Plant Physiol. (1971) 47, 595–599

Changes in Total Nitrogen, Soluble Protein, and Peroxidases in the Expanding Leaf Zone of Eastern Cottonwood

Received for publication July 20, 1970

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ABSTRACT

Nitrogen content and soluble protein and anodal peroxidase banding in acrylamide gel changed with leaf and internode development in the expanding leaf zone of eastern cottonwood (*Populus deltoides* Bartr.). Nitrogen per unit leaf area was high near the apex and decreased to a constant value at the sixth node below it. Soluble protein banding was qualitatively similar for leaves and internodes in this zone, but anodal peroxidases differed between leaves and internodes. The major leaf peroxidase band was absent from the second leaf below the apex but present in the fourth and sixth leaves; its appearance and intensification seemed to parallel the development of photosynthetic activity. The major internode peroxidase band was present in the apex, second, fourth, and sixth internodes, and intensified during internodal development. It is suggested that these two "isoenzymes" may have different functions *in vivo*.

Photosynthetic efficiency of and patterns of photosynthate distribution from eastern cottonwood (*Populus deltoides* Bartr.) leaves vary regularly with leaf age and stem position and are related to plant development (7). Of particular developmental importance is the portion of the plant in which all leaf expansion and internode elongation occur. In a single-stemmed cottonwood seedling, this zone is bounded by the shoot apex and the first fully expanded leaf. Near the lower boundary, leaf photosynthetic efficiency reaches a maximum and the proportion of current photosynthate exported downward from the leaf begins to exceed the proportion exported upward. These events immediately precede the appearance of secondary vascular tissue in the petiole and stem.

The physiological, biochemical, and anatomical correlations between leaf and stem growth in this zone are not well understood. The objectives of the study reported here were to compare biochemical changes in leaves and stem internodes in the zone of leaf expansion and to relate them to changes in organ size and physiology. Specifically, nitrogen content and soluble protein and peroxidase patterns in acrylamide gel were compared for leaves and internodes taken serially below the apex. In addition, similar data were obtained for leaves and internodes taken at different times during development at one stem position from several seedlings. Work with other species (2, 14–16, 19, 21) has related changes in nitrogen content and soluble protein and isoenzyme patterns to development. Peroxidase was chosen because of its postulated role in lignification (*e.g.*, 17), hormone metabolism (18), and its apparent inverse quantitative relationship with growth (6, 13). Evidence is presented in this paper indicating close relationships between changes in the biochemical parameters measured and the development of leaves and internodes in eastern cottonwood seedlings.

METHODS

Cottonwoods were grown from seed to harvest in growth rooms under constant conditions as in a previous study (7). To maintain uniformity, the cotyledons and the four leaves immediately above them were removed and leaves were numbered from the apex to the first remaining basal leaf. The "apex" was defined as the apical dome and young appressed leaves. The oldest leaf less than 2 cm long was designated as the first leaf below the apex.

Leaf and internode size and nitrogen concentration were determined within the zone extending nine nodes below the apex, for seedlings having 9 and 18 total leaves. To compare results from the 9- and 18-leaf seedlings with a true aging series, leaves at the 10th node from the base and their subtending internodes were harvested from a series of seedlings, beginning with 10-leaf seedlings (Fig. 1). This series is called the "horizontal" series, to distinguish it from the 9- and 18-leaf vertical series. This study was replicated three times, using a total of 30 seedlings.

Plant parts were weighed immediately after harvest and again after 24 hr in a 70 C oven. The leaves were traced before drying, and their areas were determined with a dot grid. Nitrogen was determined by the Dumas method, with a Coleman semimicro nitrogen analyzer. The dried material was ground either in a Wiley mill or a Duall homogenizer and combusted immediately. During the experiment, glycine (Mann Research, analysis 18.4% nitrogen) standards were run daily. The mean of 19 such determinations was 18.4% nitrogen with a standard error of 0.08.

Soluble protein and peroxidase electrophoretic patterns were determined, on a second set of seedlings, for a similar but restricted design. The apex, and the 2nd, 4th, and 6th leaves and subtending internodes were taken from seedlings having 9, 11, 13, 15, and 18 leaves. For each determination, 4 seedlings were pooled; 20 seedlings were used in this portion of the study. Samples from each seedling size were prepared and run on the same day. Immediately before electrophoresis, four freshly harvested plant parts from each

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sample position were ground together in a Duall homogenizer at 4 C in pH 7.4, 0.05 M tris-HCl buffer, 0.1 M in sucrose, and then centrifuged at 0 C for 1 hr at 105,000*g*. Electrophoresis (1) of the 105,000*g* supernatant fraction in a discontinuous acrylamide gel slab was done immediately after centrifugation. Soluble protein concentration of the supernatant fraction was estimated (11), with bovine serum albumin dissolved in extraction buffer as a standard, and 0.1 ml of supernatant fraction was applied to each gel slot. One gel slab accommodated three replications each of the extract of the apex and the 2nd, 4th, and 6th leaves and internodes.

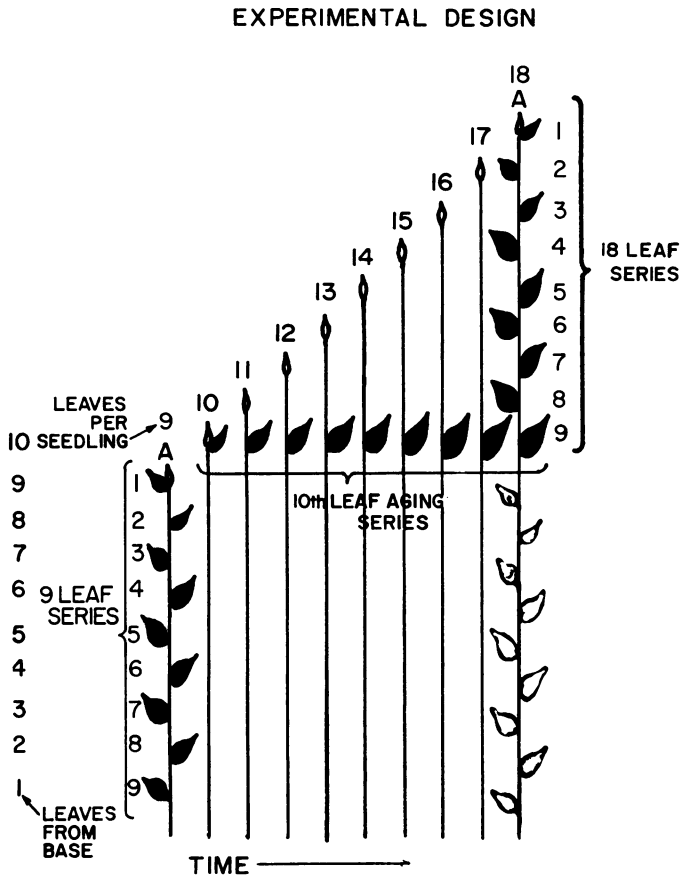

FIG. 1. Experimental design for sampling the expanding leaf zone of cottonwood seedlings having 9 and 18 total leaves, and for an aging sequence at the 10th node. Leaves rendered black, together with their subtending petioles and internodes, were harvested from each of three replications for determination of size and nitrogen content.

Total protein was stained in the gels with Coomassie brilliant blue (2.5 g/liter in 5:5:1 water-methanol-acetic acid) for 30 min and then destained for 48 hr in 5:5:1 water-methanol-acetic acid circulated through charcoal. Peroxidase activity was located with *o*-dianisidine by the method of Brewbaker *et al* (3). Densitometer traces of all gels, prepared with an E-C densitometer, were used for comparing staining intensity among bands. R_F values were calculated with bromophenol blue as a front marker. Peak areas were calculated for the major leaf and stem peroxidase bands and were used as indicators of peroxidase activity.

RESULTS

Organ size, nitrogen content, and electrophoretic patterns of soluble protein and peroxidase exhibited related quantitative developmental trends. Leaf petiole and internode dry weights (Table I) and leaf area (Table II) were similar in that they increased down to the eighth or ninth node in the 18-leaf seedlings and the horizontal sequence, and down to the fifth to seventh nodes in the 9-leaf seedlings. Fresh and dry weight trends were similar; therefore, only dry weights are presented. Internode and petiole length, however, increased only down to the fifth and sixth nodes in the 18-leaf seedlings and in the horizontal sequence, and only to the fifth node in the 9-leaf seedlings (Table II). On the smaller seedlings, leaves and internodes matured more quickly, whereas the leaves and internodes of the older seedlings eventually became larger. Because all leaves of the 9-leaf seedlings were included, their oldest leaves were probably past their peak of photosynthetic efficiency, but they had not yet begun to yellow or abscise.

Leaf and internode nitrogen contents, expressed as percentage of dry weight, were similar for all three developmental sequences (Table III). Leaf nitrogen concentration decreased down to the sixth node below the apex. Petiole and internode nitrogen concentrations varied less regularly with

Table I. *Leaf, Petiole, and Internode Dry Weights for Expanding Leaf Zone of Cottonwood Seedlings*

"Vertical" refers to organs taken serially down from the apex from seedlings with 9 and 18 leaves. "Horizontal" refers to samples taken from a single stem position from seedlings of different sizes (see Fig. 1 and text). All data in this and Tables II and III are means of three replications.

Sequence and Plant Part	Apex[1]	Dry wt at Node:								
		1	2	3	4	5	6	7	8	9
		mg								
Vertical										
9 Leaf	3.6	5.9	16.8	36.1	76.1	90.0	86.5	81.1	50.8	32.4
Petiole				2.5	4.4	4.7	4.3	3.7	2.2	1.3
Internode				3.7	6.4	8.8	9.6	10.8	8.0	6.0
18 Leaf	10.4	8.3	18.1	24.0	49.8	94.9	130.0	174.2	193.0	190.0
Petiole				3.1	5.3	8.1	9.6	10.5	11.1	10.7
Internode				6.2	9.4	14.8	19.1	22.8	27.9	29.9
Horizontal										
Leaf	3.6	7.0	18.1	33.2	64.5	121.3	132.3	138.5	186.8	190.0
Petiole				3.1	4.8	9.3	8.1	8.9	10.2	10.7
Internode				5.0	10.8	15.3	21.4	22.5	32.8	29.9

[1] Apex as defined in "Methods."

Table II. *Leaf Area and Petiole and Internode Length for Expanding Leaf Zone of Cottonwood Seedlings*

Sequence and Plant Part	Node								
	1	2	3	4	5	6	7	8	9
Vertical									
9 Leaf area, cm²	0.9	3.8	13.6	30.1	37.1	35.0	29.7	18.8	11.4
Petiole, cm			1.8	2.5	2.8	2.7	2.4	1.7	1.1
Internode, cm			0.9	1.4	1.5	1.4	1.3	0.9	0.7
18 Leaf area, cm²	0.6	2.3	5.7	15.1	32.3	45.3	63.0	68.2	69.1
Petiole, cm			2.1	2.8	3.3	3.7	3.9	3.7	3.6
Internode, cm			1.0	1.4	1.8	1.9	1.9	1.9	1.9
Horizontal									
Leaf area, cm²	1.1	3.6	9.9	21.6	40.6	50.7	76.4	65.1	69.1
Petiole, cm			1.9	2.7	3.8	3.5	3.5	3.5	3.6
Internode, cm			0.9	1.7	2.2	2.0	2.1	2.2	1.9

Table III. *Leaf, Petiole, and Internode Nitrogen Content for Expanding Leaf Zone of Cottonwood Seedlings*

Sequence and Plant Part	Apex	Nitrogen Content at Node:								
		1	2	3	4	5	6	7	8	9
		% dry wt								
Vertical										
9 Leaf	7.2	6.5	6.0	6.0	4.9	4.2	3.9	3.8	3.5	3.4
Petiole				3.6	2.5	2.6	2.8	4.1	3.6	9.1
Internode				2.6	4.3	4.5	4.2	3.9	3.6	2.9
18 Leaf	7.2	5.5	5.6	5.6	5.4	5.1	4.0	3.7	3.8	3.7
Petiole				4.9	3.0	3.0	3.0	2.4	2.4	2.7
Internode				3.6	3.1	3.2	3.7	3.4	2.8	3.6
Horizontal										
Leaf	7.2	5.2	5.7	5.5	4.8	4.6	3.8	3.4	3.5	3.7
Petiole				3.0	2.4	2.5	2.6	2.0	1.7	2.7
Internode				3.6	3.1	3.2	3.7	3.4	2.8	3.6

node number, although petiole nitrogen concentrations increased sharply at nodes 7 through 9, particularly in the 9-leaf seedlings. However, patterns of nitrogen per unit of leaf area were similar for all three sequences and values were constant and nearly identical from the sixth through the ninth nodes, indicating that there was no massive net loss of nitrogen even from the lower leaves of the 9-leaf seedlings.

The electrophoretic separation of protein soluble in alkaline buffer was done for similar plants but for fewer plant parts. However, trends were apparent (Fig. 2). Qualitatively, protein banding patterns differed little between leaves and internodes, among developmental stages, or among seedlings of different sizes. However, although the total number and relative position of the bands changed little with seedling size, quantitative differences were evident. The staining intensity of the most densely staining bands increased as seedling size increased. Also, as leaf age increased, staining intensity of the most prominent band increased. This was not due solely to an increase with age in the total amount of protein applied per gel slot (see quantities of protein per slot in Fig. 3).

In contrast to total protein, leaf and internode peroxidase patterns differed qualitatively. The major peroxidase band occurred between R_F 0.3 and 0.4 in leaves and between R_F 0.5 and 0.6 in internodes (Fig. 2). No activity in the position in the major leaf band was detectable in either the apex or the second leaf, although densely staining bands were present in the fourth and sixth leaves. Intensification with development is shown in Figure 3, in which densitometer peak area per unit of soluble protein applied to the gel has been plotted over node number for both the internode and leaf enzyme with all seedling sizes pooled. Intensification of activity with organ development appeared earlier in the major internode band and with greater activity per unit of protein. The major internode peroxidase was present in detectable quantities in the apex and the second, fourth, and sixth internodes, whereas it was barely discernible in the second leaf, and undiscernible in the fourth or sixth leaves, suggesting that isoenzyme was lost during leaf development. Two and sometimes three additional bands of peroxidase activity were present in at least trace amounts at high R_F values in all samples. The intense staining of the application slots of fourth and sixth leaves suggests the presence of peroxidases with isoelectric points at or slightly above pH 8.9. However, when extracts from the fourth and sixth leaves were run toward the cathode, no distinct bands of peroxidase activity appeared. The application slots were again intensely stained.

DISCUSSION

The pattern of developmental change in tissue nitrogen, soluble protein, and anodal peroxidases were delineated for a growth zone of eastern cottonwood where events critical to vascular development occur. When organ size, nitrogen content, and protein and peroxidase changes were considered simultaneously with respect to development, the following sequence of events appeared. For the 18-leaf seedlings, leaf area and weight reached a maximum at the eighth internode below the apex. However, petiole and internode length as

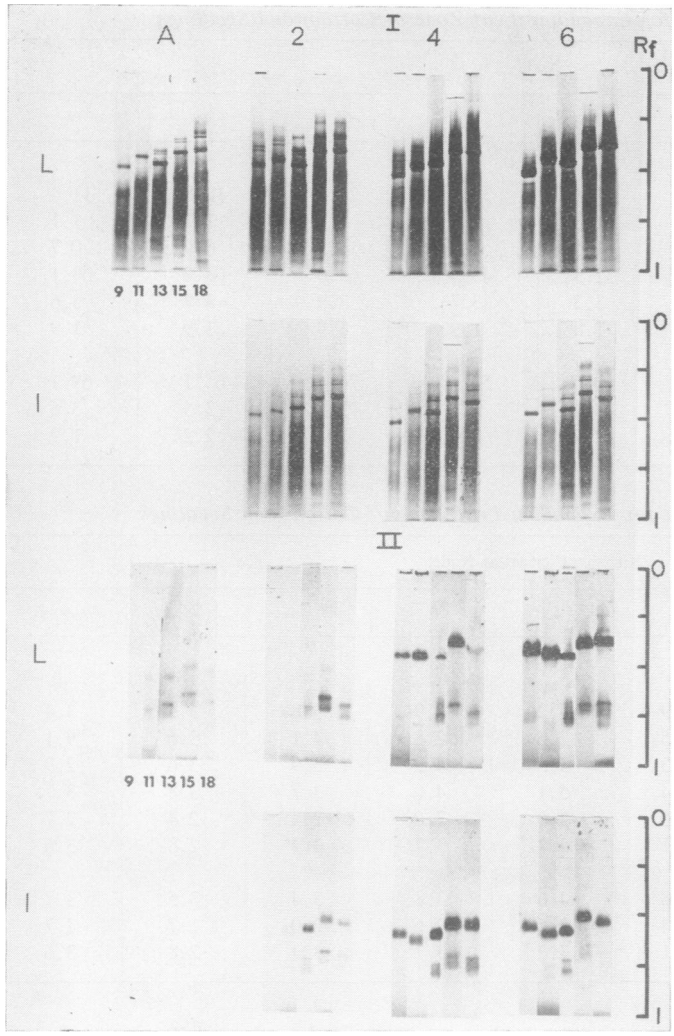

FIG. 2. Soluble protein (I) and peroxidase (II) electrophoretic patterns for the apex, and the 2nd, 4th, and 6th leaves (L) and internodes (I) below it, from cottonwood seedlings having 9, 11, 13, 15, and 18 total leaves. R_F scale spans the distance between application slot and electrophoretic front. All runs were toward the anode. One of three replications is presented.

well as nitrogen per unit of leaf area and per unit of dry weight reached constant values at the sixth internode. Nitrogen per unit of leaf area, in particular, remained constant at about 10 mg/dm² below the sixth internode in all three developmental series. Thus, a constant nitrogen concentration was established in both leaf and stem tissues approximately at the position at which internode elongation stopped, even though leaf expansion apparently continued through two more plastochrons. Just before the constant ratio of nitrogen to leaf area was established, a marked change in leaf peroxidase patterns occurred. A faint, minor band in the position of the major internode band disappeared and what subsequently was the major leaf band appeared. The sequence was thus: peroxidase change in leaves → constant ratio of nitrogen to tissue and cessation of internode and petiole elongation → cessation of leaf expansion. This cannot be regarded as a sequence of cause and effect, but rather indicates the order of developmental transitions.

Soluble protein patterns revealed by staining with Coomassie blue were qualitatively similar in all parts of the expanding leaf zone examined, indicating the general metabolic similarity between leaves and internodes in this zone. Other organs of eastern cottonwood, roots and older internodes, for example, yield different total protein banding patterns (Gordon, J. C., unpublished). Vascularization and cell expansion and elongation are occurring in both leaves and internodes, so that some similarity of protein complement is to be expected. However, as suggested for organs of *Tulipa* by Barber and Steward (2), quantitative differences in soluble protein bands between leaves and internodes probably indicate the relative importance of particular proteins or groups of proteins to the differentiation of leaves and stems.

Protein and, by implication, metabolic differentiation between leaves and internodes are further indicated by the different gel position of the major anodal peroxidase band of each. Interestingly, the position of the major leaf peroxidase coincided with the most densely staining protein band revealed by Coomassie blue. This band may have been the "Fraction 1" chloroplast protein reported by Laycock *et al.* (10). Macnicol (12) has shown that the major leaf and stem peroxidases of Alaska pea (*Pisum sativum* L.) are not likely to be isoenzymes in the proper usage of the term, and he has suggested a photosynthetic role for the leaf peroxidase. Results from our laboratory (D. I. Dickmann, unpublished) indicated that net photosynthetic activity was absent in the second leaf down from the apex, where it was present in the third and fourth leaves. The appearance of photosynthetic activity thus roughly coincided with the appearance of the major leaf peroxidase band.

From the data of this study and from demonstrations of some substrate specificity in "isoperoxidase" of other species (4), it is possible to hypothesize that in eastern cottonwood, as in pea (12, 14), major leaf and stem peroxidases

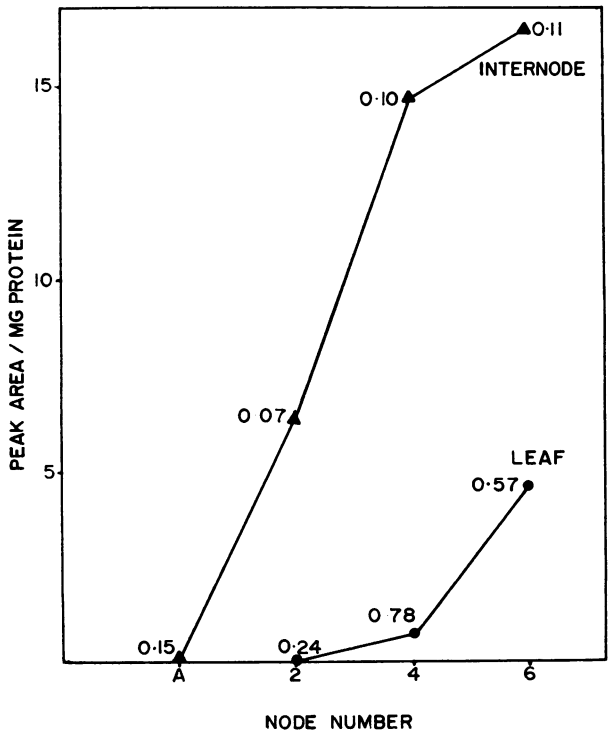

FIG. 3. Area of major leaf and internode peroxidase densitometer peaks per unit of soluble protein applied to the gel, plotted with node number down from the apex. Points are means, with all seedling sizes pooled. Mean quantity of protein (mg) applied per gel slot is indicated by each point.

have different functions *in vivo*. Several reports relate isoperoxidase induction and repression to hormone action *in vitro* (8, 9, 20), and others implicate peroxidase in lignification and IAA destruction (17, 18). More detailed information on the specific developmental roles of peroxidase is not available beyond the studies that relate high peroxidase activity to reduced growth (5, 13). Experiments to explore further the developmental role of peroxidase in eastern cottonwood are in progress.

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