DIFFERENTIATION OF ISOLATED INTERFASCICULAR TISSUE OF RICINUS COMMUNIS L.

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SUMMARY

From hypocotyls of 6, 7, or 8 days old castor bean seedlings which *in situ* start to develop an interfascicular cambium on the 11th or 12th day, strips of interfascicular tissue were separated from adjacent vascular tissue by means of radially inserted metal blades or were excised and cultured in a basal medium containing mineral salts and sucrose. Since under these conditions cambial development proceeded normally, it is concluded that the vascular tissue does not supply the parenchymatous cells of the interfascicular tissue with a specific inductive factor. Addition of IAA, GA_3, or kinetin to the medium enhanced development. It is argued that this is not the consequence of chemical substitution for a stimulatory influence from the adjacent vascular tissue, but rather a compensation for (i) production of growth substances by the dividing cambial cells, partially leaking out under the conditions of the culture, or for (ii) stimulatory substances originating *in vivo* from the apical parts of the plants.

1. INTRODUCTION

In a previous paper (SIEBERS 1971a) experiments were reported in which small strips of young and undifferentiated interfascicular tissue were excised and replaced in reverse between the vascular bundles of a hypocotyl. The results indicated that the future cambial cells were already determined and the radial polarity impressed upon them many days before the first cambial activity could be demonstrated. On this basis the hypothesis was put forward that the initiation of the interfascicular cambium is completed in a very young stage in the development of the shoot, i.e. the stage in which a primary meristem ring is present.

Initiation at an early stage, however, does not exclude the existence of a stimulatory influence from the vascular tissue on the events in the interfascicular region. That the vascular tissue, e.g. the phloem, can exert a decisive influence on the mitotic activity of surrounding parenchyma cells was demonstrated as early as 1913 by HABERLANDT. Study of the nature of the hormone inducing cell division, present in vascular tissue but absent in pith parenchyma (JABLONSKI & SKOOG 1957), led to the well-known discovery of the cytokinins (MILLER *et al.* 1955). According to SHELDRAKE & NORTHCOTE (1968a, b) cytokinins and auxins can be formed as a result of autolysis in the differentiating vascular tissues. It would not be surprising, therefore, to find a stimulatory influence of hormonal character originating from the vascular tissue and acting on the development of the interfascicular cambium. Such an influence could account for the observation, mentioned in many textbooks of plant anatomy, that formation of the

interfascicular cambium starts in the vicinity of the vascular bundle and proceeds to the space between the bundles.

The simplest way of demonstrating the existence of such an influence would seem to be to follow the development of interfascicular tissue after its separation from adjacent vascular tissue by means of two radially inserted strips of impermeable material, but this method does not exclude diffusion of substances from other directions than the tangential. A more reliable method is to culture isolated blocks of interfascicular tissue in a suitable medium.

If the initiation process can proceed normally under these conditions, the existence of a special stimulatory influence from the vascular bundle would be disproved. If, however, the process is inhibited or delayed, it could be concluded that the vascular tissue is involved in some way in the development in the interfascicular cambium. The addition of phytohormones, such as kinetin or auxin, to the medium to simulate the postulated stimulation, was expected to shed light on the way in which the influence is mediated.

Under the conditions used to culture seedlings the first demonstrable cambial activity in the interfascicular tissue takes place on the 11th or 12th day. The results reported here describe experiments in which use was made of 6, 7, or 8 days old hypocotyls exhibiting the differentiation of only primary vascular tissue.

If it is accepted that the initiation process is already determined at the primary meristem ring stage, it is possible that the vascular bundle exerts its postulated influence in a younger stage of the development of the hypocotyl. Conclusive evidence can then be obtained by using hypocotyls from embryos in which no differentiating vascular tissue is present. The results of culture experiments on this point will be reported in another paper (SIEBERS 1971b).

2. METHODS

2.1. Culture of sterile plants

Castor bean seeds (*Ricinus communis* L., received under the name *R. sanguineus* from Vilmourin-Andrieux, Paris) were sterilized for 2 minutes with a 1 per cent bromine solution. For sterile culture, seeds were placed in a specially designed culture unit (*fig. 1*) consisting of a glass funnel (F) filled with 250 ml gravel resting on a stainless steel gauze disk and mounted on a 250 ml erlenmeyer flask (E) provided with 100 ml Hoagland solution. The funnel reached to 1 cm below the solution level. Between the funnel and an inverted beaker (B) a ring of cotton wool was placed. Via an air inlet (I) plugged with cotton wool air was automatically pumped into the flask twice daily for 10 minutes, thus pushing part of the solution into the gravel, after which intensive aeration was applied. A capillary tube (C) in the air inlet provided for equal pressure in all culture flasks linked to the pump and caused the solution in the gravel to run back when the pump stopped. After autoclaving at 110 °C for 30 minutes, per holder three sterilized seeds were inserted in the gravel. The units, 10 per experiment, were placed in a growth chamber under conditions of light and temperature as already des-

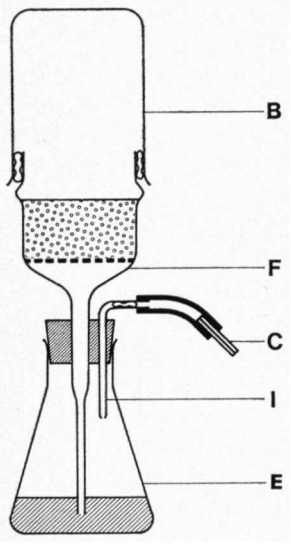

Fig. 1. Sterile culturing unit for seedlings. B = inverted beaker, F = funnel with gauze and gravel, C = capillary tube, I = air inlet, E = flask with Hoagland solution.

cribed (SIEBERS 1971a). After an 8-day culture period 10 seedlings with a hypocotyl length of about 7 cm were selected for use.

2.2. Excision and culture of interfascicular and fascicular tissue blocks

Interfascicular or fascicular tissue blocks about 0.8 mm wide were prepared aseptically under a dissection microscope by making two perpendicular cuts along the diameter of a 2 mm segment taken from the basal part of a hypocotyl. Six segments were prepared from each hypocotyl.

The isolated tissue blocks were transferred to 100 ml erlenmeyer flasks each containing 10 ml culture medium and 2 tissue blocks, cultured under constant stirring on a gyrorotary shaker (60/min) under the same light and temperature conditions as the seedlings. The medium consisted of a half-strength Heller solution fortified with 4 per cent sucrose. The pH was adjusted to 6.0 before autoclaving. All phytohormones were prepared in separate stock solutions, adjusted to pH 6.0, cold sterilized by ultrafiltration, and added to the autoclaved medium.

2.3. Anatomical observation

At the end of the culture period, which varied from 8 to 21 days, the tissue blocks were examined for anatomical differentiation. Since the original longitudinal polarity is considered to be maintained in the isolated tissue blocks (which could be concluded from the fact that differentiation usually had made more progress on the originally basal than on the apical side), per tissue block two hand-cut sections, taken 0.5 mm from the basal and the apical wound surfaces, were studied. For tissue blocks showing characteristic differentiation the remaining

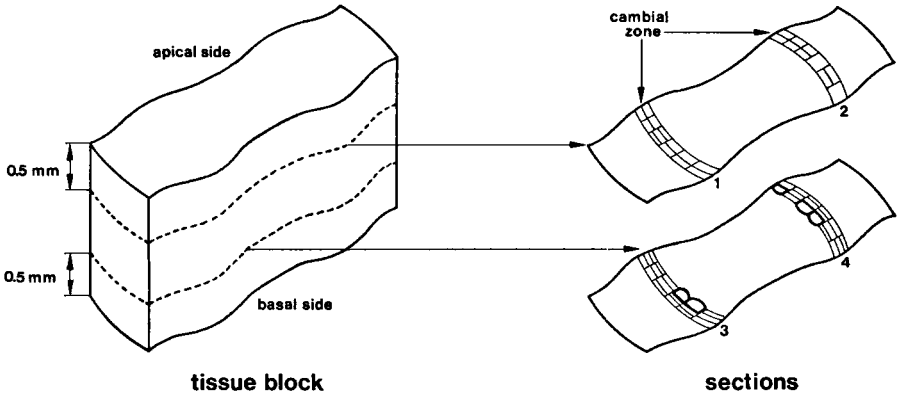

Fig. 2. Schematic representation: (i) tissue block after culturing and (ii) sections showing cambial zones.

central part was fixed, dehydrated, and embedded in Pegosperse 1005 (Glyco Chemicals, New York). Sections (12μ thick) were stained with a triple stain (astra blue, auramine, and safranine) according to MAÁCZ & VÁGÁS (1963). The hand-cut sections were immediately studied to determine type and number of cell walls in the cambial layer and the number of tracheary elements of both cambial and non-cambial origin. Because each section included two parts or zones of the cambial ring, each tissue block offered four zones for analysis (*fig. 2*). Necrotic zones or zones containing an undesired type of tissue as a result of incorrect excision of the tissue block, were discarded.

Since all cambial cells within a zone had passed through a more or less identical number of mitoses, the cambial development per zone was determined by estimation of the mean number of cell walls formed per cambial cell. The secondary xylem elements, which show a more group-wise differentiation, were individually scored and counted per zone, as was the case for the tracheary elements of non-cambial origin. Only those cells were scored as tracheary element that exhibited pit structures in the thickened cell walls. Because of difficulties associated with phloem identification, cambial production was evaluated only on the basis of xylem differentiation.

3. RESULTS

3.1. Isolation by radial strips

Experiments were performed on 6, 7, or 8 days old seedlings. Two pieces of stainless steel razor-blade measuring 2 × 4 mm were radially inserted into the base of a hypocotyl to isolate a block of interfascicular tissue (2 mm high, 0.8 mm wide) from the neighbouring vascular tissue. The best position for the strips proved to be 0.4 mm from either side of a particular epidermal groove, identified from its greater depth just at the level of the cotyledons and followed down-

ward to the experimental area. Anatomical analysis 9 days after the operation revealed that the operation was without any consequence for cambium initiation and further differentiation.

3.2. Preliminary culturing experiments

In a series of preliminary experiments conditions for culturing interfascicular tissue blocks were investigated. In a medium containing only mineral salts and sugar (glucose 2 per cent) no growth or differentiation was observed. Therefore, several additions, e.g. casein hydrolysate (50 and 500 ppm), yeast extract (100 and 1000 ppm), vitamins (according to JACQUIOT 1950, and WHITE 1943), and coconut milk (10 per cent V/V) were tested for their ability to improve the results.

Except for the last addition, which caused abnormal differentiation in the sense of a number of disoriented cell divisions in the cambium and a callus-like outgrowth on the wound surface, cambial development was not procured with any of these substances. Replacement of glucose by sucrose and increase of the concentration to 4 per cent resulted in at least a number of cases in roughly normal cambial development and subsequent differentiation of some secondary xylem elements (*fig. 3*). Furthermore, the condition of the parenchymatous cells of especially the pith, which had been mainly colourless and somewhat necrotic, appeared to be improved. During the course of the investigation the results continued to improve steadily, possibly due to greater technical skill facility. Anatomical observations were made after an 8-day culture period. Longer periods did not result in any marked increase in differentiation.

The introduction of vascular tissue to the medium – in the form of two 2-mm thick hypocotyl segments – had no effect on the degree of differentiation in the interfascicular tissue blocks. "Mixed" tissue blocks, which were half interfascicular and half fascicular (*fig. 5*), did not show an earlier or more pronounced development of interfascicular cambium as compared with tissue blocks containing only interfascicular tissue (*fig. 4*).

3.3. Effects of IAA, GA_3, and kinetin on the differentiation of excised and cultured interfascicular tissue blocks

From each main group of growth substances one representative was chosen to test its stimulatory effect on the differentiation of the isolated tissue blocks. The results concerning orientation and mean number of cell divisions in the cambial layer and the number of tracheary elements of both cambial and non-cambial origin are shown in *table 1*.

In most experiments at least some of the tissue blocks were partly or entirely necrotic at the end of the culture period. The percentage of dead tissue blocks varied greatly: from 0 to 40. Tissue blocks cultured with kinetin in the medium were relatively free of necrosis and frequently showed more intensely coloured chloroplasts and anthocyanin-containing vacuoles than did the IAA or GA_3 material.

Without addition of phytohormones, about half of the tissue blocks were capable of some cambial development. The orientation of the newly formed cam-

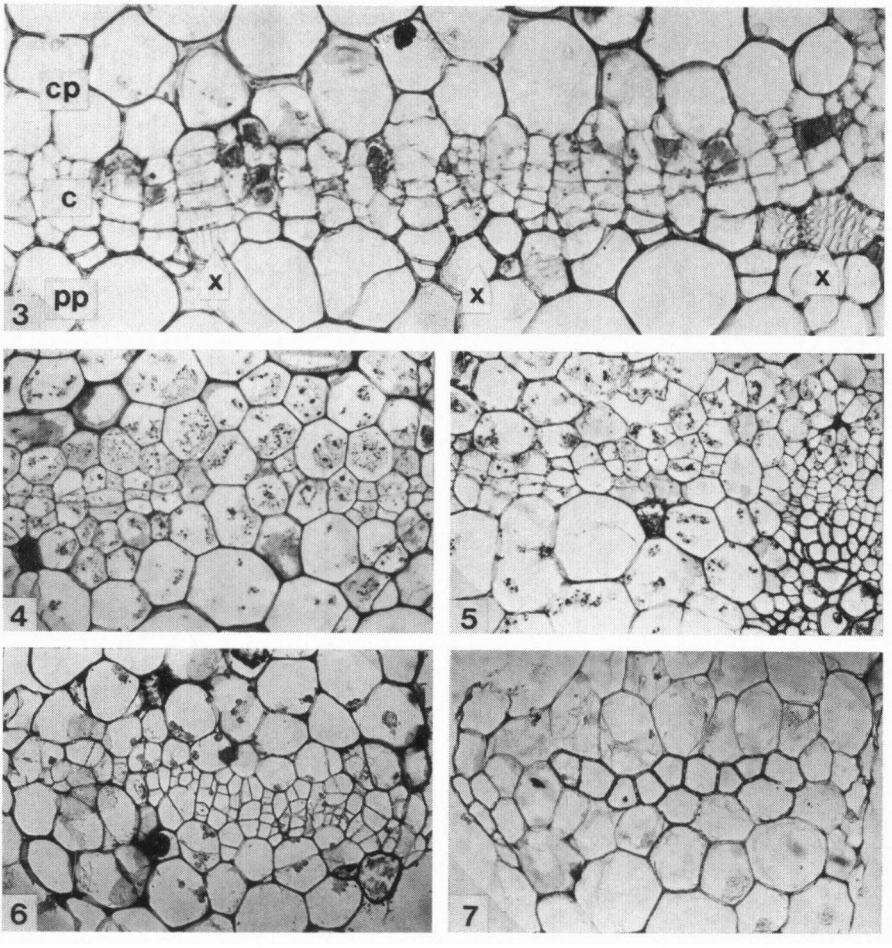

Fig. 3–7. Differentiation in excised tissue blocks taken from 8 day old seedlings and cultured in different media.

Fig. 3. Cambial development and xylem differentiation in an interfascicular tissue block cultured for three weeks on a basal medium. × 172.

Fig. 4. Beginning of cambial development in an interfascicular tissue block cultured for two weeks on a basal medium. × 87.

Fig. 5. Same experiment as in Fig. 4. but with a block of half fascicular (right) and half interfascicular (left) tissue. × 87.

Fig. 6. Disorientated cambial divisions in a block of interfascicular tissue cultured for 8 days on a basal medium supplemented with kinetin (2.5 ppm). × 87.

Fig. 7. Xylogenesis in a block of interfascicular tissue cultured for 8 days on a basal medium supplemented with IAA (12.5 ppm), photographed with polarized light. Note the direct transformation of the cambial layer into tracheary elements. × 87.
c = cambium, x = secondary xylem elements, cp = cortex parenchyma, pp = pith parenchyma.

Table 1. Influence of IAA, GA_3, and kinetin on the differentiation of interfascicular tissue blocks taken from 8 day old seedlings and cultured for 8 days. For each of the cambial zones (4 per tissue block when none were discarded) the mean number of tangential walls was estimated and the total number of tracheary elements counted. Per treatment, mean (mn) and maximal (mx) values are given.

	phyto-hormone	observed zones (total number)	no. of zones with disoriented cambial walls	cambial walls		tracheary elements of cambial origin		other tracheary elements	
	ppm		%	mn	mx	mn	mx	mn	mx
	0.0	103	6	1.0	4	2.8	19	0.0	0
	0.1	91	29	2.6	6	7.1	30	1.4	17
IAA	0.5	115	48	3.2	6	9.4	29	0.8	10
	2.5	100	39	1.0	6	8.1	21	0.6	11
	12.5	48	0	0.0	1	4.0	14	0.3	8
	0.0	108	2	0.7	4	2.0	18	0.0	0
	0.1	126	6	1.6	5	9.0	30	0.0	5
GA_3	0.5	163	11	1.4	4	6.0	27	0.1	9
	2.5	100	10	1.1	5	3.0	29	0.0	3
	12.5	140	9	1.3	5	4.1	30	0.1	3
	0.0	96	1	0.4	3	0.9	15	0.0	0
	0.1	127	28	2.5	6	5.7	26	0.0	0
kinetin	0.5	123	26	2.1	6	4.8	28	0.0	2
	2.5	141	41	1.9	6	5.7	27	0.3	16
	12.5	92	53	1.4	4	4.6	28	0.0	0

bial walls, however, was not always tangential as occurs in the situation *in vivo*. The addition of phytohormones enhanced depolarization, which was particularly striking at moderate concentrations of IAA and high concentrations of kinetin. Since disorientation seriously hampered determination of the number of cell divisions, only data for approximately tangential walls were used.

As can be seen from the number of cambial walls, all the phytohormones used can stimulate cambial development and xylem differentiation. Especially in terms of the maximum effect, IAA, GA_3, and kinetin reached about the same level, i.e., 5 or 6 tangential walls (*in vivo* 8 to 10 tangential walls are formed before xylem differentiation sets in). Although it may be said that in general all phytohormones have the same effect, some differences do occur. IAA and kinetin exhibited very high percentages of disorientated cambial walls (*fig. 6*). Tissue blocks cultured in the presence of IAA exhibited a marked xylogenesis in the parenchyma cells of predominantly the pith area, resulting in a number of tracheary elements of non-cambial origin. High IAA concentrations do not favour xylogenesis to the same degree as do low concentrations, except for the cells of the cambial layer; and because mitotic activity is completely inhibited at high concentrations, the cambial layer is directly converted into a layer of xylem elements without prior tangential cell division (*fig. 7*). From the morphogenetic

Table 2. Influence of phytohormone combinations on the differentiation of interfascicular tissue blocks taken from 8 days old seedlings and cultured for 15 days.

added phytohormone (0.1 ppm)	observed zones (total number)	no. of zones with disoriented cambial walls	cambial walls		tracheary elements of cambial origin		other tracheary elements	
		%	mn	mx	mn	mx	mn	mx
none	31	22	2.3	4	11.6	21	0.3	4
GA_3 + kinetin	22	5	5.0	7	25.6	41	0.9	13
GA_3 + IAA	23	29	4.7	7	21.0	35	1.8	9
IAA + kinetin	19	57	4.2	7	15.8	32	6.5	45
GA_3 + IAA + kinetin	25	38	4.2	8	20.0	36	2.1	11

point of view GA_3 gave the best results: disorientation was minimal and transformation of tracheary elements from cortex and pith parenchyma was virtually absent.

3.4. Effect of phytohormone combinations

In one experiment phytohormones (dosage: 0.1 ppm) were tested in combination: GA_3-kinetin, GA_3-IAA, kinetin-IAA, and GA_3-kinetin-IAA. As compared to the results obtained with the basal medium, all combinations reached approximately the double number of tangential walls in two-week cultures. The effect of the GA_3-kinetin combination proved to be slightly better, which was also demonstrated by the number of secondary xylem elements. From the morphogenetic point of view this combination appeared to excel the others: disorientation of the cambial walls was minimal and even lower than in the control experiments, and, obviously due to the absence of IAA, tracheary elements of non-cambial origin occurred only exceptionally (*table 2*).

4. DISCUSSION

In the process by which the interfascicular cambium is formed, selected cells of the medullary rays must be informed that they are to become meristematic. In addition, polarity has to be established so that the newly formed cell walls will be oriented tangentially and the cambial derivatives, *i.e.* the xylem and the phloem, will be laid down in the proper direction. Once this information is obtained, the initiation process is in fact determined. Whether it actually will give rise to a well-developed cambium depends on the presence of one or more stimulatory influences, acting at given moments during the initiation process.

As postulated earlier (SIEBERS 1971a), the initiation of the interfascicular cambium is supposed to be determined in the very young shoot, i.e. at the stage in which the primary meristem ring is present. The following discussion deals with the question of whether the actual development of the interfascicular cambium

is dependent on or markedly influenced by stimuli originating in adjacent vascular tissues.

That parenchymatous cells can be forced to divide by a stimulatory substance originating from the phloem was first demonstrated by HABERLANDT (1913), who applied his findings to the problem of the initiation of the interfascicular cambium. According to BÜNNING (1953) it is generally accepted that the cambium of the vascular bundle can exert a stimulatory influence on the medullary ray cells leading to the development of the interfascicular cambium. BÜNNING argues that this influence can also be involved in the determination of the type of cell differentiation. From the work of JABLONSKI & SKOOG (1954), MILLER *et al.* (1955), and SHELDRAKE & NORTHCOTE (1968a, b) we can anticipate that if there is a stimulatory influence from the vascular bundle, it is mediated by cytokinin or auxin action.

The existence of a stimulatory influence in the particular case of the interfascicular cambium is only inferred from descriptive evidence. HABERLANDT (1924) described the initiation of the interfascicular cambium as a process that starts in the vicinity of fascicular cambium and proceeds to the space between the vascular bundles. This progressive development suggests an influence originating from the fascicular cambium. BÜNNING (1956), in a discussion of his paper on general processes of differentiation, confirmed such progressive development for *Aristolochia.*

The illustrations in the literature pertaining to the onset of cambial activity show that (*table 3*), although in a majority of cases the fascicular cambium pro-

Table 3. Time course of development of fascicular and interfascicular cambium, derived from Figures in the literature and based on the number of tangential walls of the cambia. F = fascicular cambium leading; FI = fascicular and interfascicular cambia equally developed; P = progressive and S = simultaneous development of the interfascicular cambium; P/S = both forms of development observed.

Author	Material	Observed development	
SACHS, 1873*	*Ricinus communis*, hypocotyl	F	S
STRASBURGER, 1898**	*Aristolochia durior*, shoot	F	S
PALLADIN, 1914***	*Ricinus communis*, hypocotyl	F	S
HABERLANDT, 1924	*Begonia fuchsioides*, shoot	F	S
DUNCKER, 1935	*Helianthus annuus*, epicotyl	F	S
POPHAM, 1952	*Helianthus annuus*, shoot	FI	S
ESAU, 1960	*Lotus corniculatus*, shoot	FI	S
KAUSSMANN. 1963	*Aristolochia sipho*****, shoot	F	S
idem	*Helianthus annuus*, shoot	FI	S
BRAUNE *et al.*, 1967	*Aristolochia macrophylla*****	F	P
BIEBL & GERM, 1967	*Aristolochia macrophylla*****, shoot	F	P/S
NULTSCH & GRAHLE, 1968	*Aristolochia sipho****, shoot	F	P

* in REINDERS & PRAKKEN (1964)
** in VON DENFFER (1962)
*** in FAHN (1967)
**** synonym of *A. durior Hill.*

ves to lead to the interfascicular cambium, progressive development could actually be observed in some cases in *Aristolochia durior*. However, many textbooks contain the general statement that the interfascicular cambium extends laterally from the sides of the vascular bundle (EAMES & MACDANIELS 1947; BRIMBLE 1953; BURNS 1964; ESAU 1965; BRAUNE *et al.* 1967). The grounds on which the existence of a stimulatory influence is postulated therefore appear to be rather limited. Furthermore, the observed sequential occurrence of tangential walls in cambial development does not necessarily prove that the leading cambial cell exerts an influence on the more slowly developing neighbouring cell. This phenomenon might also be explained by a difference in the internal rate of development of the individual cells.

The results presented here provide strong evidence that initiation of the interfascicular cambium does not depend on the presence of the vascular bundle. Separation of interfascicular and fascicular tissue by means of a metal strip five days before the first mitotic activity *in vivo* could be demonstrated has no consequence for the cambial development on either side of the metal strip. Cultured interfascicular tissue blocks isolated at the same age can exhibit an almost normal initiation of the cambium, even in the absence of phytohormones in the medium. Both these findings confirm earlier results (SIEBERS 1971a) showing that the initiation process is determined in an early stage of development, and that the existence of an inductive influence in the sense given by BÜNNING must be rejected.

Tissue blocks containing vascular tissue did not give better results than pure interfascicular blocks, so it may be concluded that the vascular tissue did not supply the cambial cells with some indispensable stimulatory factor. This is not in contradiction with the observed cessation of cambial activity after about two weeks, which may be due to the absence of factors that originate *in vivo* from other parts of the plant, e.g. the apex or young leaves. It is certainly relevant that decapitation, including the cotyledons, has a dramatic effect on cambium initiation (SNOW, 1935). Among the substances capable of substituting for the influence of the apical parts and restoring normal cambium initiation, auxin is mentioned the most frequently (LIBBERT & URBAN 1967). Another possibility to explain cessation of cambial development in the cultured tissue blocks might be that stimulatory factors, produced by cambial cells once they have started to divide and capable of stimulating further mitotic activity and differentiation *in vivo* (SÖDING 1937, 1961), at least partially leak out into the medium under our experimental conditions, before having influenced cambial development. Reports concerning the presence of stimulatory substances such as IAA (DÖRFFLING 1963) and cytokinin in the cambium (NITSCH & NITSCH 1965) are in agreement with this assumption. The stimulation by phytohormones observed in our cultures makes a shortage of stimulatory substances, due either to the absence of the apical parts or to leakage, indeed likely.

IAA, GA_3, and kinetin proved to stimulate cambial development to about the same maximum level. ROBARDS *et al.* (1969), testing different growth substances on disbudded willow stems, observed the same effects and suggested that this

may be due to the impossibility of depleting the stem of the synergistic endogenous growth substances. It is unlikely that this suggestion would hold for the small tissue fragments used in our experiments unless the growth substances under discussion were produced by the cambial cells in the course of the culture period instead of having been already present in the parenchymatous cells at the beginning of the experiment.

Although in our experiments IAA and kinetin exhibit stronger stimulatory properties than GA_3, their use involves a serious disadvantage, i.e. the disturbing activity with respect to the orientation of the cambial divisions. Moreover, IAA caused an abnormal xylogenesis, well known in this kind of explants (EARLE 1968). At higher concentrations the xylogenetic effect decreased but obviously not to the same extent for the cells of the cambial layer which, as a result of a simultaneous inhibition of the mitotic activity, exhibited a direct differentiation to xylem elements without prior cell division. GA_3 gave relatively few side-effects, and when applied in low concentrations and in combination with kinetin, was very successful in stimulating the development *in vivo*. In connexion with the favourable action of gibberellin it is interesting to mention experiments, now in progress, which suggest that sugar is sufficient to trigger cambium initiation in the entire hypocotyl and that further activity of the cambium is controlled by gibberellins produced in the cotyledons rather than by auxins produced in the expanding young leaves or buds.

The present results do not permit exact conclusions about the stimulatory activity of phytohormones on the development of the interfascicular cambium *in vivo* due to the method of culturing very small tissue blocks, which in fact have some serious disadvantages such as leakage of possibly stimulating substances into the medium and the unknown effect of relatively large wound areas.

In conclusion it should be pointed out that our results give no evidence indicating the existence of an inductive factor originating from the fascicular cambium and acting on the cells in the interfascicular region. A stimulatory influence cannot be excluded but if it exists, it must have its origin in other parts of the plant and not in the adjacent vascular bundle tissue. The incidentally observed progressive development of the interfascicular cambium may be regarded as the result of accidental circumstances, such as food stress, which favour primary division of those cells of the cambium bordering the stream of assimilates in the vascular bundle.

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