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Role of Cytokinin in Differentiation of Secondary Xylem Fibers

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ABSTRACT

The differentiation of secondary xylem fibers was studied in cultured hypocotyl segments of *Helianthus annuus* L. It is shown that cytokinin is both a limiting and controlling factor in the early stages of fiber differentiation. In the absence of kinetin or zeatin, there was no fiber differentiation. However, cytokinin could induce fiber differentiation only in the presence of indoleacetic and gibberellic acids. First fibers were observed in the tissue after 12 days in culture, and their number increased linearly during the following 2 weeks. At low cytokinin levels, there was a positive correlation between cytokinin concentration in the medium and the number of fibers formed in the explants. A similar correlation was also found at low gibberellic acid concentrations. At high concentration, zeatin was more effective than kinetin. It seems that later stages of fiber differentiation can occur in the absence of cytokinin. It is proposed that the mechanism which controls and determines the early stages of fiber differentiation is based on an interaction of three major hormonal signals: indoleacetic acid plus gibberellic acid from the leaves with zeatin from the root apices.

Fiber differentiation is dependent on signals originating in the leaves (1, 2, 16). The signals for fiber differentiation flow in a strictly polar fashion from the leaves to the root and induce fibers along their flow (1, 2). It has recently been shown that the role of the leaves in primary phloem fiber differentiation in *Coleus* can be fully replaced, both qualitatively and quantitatively, by exogenous application of combined IAA and GA_3 (3).

The nucleus of the fiber cell divides several times during fiber development (7, 8, 11, 14). Nuclear divisions may be followed by cytokinesis (14, 15). This general phenomenon, occurring early in the gradual process of fiber differentiation, indicates that cytokinin, a known cell division factor, is probably another signal involved in fiber differentiation. As cytokinins are produced by root apices (9, 20), it is suggested that the mechanism controlling differentiation of fibers combines an interaction between leaf and root signals.

To investigate the effect of cytokinin on fiber differentiation in the stem, a root-free experimental system is required. Fiber differentiation is a gradual process occurring over a period of a few weeks, while the formation of adventitious roots takes only a few days. To overcome this problem, small hypocotyl segments grown on solid medium were used, in which fiber differentiation could be induced without accompanied root formation. In addition, this *in vitro* system enabled the study of the direct effect of cytokinin on fiber differentiation with no possible indirect effect of cytokinin mediated by increasing the physiological levels of IAA and GA_3 produced by the leaves.

The work reported here is an attempt to elucidate the role of kinetin and zeatin in the differentiation of secondary xylem fibers in cultured hypocotyl segments of *Helianthus*.

A preliminary account of some of these findings was published previously (5).

MATERIALS AND METHODS

Hypocotyl segments of *Helianthus annuus* L. seedlings were used in this study. Seeds were sterilized with 5% NaOCl for 15 min, rinsed repeatedly with sterile distilled H_2O, and incubated in humid sterile Petri dishes for 3 d in the dark. Hypocotyl segments 5 mm long were longitudinally divided and each half was placed with the longitudinally cut surface down in contact with the medium. The explants were placed in culture tubes (25 mm diameter, 75 mm long) containing 8 ml of Murashige and Skoog (13) medium and maintained at a temperature of 25 ± 1°C in the dark. If adventitious roots appeared, they were removed.

In each experiment, 10 one-half hypocotyl segments were used per treatment. All the experiments were repeated three times. Kinetin was used in all the experiments. Zeatin was used in the experiment shown in Figure 4.

The hypocotyl segments were harvested at various intervals during the 40-d culture period of the time course experiment (Fig. 1). Experiments on the effects of the growth regulators lasted 30 d (Figs. 3 and 4). To determine cytokinin requirement during fiber differentiation, the 30-d experimental period was divided into two subculture periods of 15 d, during which time the explants grew in either the presence or absence of kinetin (Table I).

Thin longitudinal hand sections were prepared from the explants which had been fixed with FAA (formalin-alcohol-acetic acid). The sections were stained with 2% lacmoid in 96% ethanol for 2 to 5 s, washed with tap water, and then mounted on slides in 60% sodium lactate for observation (4).

The secondary xylem fibers were counted under light micro-

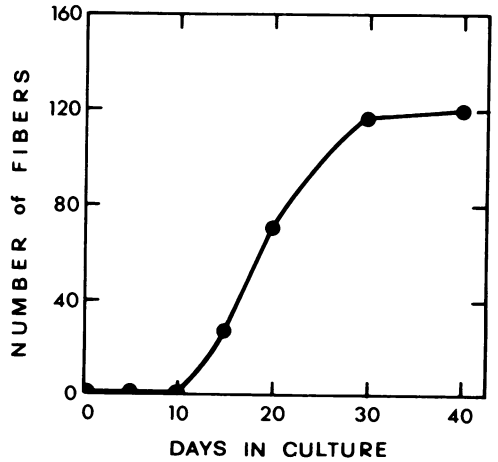

FIG. 1. Time course of secondary xylem fiber differentiation in hypocotyl segments of *Helianthus* cultured on medium containing 0.5 μg/ml IAA, 0.2 μg/ml kinetin, 0.1 μg/ml GA_3, and 3% (w/v) sucrose. Each point represents an average of five replicates from 10 explants.

scope. The area to be counted was defined as the field of vision at 200-fold magnification. In each explant, the average number of fibers was calculated from five random counting areas. All counts and measurements were made from coded slides to eliminate possible subconscious bias. Statistical terminology and tests of significance were by Students' *t* test and according to Sokal and Rohlf (18).

RESULTS

Time Course of Secondary Xylem Fiber Differentiation in Culture. Figure 1 shows that the course of secondary fiber development in hypocotyl segments of *Helianthus* grown *in vitro* can be divided into three stages. The first phase lasts 10 to 12 d during which many cell divisions occur in the explant. The cambium and the fiber initials are formed in this phase, but no fibers with secondary walls can be detected. The second phase is characterized by a linear increase in the number of secondary xylem fibers and lasts about 2 weeks. The last phase is the stationary stage, in which overall growth of the explants, as well as any further differentiation of xylem fibers tapers off. However, in the latter phase, new xylem fibers continue to differentiate in the callus which formed on the surface of the hypocotyl segments.

In the middle of the explants, fibers were oriented in the longitudinal direction, while at the periphery of the hypocotyl segments and in the callus formed at their ends, the xylem fibers differentiated in nodules. Secondary xylem fibers were easily detected by their lignified walls stained blue by the lacmoid and by their typical small pits compared with the reticular secondary wall thickenings formed in the tracheary elements (Fig. 2).

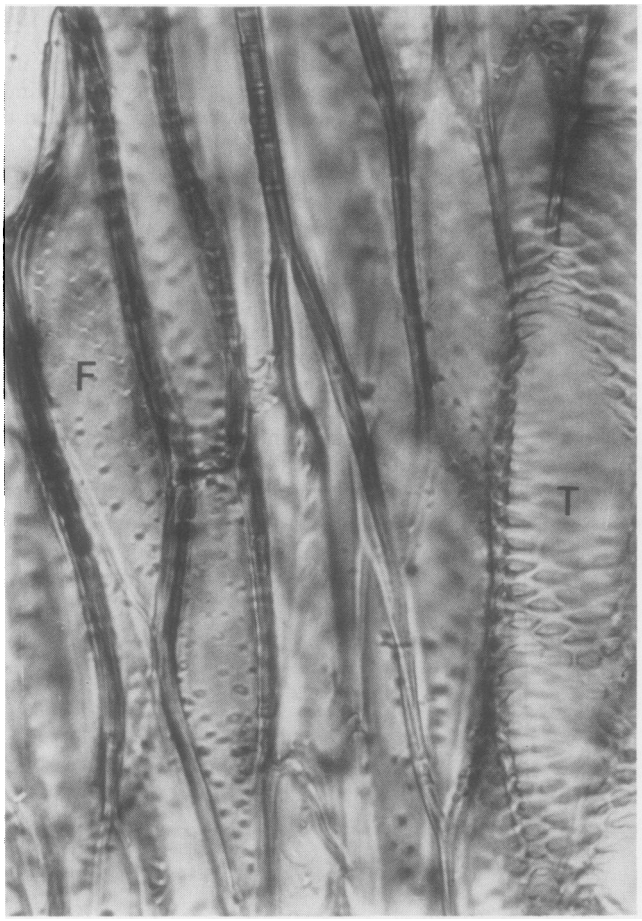

FIG. 2. Longitudinal view of secondary xylem fibers in thick and stained tissue prepared from a 30-d-old cultured hypocotyl segment of *Helianthus*. The tissue developed on the same medium reported in Figure 1. The fibers are recognizable by their thick walls with small pits compared with the reticulated wall thickenings of the tracheary elements. F, fiber; T, tracheary element. (× 600)

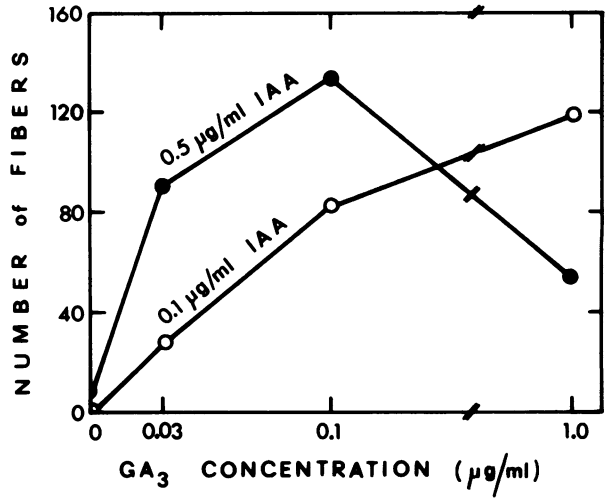

FIG. 3. Effect of GA_3 on the differentiation of secondary xylem fibers in cultured hypocotyl segments of *Helianthus* after 30 d in the presence of 0.1 μg/ml IAA or 0.5 μg/ml IAA. Kinetin concentration in the medium was 0.2 μg/ml. Each point represents an average of five replicates from 10 explants.

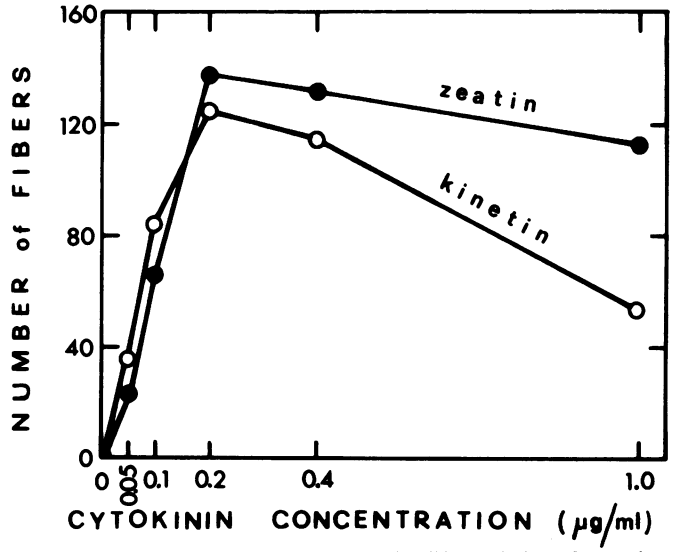

FIG. 4. Effect of zeatin and kinetin on the differentiation of secondary xylem fibers in cultured hypocotyl segments of *Helianthus* after 30 d in the presence of 0.5 μg/ml IAA and 0.1 μg/ml GA_3. Each point represents an average of five replicates from 10 explants. At high concentration (1.0 μg/ml), the difference between the number of fibers induced by zeatin and kinetin was significant ($P < 0.05$).

Effect of IAA and GA_3 on the Differentiation of Secondary Xylem Fibers. To study the role of cytokinin in xylem fiber differentiation, it was necessary to determine the optimal concentrations of IAA and GA_3 in the medium. It was found that IAA alone in the presence of cytokinin, sufficed to cause the differentiation of a few secondary xylem fibers. Figure 3 shows that at low auxin concentration (0.1 μg/ml) there is a positive correlation between GA_3 concentration in the medium and the number of secondary xylem fibers formed in the explants. The optimal medium for fiber differentiation contained 0.5 μg/ml of IAA at 0.1 μg/ml GA_3.

Table I. *Effect of Cytokinin on Secondary Xylem Fiber Differentiation in Vitro over a 30-Day Culture Period*

Values are mean ± SE. Sample size was 10 for each treatment. There was no significant difference in the number of secondary xylem fibers under treatments A and C. This was also true for the final fresh weight under any of the four treatments. Kinetin concentration was 0.2 μg/ml. All culture media contained 0.5 μg/ml IAA and 0.1 μg/ml GA_3.

Treatment	First 15 d	Last 15 d	No. of Fibers	Final Fresh Wt
				mg
A	+ kinetin	+ kinetin	123.4 ± 13.7	994 ± 291
B	− kinetin	+ kinetin	0 ± 0	613 ± 179
C	+ kinetin	− kinetin	117.5 ± 15.3	736 ± 256
D	− kinetin	− kinetin	0 ± 0	595 ± 164

Effect of Cytokinin on Secondary Xylem Fiber Differentiation. Both zeatin and kinetin were found to stimulate the differentiation of fibers in the explants, but only in the presence of IAA and GA_3 (Fig. 4). In the absence of cytokinin, there was no fiber differentiation whatsoever even in those explants that showed adventitious root formation. Increase in concentration of any of the cytokinins up to 0.2 μg/ml boosted the number of xylem fibers in the hypocotyl segments. The effects of zeatin and kinetin on fiber differentiation were similar at low concentrations, but different at high concentration. At 1.0 μg/ml, the number of fibers induced by kinetin was much lower than for zeatin (Fig. 4).

Table I demonstrates that for fiber differentiation to occur in the explants, cytokinin is required during the first 2 weeks of culture. In the absence of kinetin in the first subculture period, no fibers differentiated (treatment B). Evidently, the absence of cytokinin limits fiber initiation on the profibrilar phase when many nuclear divisions were observed in the tissue. On the other hand, differentiation of many secondary xylem fibers continued in the absence of cytokinin in the last 2 weeks (treatment C), suggesting that there is no requirement for cytokinin in the later stages of fiber maturation.

DISCUSSION

The present study is the first to illustrate the role of cytokinin as a limiting and controlling factor in fiber differentiation. In the absence of cytokinin there was no fiber differentiation. Cytokinin, in the presence of auxin and gibberellin, stimulated the differentiation of a considerable number of secondary xylem fibers in cultured hypocotyl segments of *Helianthus*. Maity *et al.* (12) found that kinetin increased the length of secondary xylem fibers in cultured wood of *Adhatoda vasica*. It seems that the basic role of cytokinin as a main factor in fiber differentiation could not be ascertained earlier because most of the previous experiments were done on plants with intact roots (3, 16, 17, 19).

Cytokinins were found to be involved in the control of vascular differentiation with both zeatin and kinetin inducing tracheary element formation in cultured explant pith of *Lactuca*, the zeatin being more effective (6). Zeatin stimulated phloem regeneration in rootless *Coleus* stumps, whereas kinetin was ineffective (10). In the present study, zeatin and kinetin exerted similar effects on secondary xylem fiber differentiation, but at high concentrations zeatin was found to be more effective.

IAA and GA_3, produced by the leaves, are known to control fiber differentiation (3). The present results show further that early stages of fiber differentiation are cytokinin dependent. Thus, the differentiation of fiber initial seems to be controlled by an interaction between root signal with leaf signals. This study also shows that advanced stages of fiber maturation in *Helianthus* can occur in the absence of cytokinin.

The differentiation of nodules of xylem fibers in the callus formed on the ends of cultured hypocotyl segments is probably due to the growth conditions which exist in the culture tubes, in which the three hormones supplied through the medium come from one direction only. This result emphasizes the possibility that the organized differentiation of fibers in the intact plant is determined by a two-ended control mechanism, in which polar flow of auxin and gibberellin come from the leaves (3) and cytokinin from the root apices. These signals are part of the general positive feedback control between the shoot and the root tissues, which determines the development and form of the plant. It is therefore suggested that the basis for correlations between the development of the plant body and the differentiation of its supportive tissues is based on their common dependence on the same shoot/root feedback control signals.

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