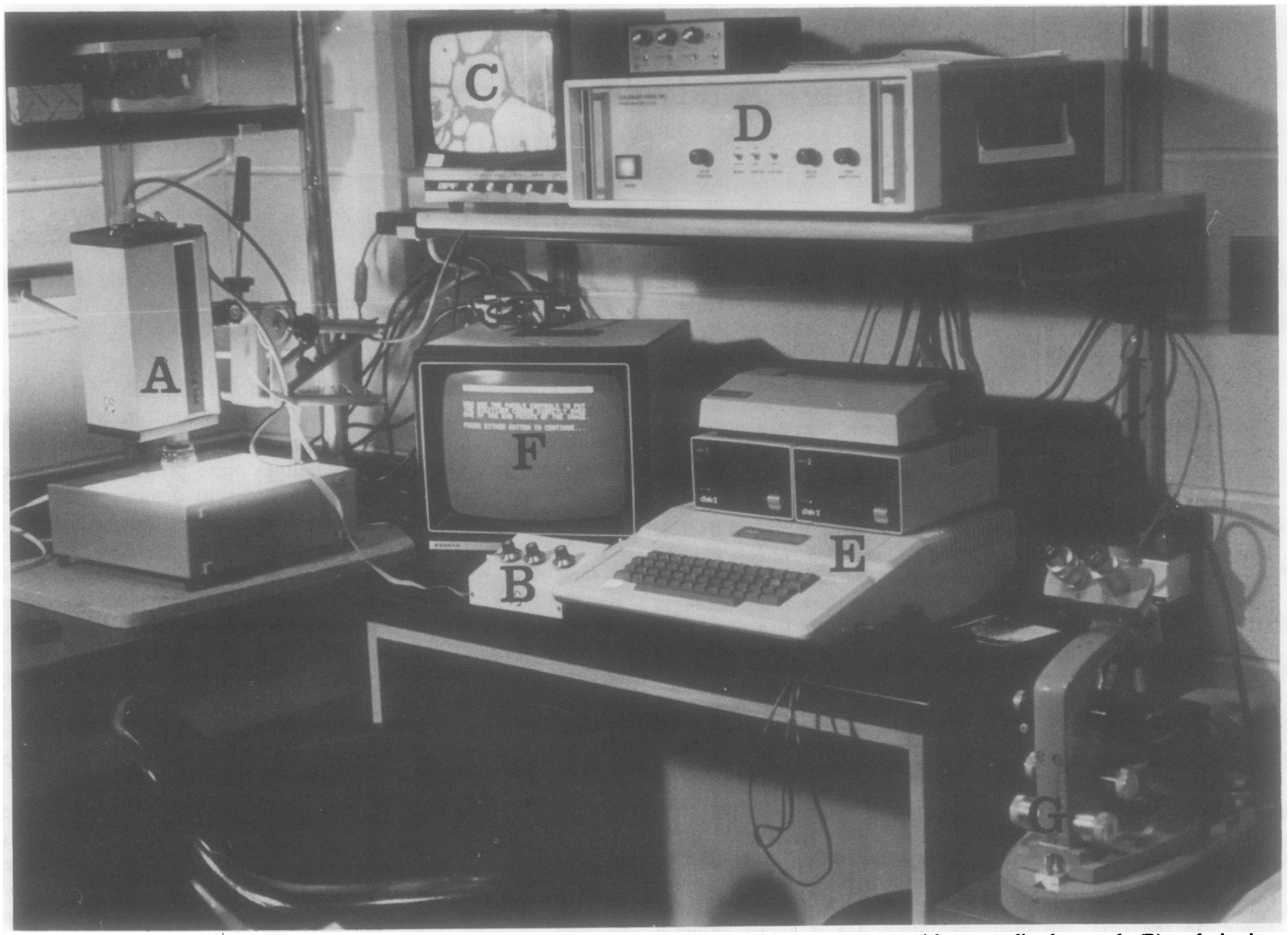

FIG. 1. Overall view of the DARWIN image analysis system. The components are: video camera (A) with externalized controls (B) and viewing CRT (C), digitizer (D) and microcomputer (E) with its CRT (F). The camera can be mounted on the Trinocular microscope (G).

position and the number of contiguous pixels of similar density is recorded. The program then compares the position with the position of previous pixels in the preceding columns. If a match of contiguous rows and columns is confirmed, these pixels are added to an existing area-position file. If no match is found, a new file, identifying a new object, is created. The low resolution reconstruction is based on the six 'maximum' points of the object's position. These points are: first pixel, first column; last pixel, first column; highest pixel, between first and last columns; lowest pixel, between first and last columns; first pixel, last column; last pixel, last column. In the future, we intend to modify this reconstruction to a high resolution reconstruction based on all perimeter points of the object.

Topographer I was used to count and measure the areas of the lumens of tracheids and resin canals in cross-sections at a magnification of × 200 and × 15, respectively. The cross-sectional areas of bark and xylem were determined in a similar fashion at × 2, and the proportion of the former to the latter was also automatically reported by the computer.

The second program, 'Area,' measures the total area of a contrast-distinctive topographical feature or features. If only one feature is measured, it also reports the horizontal width of the object. The logic for the Area program is similar to that of Topographer described above. Inasmuch as only one object is scanned, there is no need to sort and create separate position-area data files for many objects. The length of the object is determined when the axis to be measured is lined up perpendicularly to the scan columns. The distance between the first and last columns is the length of the axis desired. Alignment of the object on the digitizer screen is easily accomplished by rotating the camera or specimen. Tracheids were lined up along the horizontal axis and measured at × 100, using this program. The area program was also used to measure the total cross-sectional area of the cell wall matrix in a field of 306 mm^2.

RESULTS AND DISCUSSION

The Ethrel-treated seedlings were shorter and their stems thicker than the nontreated controls (Table I). There is also a reduction in the length of needles and a reduction in overall fresh weights in response to the Ethrel treatment. The only exception is an increase observed in the fresh weight of the hypocotyl. The increase is a result of an increase in the fresh weight of the bark. There is no change in the fresh weight of the xylem. However, a reduction becomes evident in the dry weight of Ethrel-treated xylem compared to the dry weight of control xylem. The greater decrease in the dry weight of Ethrel-treated xylem may be a result of a larger quantity of volatile resins produced by the larger resin canals.

The image analyzer gave excellent rapid quantification of the anatomical features under observation. The data derived from these analyses were obtained by the image analyzer and are reported in Table II. The tracheids of the Ethrel-treated seedlings

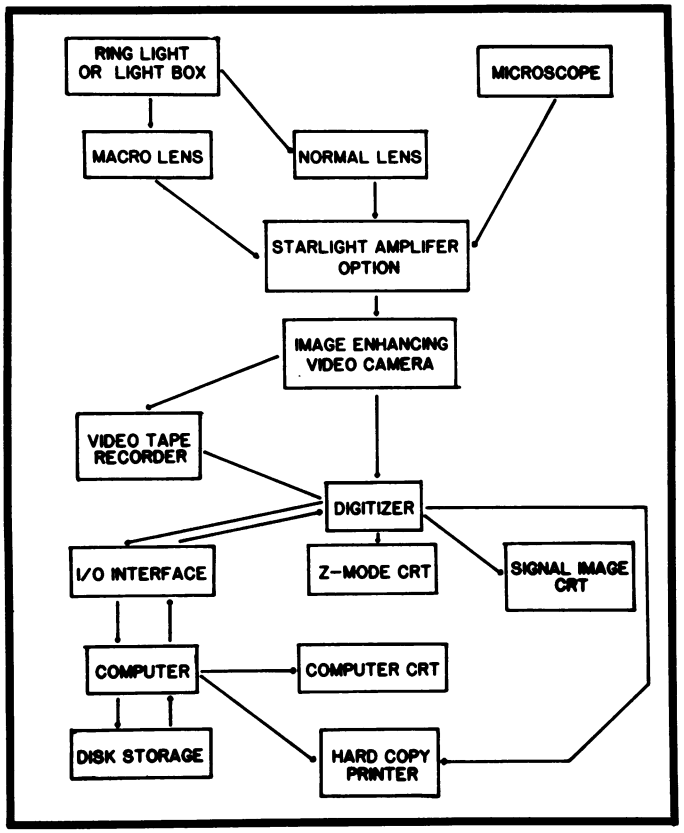

FIG. 2. Block diagram of the DARWIN image analysis system. The 'Starlight Amplifier' allows for measurements under very low light conditions. This option can allow for a 100,000-fold increase in the ambient light signal.

Table I. *Morphological Data and Fresh and Dry Weights of 6-Month-Old Loblolly Pine Seedlings*

Each datum represents the mean of 30 seedlings and is followed by its SE.

	Control	Ethrel
Height (mm)	109.9 ± 20.3	55.4 ± 15.6
Diameter at base of hypocotyl (mm)	3.6 ± 0.5	4.6 ± 0.9
Needle length (mm)	49.0 ± 5.0	37.5 ± 5.3
Root fresh wt (g)	1.99 ± 0.06	1.53 ± 0.09
Root dry wt (g)	0.54 ± 0.02	0.32 ± 0.02
Epicotyl fresh wt (g)	2.91 ± 0.10	1.64 ± 0.08
Epicotyl dry wt (g)	0.88 ± 0.03	0.48 ± 0.02
Hypocotyl		
Xylem fresh wt (g)	0.29 ± .01	0.29 ± 0.02
Xylem dry wt (g)	0.12 ± 0.01	0.08 ± 0.01
Bark fresh wt (g)	0.24 ± 0.01	0.42 ± 0.02
Bark dry wt (g)	0.08 ± 0.01	0.15 ± 0.01

are shorter and smaller in cross-sectional area. The decrease in the lumens of tracheids from Ethrel-treated material is mostly due to an increase in the thickness of the cell wall (Table II). The ratios of control to Ethrel-treated lumen size is 1.39, and the reverse ratio of cell wall material is 1.28. Thus, almost all of the decrease in cell lumen size can be accounted for by the increase in cell wall thickness. There is also an increase in the total cross-sectional area of the xylem occupied by resin canals in the Ethrel-treated seedlings (Table II). Computer reconstructions of the resin canal lumens and tracheid lumens in the cross-sections of both

Table II. *Anatomical Data of 6-Month-Old Loblolly Pine Seedlings*

Each datum represents the mean of four scan fields per section of each of 10 sections (one/plant) followed by its SE.

	Control	Ethrel
Cross-sectional area of bark as a % of the total cross-section area	38.0 ± 0.7	68.2 ± 1.9
Cross-sectional area of xylem as a % of the total cross-sectional area	62.0 ± 0.9	31.8 ± 1.9
Cross-sectional area of resin canals as a % total cross-sectional xylem area	9.7 ± 0.5	16.9 ± 1.1
Tracheid cross-sections area (μm^2)	160.6 ± 19.2	115.7 ± 4.6
Tracheid length (μm)	966.5 ± 13.2	695.9 ± 25.4
Area of sectioned cell wall matrix (μm^2)[a]	12,488 ± 322	15,895 ± 472

[a] This measurement is related to the thickness of the cell walls.

Ethrel-treated and control hypocotyls, and their respective cross-sections are compared in Figures 3 and 4. It should be noted that the computer reconstructions are not faithful replicates of the shapes or areas of the original images. This is because of the low resolution nature of the reconstruction sub-routine of the program, but this does not interfere with the accurate measuring function of the program. The reconstruction is only used for comparison with the original image to insure that the scan has recognized and measured all of the desired topographical features.

The decrease in tracheid dimensions and increases in resin canals in response to Ethrel treatment is in agreement with previous reports (2, 10). It is of interest to note the reversal in the ratio of wood to bark production (Table II; Fig. 3) at the concentration of Ethrel used in this experiment. These results are different than those reported by Barker (2) in saplings of *Pinus radiata* L. treated with lower concentrations of Ethrel. He reported an increased production of both xylem and phloem in roughly the same proportions in reponse to the Ethrel treatment. Ethylene is known to inhibit the basipetal transport of auxin (1). In woody plants, auxin activates xylem formation but has no effect on phloem initiation. GA initiates the formation of both phloem and xylem. High auxin-to-GA ratios favor xylem development, while low auxin to GA ratios favor phloem development (3, 4, 8). Although the previous work on auxin to GA levels has been on woody angiosperms, it is possible that a similar system functions in gymnosperms. The high levels of Ethrel used in this experiment may have affected the auxin to GA ratios by reducing auxin transport, resulting in a reduced xylem production and favoring phloem differentiation. It is not now apparent if the Ethrel-produced ethylene is directly responsible for the increased cortical tissue or if this is also a result of an imbalance in auxin to GA ratios induced by the treatment. The stimulation of xylem production by low concentrations of Ethrel, reported by Barker (2) in *P. radiata*, appear to be a direct ethylene effect. The changes observed here in a reduction of xylem production with an increase in phloem and cortical cells may involve more complicated phytohormonal interactions. The understanding of the role of ethylene in the differentiation of the vascular cambium of woody plants is necessary in interpreting the response of trees to environmental stresses. This is especially true, in the light of the increasing number of reports of stress-induced endogenous ethylene production.

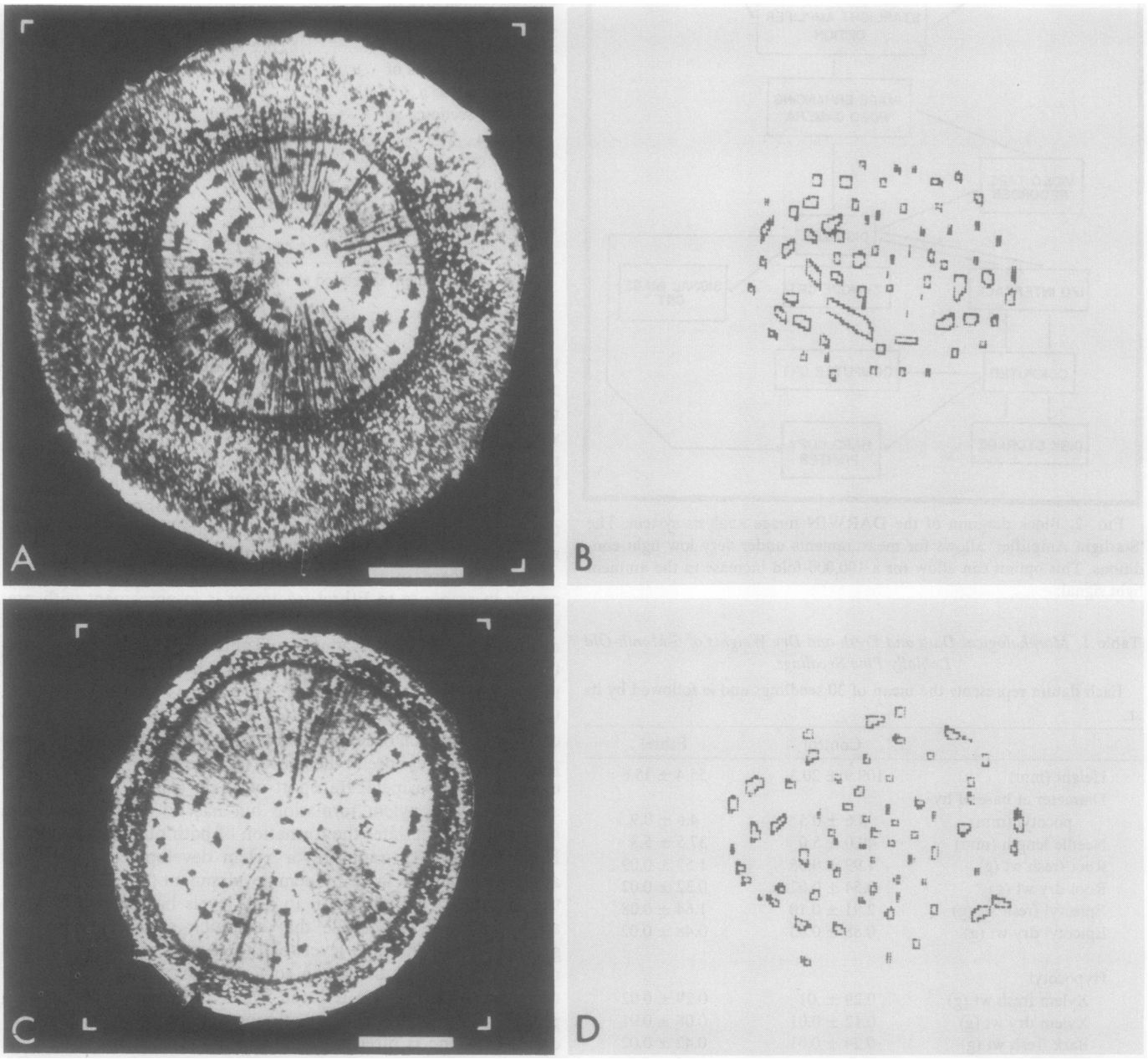

FIG. 3. Comparison of control (A) and Ethrel-treated (C) hypocotyl cross-sections and their computer-generated reconstructions of resin canals in the cross-sections (B and D). The white corners delimit the scanning 'windows.' The white bar represents 1 mm.

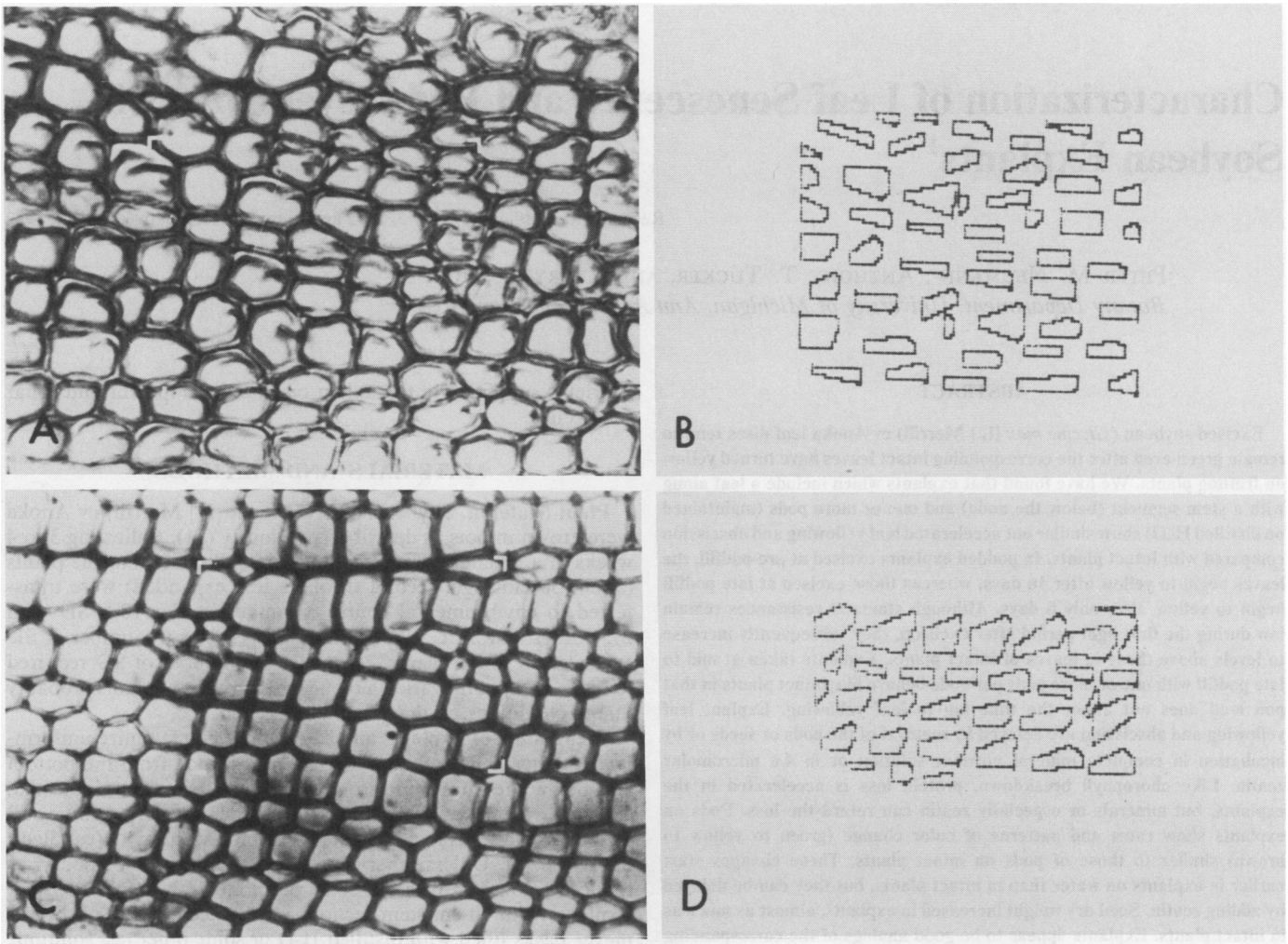

FIG. 4. Comparison of control (A) and Ethrel-treated (C) hypocotyl cross-sections and their computer-generated reconstructions of trachaid lumens in the cross-sections (B and D). The white corners delimit the scanning 'windows.' Magnification, × 400.

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