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Changes in auxin level in the course of growth of a sunflower crown-gall suspension culture

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Abstract

The auxin level in the cell mass and culture medium was determined by means of the *Avena* straight coleoptile test in various periods of the suspension culture cycle of the sunflower crown-gall tumour. The investigations were performed in the course of the zero passage (PO) and first one (P1), differing in their time of duration of maximum growth and its intensity. In both passages the intra- and extra-cellular auxin levels reach values of the same order. At the beginning of the maximal growth phase the activity corresponding to IAA in the cells prevails over that of the other auxin-like compounds. This disproportion diminishes with further development of the culture, and with the beginning of the stationary phase the cellular IAA level is lower than that of the remaining auxin-like compounds. The short phase of maximal growth (PO) occurs with an auxin level decreasing in the cell mass and increasing in the medium, and towards the end of the cycle these levels become equal. During the long phase of maximal growth (P1) the total amount of auxins in the cells increases and is 2-3 times higher than in the medium, whereas IAA in the cells remains at a constant level. These results suggest that the participation of IAA in the intracellular pool of auxin-like substances is decisive for the mitotic activity of the cells and maintenance of growth in the culture.

Key words: crown-gall, sunflower, suspension culture, auxin level.

INTRODUCTION

It has repeatedly been observed that crown-gall cells produce excessive amounts of auxins and cytokinins (Sequeira 1973, Miller 1974, Nakajima et al. 1979). One of these tumour cytokinins has been identified as ribosyl-trans-zeatin (Einset 1980, Scott et al. 1980), whereas the main crown-gall auxin, indispensable for its growth is

indole-3-acetic acid (IAA) (Sequeira 1973). The mechanism of hormonal regulation of crown-gall tumour growth has not been elucidated so far, however. Neither is it clear whether there exists a direct relation between the given auxin level in the tumour cells and the intensity of their growth. In such investigations cell cultures seem particularly convenient since in them changes in the intracellular hormone level and in the surrounding medium can be followed. But few papers have appeared to date concerning quantitative changes in hormone content in the growth cycle of crown-gall cells culture, and the results are controversial (Atsumi and Hayashi 1978, Nakajima et al. 1981).

In the present study the auxin content in the cells and medium of the suspension culture of sunflower crown-gall was investigated. Transfer of the tissue from solid to liquid medium entails considerable changes in cell metabolism, and a certain time is required for adaptation of the cells to the new growth conditions. In this period (PO) the course of growth of the culture does not correspond to the model of tissue growth on agar or to the model typical for the specific stabilised cell culture (P1 and further). Earlier the auxin level in sunflower crown-gall tissue was studied in agar culture (Chirek 1979). Results of analogous experiments with suspensions of cells of the same tissue allow a fuller characteristic of auxin content in the tumour under study.

MATERIAL AND METHODS

Sunflower tumour tissue was isolated in 1978 from crown-gall induced on the stem of *Helianthus annuus* L. var. Borowski prążkowany by infection with *Agrobacterium tumefaciens* (CCM 1037). The tissues were cultured on Murashige and Skoog culture medium (1962) free of auxin and cytokinin (M-S) at a thiamine level of 0.4 $mg \cdot dm^{-3}$. The suspension culture (referred to as PO) was obtained by placing about 3 g of loose tissue in 70 cm^3 of liquid M-S medium with an increased thiamine level (1 $mg \cdot dm^{-3}$). After 28 days of culture (rotating shaker, 110 $cycles \cdot min^{-1}$, amplitude 50 mm, darkness, $25 \pm 1°C$) 20 cm^3 of the suspension (density ca. 10^5 $cells \cdot cm^{-3}$) was transferred to 50 cm^3 of fresh medium to obtain the P1 cell culture with outset density of about 3×10^4 $cells \cdot cm^{-3}$.

The culture growth was evaluated according to the number of cells in 1 cm^3 of suspension and cell fresh weight ($g \cdot 100$ cm^{-3}). The cell mass was obtained by filtration of the suspension from several culture flasks.

The auxins were extracted with 80 per cent methanol from 5-6 g of fresh cell mass within 3 h at 4°C with twofold change of alcohol. Then the methanol was evaporated at 35°C (under vacuum), the residue was acidified with 1 N HCl to pH 3, separated threefold with ether and then

2 per cent $NaHCO_3$ and once more with ether at pH 3 (Eliasson 1969). The culture medium was concentrated under vacuum at 45°C, acidified, extracted with ether and the subsequent procedure was the same as with cells. The extract obtained containing auxins was separated by thin layer chromatography on silica gel in an isopropanol:water:ammonia (10:1:1) system. Auxin activity of the successive chromatogram zones was determined, in *Avena* straight coleoptile tests as described earlier (Chirek 1979) and the auxin level (both in cells and in medium) was expressed in IAA μg equivalents $\cdot kg^{-1}$ of fresh cell weight.

RESULTS

Growth. The growth dynamics of the culture examined in PO and P1 stages is shown in Table 1 and Fig. 2. In PO the culture attains the phase of maximal growth as late as after 3 weeks. The drastic increase of the cell number and of fresh weight characteristic for this phase lasts about seven days. Then the culture passes to a stationary phase, as indicated by the subsiding mitotic activity of cells and the decreased rate of biomass increment. In P1 an intensive increase of the cell number was observed between the 14th and the 35th day. Maximum increase of the cell number is noted in the fourth and of fresh weight in the fifth week of culture. During five weeks of growth the suspension does not attain the stationary phase. Thus, the culture shows in P1 a two times longer than in PO phase of intensive growth, and although the initial density is lower, it yields a higher cell crop.

Table 1

Growth of sunflower crown-gall suspension cultures

Age of culture in weeks		2	3	4	5
Passage 0	cell number, $\times 10^4 \cdot cm^{-3}$	4.1±0.43	5.6±0.57	10.5±1.11	11.2±2.11
	fresh weight, $g \cdot 100\ cm^{-3}$	—	5.2±1.54	10.6±2.67	12.0±3.13
Passage 1	cell number, $\times 10^4 \cdot cm^{-3}$	3.7±1.07	5.4±0.68	9.9±2.29	12.9±3.08
	fresh weight, $g \cdot 100\ cm^{-3}$	1.8±0.10	4.0±0.41	6.9±0.93	12.1±2.51

The results represent mean values ± S.E. from 4-7 analyses

Auxin level was determined in the cell mass and culture medium after 3, 4 and 5 weeks of growth. Auxin activity in the *Avena* straight biotest was found in several zones of the chromatogram: I — Rf 0-0.3,

II — Rf 0.3-0.5, III — Rf 0.8-1.0. According to the standard position of IAA and earlier data (Chirek 1979) it is supposed that IAA is the main active compound in zone II. The activity of this zone was adopted as measure of the approximate IAA level, and further the expression "IAA level" is used in this sense. An example of auxin activity distribution in the suspension culture of sunflower crown-gall (P1) is shown in the histogram (Fig. 1). Noteworthy is the distinctly enhanced auxin activity of cell extracts with the age of the culture and the appearance of inhibitors in the early period of culture.

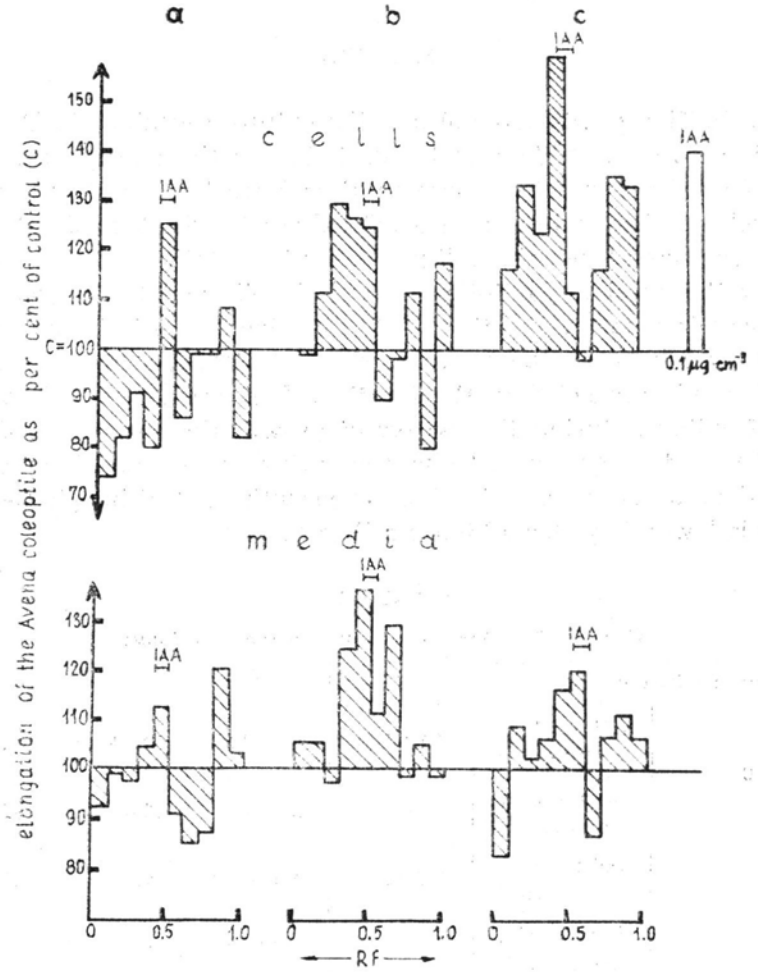

Fig. 1. Auxin activity in *Avena* bioassay of extracts from cells and media of sunflower crown-gall suspension culture at several growth stages (passage 1st — P1). a, b, c — show activity of 3-, 4-, 5-week old cultures, respectively; a — extract from 7 g of cells and 230 cm^3 of medium, b — 6 g and 215 cm^3, c — 6 g and 205 cm^3, respectively. IAA — position of standard IAA. Elongation produced by standard solution of IAA (0.1 $\mu g \cdot cm^{-3}$) is indicated on the right side of the histogram

Changes in the auxin level in cells and medium during suspension culture growth of sunflower crown-gall are illustrated in Table 2 (in μg-equ. IAA·kg^{-1} fresh cell weight) and in Fig. 2 (in μg-equ. IAA·10^{-9} cells). In PO the highest auxin-like substances level in the cell material was recorded at the beginning of the phase of maximal growth (3-week suspension). During further growth of the culture the level of these compounds in the cells decreases reaching in the stationary phase a two times lower value. The direction of the changes in auxin-like substance content in the medium has an opposite course: it is low in the 3-week culture and increases more than threefold during the following two weeks. Thus, the total auxin content in the studied culture during the growth cycle changes but slightly (Table 2). The proportion of IAA in the whole pool of auxin-like compounds decreases in the period discussed by about 60 per cent to 40 per cent, both in the cells and in the medium, although the absolute IAA quantity increases in the nutrient solution.

Thus, the cells in PO entering the phase of maximal growth have at their disposal a considerable provision of endogenous auxins, among which IAA prevails. These auxins must have formed and accumulated in an earlier period of the cycle.

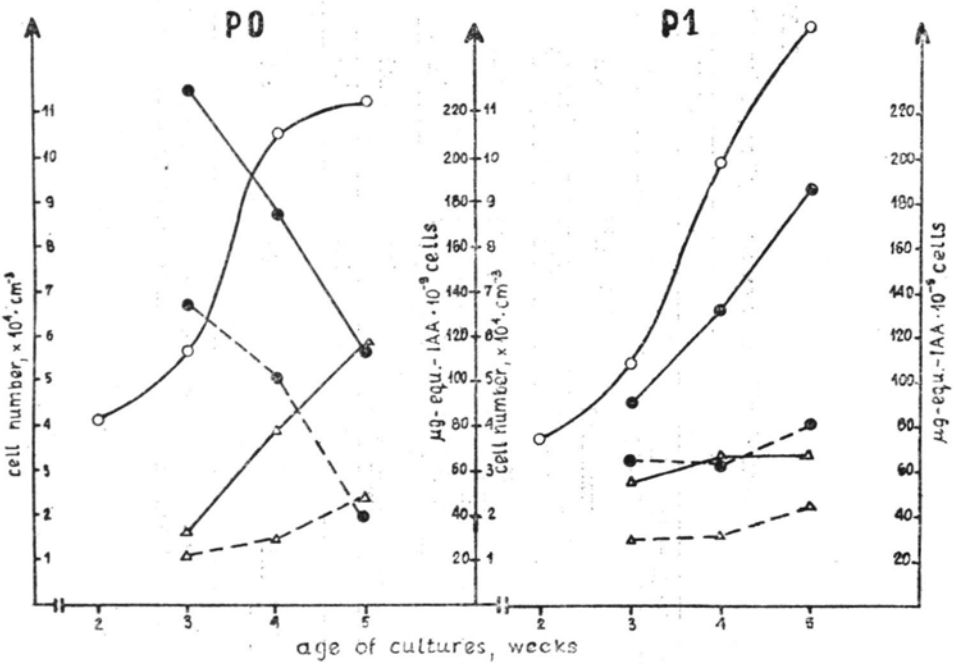

Fig. 2. Changes in auxin level in sunflower crown-gall suspension cultures in the course of growth. Cell number o——o; total auxin level: in cells ●——●, in medium Δ——Δ; IAA level: in cells ●– – –●, in medium Δ– – –Δ

Table 2

Auxin level in sunflower crown-gall suspension cultures (in µg-equ.IAA ·kg^{-1} fresh weight of cells)

		Passage 0			Passage 1		
Age of culture in weeks		3	4	5	3	4	5
Cells	total auxin level	250±86	172±32	106±38	122±33	189±41	198±48
	IAA level	145±51	100±22	37± 7	86±24	90±18	85±21
Medium	total auxin level	35±15	76±33	108±43	75±23	93±17	72±20
Cells+ Medium	IAA level	23±11	29±12	45±20	39±13	45±16	47±22
	total auxin level	285	248	214	197	282	270
	IAA level	168	129	82	125	135	132
IAA in cells / IAA in medium	ratio	6.3	3.4	0.8	2.2	2.0	1.8

Auxin level was calculated from the activity shown in *Avena* coleoptile straight test. Separated from the whole zone II with Rf 0.3-0.5 or 0.6 corresponding to IAA standard and accepted as an approximate IAA level. (This concerns also Fig. 2). The results represent mean values ± S.E. from 4-7 analyses.

In P1 of the studied culture the auxin-like substances level in the cells at the beginning of the maximal growth phase (21st day of culture) is relatively low, but increases twofold (when calculated to one cell) in the course of the two weeks of duration of this phase (Fig. 2). The level of these compounds in the medium, two times higher as compared with that in PO in the early period, only slightly rises in the course of further culture. The participation of IAA in the whole pool of auxin-like compounds in the cells is somewhat higher as compared to that in PO, and although it decreases in the course of growth of the culture, the absolute IAA level remains constant, while in the medium it slightly increases.

Therefore, in P1 cells entering the maximal growth phase dispose of a two times smaller auxin reserve than do those in PO. Nevertheless, the phase of maximal growth lasts in P1 two times longer than in PO and the yield of cells is higher. The changes in the level of auxin-like compounds observed during the discussed phase in P1 concern exclusively compounds other than IAA, whereas the content of the latter compound remains over this entire period at a constant level (Table 2), and when calculated to one cell, it slightly increases (Fig. 2).

Quantitative relations of auxins in the culture. In the early phase of maximal growth when divisions are most intensive, both in PO and in P1 the IAA level (in cells and the culture as a whole) is much higher than the level of the remaining auxin-like compounds (Table 2). With further development of the culture this disproportion gradually disappears and in the end period when divisions cease the amount of auxin-like substances other than IAA markedly prevails. This indicates that the participation of IAA in the intracellular auxin pool may be decisive for the division activity of the cells.

During the long phase of maximal growth the ratio of the total amount of auxin-like compounds to the approximate IAA level in the cells and medium remains relatively constant, close to 2 (Table 2). In the stationary phase, however, pronounced in PO the auxin-like substances level in the cells and medium becomes equal and their ratio reaches a value close to 1. Although in the stationary phase cell lysis may be expected, this, however, involves only a small number of cells. It cannot, therefore, explain the considerable increase in auxin content in the medium (Table 2). It would seem, therefore, that in the stationary phase changes occur in the physiological secretion of cells.

The here presented data indicate that continuation of growth in the culture requires the maintenance of a definite, relatively high auxin level in the cells, which would be balanced by their relatively low content in the medium.

DISCUSSION

In the studied suspension culture of sunflower crown-gall the phase of maximal growth in PO and P1 starts with widely differing auxin levels in the cells. This suggests that their absolute value may not have a decisive influence on the culture growth. According to Davies and McDaniel (1977) even an important reduction of the IAA level (caused by inhibition of its synthesis) in crown-gall cells of *Vinca rosea* does not affect the growth of these cells. Only a fall of the IAA level below the threshold concentration inhibits culture growth. The studied sunflower tumour cells (PO) entering the phase of maximal growth have at their disposal a large provision of auxins, but synthesis is not continued as indicated by the depression of the auxin level in further phases of growth. The consequence of this seems to be the short duration of the phase of maximal growth of the number of cells in PO. In P1, however, the auxin level at the beginning of the discussed phase is lower as compared to that in PO, and the duration of division activity of these cells seems to be the result of subsistence at this time of a constant IAA level.

The scarse literature data dealing with the role of IAA in the regulation of tumour cell growth in the suspension culture cycle do not supply univocal information. For instance Atsumi and Hayashi (1978), using their own IAA extraction procedure, evaluated the content of this auxin in the phase of maximal growth of the cell culture of a tumour of the sunflower var. Giant Russian as 2 $\mu g \cdot kg^{-1}$ fresh cell weight. They noted the higher IAA content (14 $\mu g \cdot kg^{-1}$) in the transition phase when cell division ceases. The above mentioned authors believe that during the phase of intensive growth there exists a balance between the production and utilisation of auxin, and that in the transition phase, owing to lower utilisation, the level of this substance increases, this leading to an enhanced destruction and, consequently, depression of the auxin content in the stationary phase. Nakajima et al. (1981), however, established that in cell culture of tobacco crown-gall var. Hicks-2, the highest IAA level in the cells (800 $\mu g \cdot kg^{-1}$) occurred in the early phase of maximal growth of the culture (evaluated in terms of biomass increment), then it diminishes by one half, and in the stationary phase falls almost to zero. These investigators suggest that IAA plays an essential role in the initiation of cell division. A similar conclusion was reached by Nishinari and Yamaki (1976) in investigations of synchronic cultures of normal tobacco cells.

The present author's own results confirm in principle the concept of the role of auxin (IAA) in initiation and maintenance of intensive cell division in the culture. It would seem that the depression of the auxin level (mainly IAA) is correlated with the short phase of intensive

growth, whereas a longer duration of this phase requires at least a certain definite IAA level. As regards the stationary phase characterised by the cessation of cell division, all the known reports of results uniformly state a considerable decrease of the auxin level in the cells (Atsumi and Hayashi 1978, Nakajima et al. 1981, Chirek et al. 1981).

Analysis of the results of the present study also suggests that in regulation of mitosis in the *in vitro* culture the ratio between the auxin level in the internal and external medium may play a role. It seems that for maintaining intensive growth of the culture a definite balance is required between the relatively high auxin (IAA) level in the cells and an appropriate auxin level in the medium. Lequay and Terrine (1975) and Lequay and Guern (1977) when investigating earlier regulation of normal cell division of *Acer* in suspension culture reached the conclusion that the appearance of divisions depend both on the threshold auxin concentration in the cells and on the specific balance between auxins in the internal and external medium. For modification of this balance changes in the intracellular pH are responsible, and these in turn depend on the population density.

Trials of establishing the factors influencing the changes in auxin level in the sunflower tumour culture will be the subject of further experiments.

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Zmiany poziomu auksyn w cyklu wzrostu kultury zawiesinowej crown-gall słonecznika

Streszczenie

Stosując test cylindryczny *Avena* oznaczono poziom auksyn w masie komórkowej i podłożu, w różnych okresach cyklu kultury zawiesinowej tumora crown-gall słonecznika. Badania prowadzono w pasażu zerowym (PO) i pasażu pierwszym (P1), różniących się czasem trwania maksymalnego wzrostu i stopniem jego nasilenia. W obu pasażach poziomy auksyn wewnątrz- i zewnątrzkomórkowych osiągają wartości tego samego rzędu. Na początku fazy maksymalnego wzrostu, aktywność odpowiadająca IAA w komórkach przeważa nad aktywnością pozostałych związków auksynopodobnych. Dysproporcja ta zanika w miarę dalszego rozwoju kultury i z początkiem fazy stacjonarnej poziom IAA komórkowego jest niższy od poziomu pozostałych związków auksynopodobnych. Krótka faza maksymalnego wzrostu (PO) przebiega przy spadającym w masie komórkowej, a wzrastającym w podłożu poziomie aktywności auksynowej i pod koniec cyklu poziomy te wyrównują się. Podczas długiej fazy maksymalnego wzrostu (P1), całkowita ilość auksyn w komórkach wzrasta i jest 2-3 razy większa niż w podłożu, podczas gdy ilość IAA w komórkach utrzymuje się na stałym poziomie. Wyniki sugerują, że udział IAA w wewnątrzkomórkowej puli substancji auksynopodobnych jest decydujący dla aktywności podziałowej komórek i utrzymania wzrostu w kulturze.