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# INFLUENCE OF THE CONDITIONS OF CULTIVATION ON THE GROWTH OF CALLUS AND CELL CULTURES OF *Ajuga turkestanica* AND THE BIOSYNTHESIS OF ECDYSTERONE

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*The influence of light on the total productivity of a tissue culture of* Ajuga turkestanica *has been studied. When calluses are grown in the absence of light the yield of biomass and its ecdysterone content increase. In a study of the influence of α-naphthylacetic acid on the productivity of a suspension cell culture it has been shown that with an increase in the concentration of the auxin the yield of crude mass rises but the ecdysterone content falls. The activation of the biosynthesis of the secondary product takes place with a retardation of the processes of growth and cell division.*

Under the conditions of a decrease in the natural reserves of useful plants, work on the production of biologically active compounds with the aid of tissue and plant cell cultures is becoming urgent.

We have previously obtained callus tissue of *Ajuga turkestanica* retaining the biosynthetic activity of ecdysterone under *in vitro* conditions [1]. With the aim of studying the possibility of a physiological regulation of the growth processes of tissue and cell cultures and of the synthesis of ecdysterone in them, we have investigated the influence of the illumination regime on the growth of *A. turkestanica* callus tissue and the yield of product and have considered the dependence of the growth of a suspension cell culture and of the accumulation of ecdysterone on the amount of an auxin in a liquid nutrient medium and also the nature of its accumulation in the dynamics of the growth of the cell culture.

We investigated callus tissue being passaged for five years. Samples of tissue grown under conditions of illumination (6000 lux with a 16-hour photoperiod) and in the dark were taken on the 30th day of cultivation. In the tissue culture grown in the light it was impossible to detect the presence of ecdysterone by the HPLC method because it was present in only trace amounts. The yield of crude mass from one flask was 11.46 g, the dry matter amounting to 0.42 g. Growing the callus tissue in the dark had a favorable effect not only on the yield of biomass but also on the biosynthesis of ecdysterone. Thus, the yields of crude mass and dry matter were 13.62 and 0.54 g, respectively, and the ecdysterone content was 0.069%.

The influence of an auxin on productivity was studied on a suspension cell culture obtained by transferring *A. turkestanica* callus tissue into a liquid nutrient medium with the following composition: 1/2 part of Murashige-Skoog mineral salts [2]; sucrose, 30 g/liter; inositol, 100 mg/liter; thiamine-HCl, 0.4 mg/liter. By the repeated selection of a finely aggregated fraction we obtained a viable, actively growing suspension. Media with different amounts of α-naphthylacetic acid (NAA) were tested: 1) with no added auxin; 2) with 1 mg/liter of NAA; 3) with 2.5 mg/liter of added NAA. Ecdysterone levels were determined in 14-day dry samples.

The results of the experiment showed that α-naphthylacetic acid affects the growth of the culture and the yield of product differently (Table 1).

Thus, in the absence of an auxin the growth of the biomass decreased but at the same time an increased yield of ecdysterone — 0.0057% — was observed. The best growth of the culture took place in a medium containing 2.5 mg/liter of NAA. However, this concentration inhibited the accumulation of ecdysterone, its yield amounting to only 0.0042%.

The dynamics of the growth of a suspension culture was studied for the following parameters: yields of crude biomass and of dry matter, density of the cell population, and viability. The synthesis of ecdysterone was observed simultaneously (Table 2).

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TABLE 1. Influence of the Auxin on the Growth of a Suspension Culture of *A. turkestanica* and on the Biosynthesis of Ecdysterone

| Concentration of NAA, g/liter | Crude mass | Dry matter | Ecdysterone content |
|---|---|---|---|
| | g/liter | | % of the dry weight |
| | 27.73 | 1.97 | 0.0057 |
| 1 | 41.43 | 2.47 | 0.0051 |
| 2.5 | 53.86 | 2.73 | 0.0042 |

TABLE 2. Indices of the Growth of a Cell Culture and the Level of Ecdysterone in the Dynamics of Growth

| Days of cultivation | Crude biomass | Dry matter | Viability, % | Density of the suspension, × 10 cells/ml | Ecdysterone content, % of the dry matter |
|---|---|---|---|---|---|
| | g/liter | | | | |
| 7 | 55.61 | 1.83 | 70.6 | 1.06 | |
| 14 | 152.13 | 10.22 | 87 | 2.68 | 0.021 |
| 20 | 268.43 | 16.98 | 80 | 2.67 | 0.029 |
| 25 | 263.39 | 13.7 | 55 | 2.40 | 0.024 |

The cell culture was grown in a medium containing 1 mg/ml of NAA and 0.002 mg/liter of Dropp. The activation of cell division began from the fourth day after transfer to fresh nutrient medium and the viability of the cells increased. In the first period of rapid cell division (7th day of cultivation) no product of secondary metabolism was detected. On the 14th day of vital activity the density of the cell population reached a maximum, and, according to HPLC, the ecdysterone content was 0.021%. By the 20th day a retardation of growth processes was observed and the culture passed into the stationary phase. A slight growth of crude mass took place through the expansion of the cells. In this period the ecdysterone content reached its maximum — 0.029% — but by the 25th day — in the degradation phase — it amounted to only 0.024%

Thus, the results of the investigations performed permit the conclusions that:

the absence of illumination has a positive effect on the growth of callus tissue and the biosynthesis of ecdysterone;

the presence of an auxin (NAA) in the nutrient medium stimulates the growth of the biomass but suppresses the biosynthesis of ecdysterone. Raising the concentration of NAA to 2.5 mg/liter leads to a high yield of biomass, while, conversely, the biosynthesis of ecdysterone takes place more actively in its absence;

the biosynthetic activity of a suspension culture of *A. turkestanica* cells reaches its maximum level in the stationary phase of the growth cycle.

## EXPERIMENTAL

**Cultivation.** Callus tissue was grown in the dark and under illumination at 26°C in Murashige-Skoog medium with the addition of 1 mg/liter of NAA and 0.002 mg/liter of Dropp. A suspension culture was cultivated in flat-bottomed flasks on shaking machines at speeds of rotation of 100 rpm at +26°C and 70% relative humidity in the dark. The density of the suspension was determined by counting cells in a Fuchs-Rosenthal chamber. The mean value was determined from the formula

$$\text{number of cells in 1 ml} = \frac{\text{number of cells in the chamber}}{3.2} \times \text{dilution}$$

Maceration with 10% chromic acid was first carried out: to one volume of culture was added four volumes of the acid, and the mixture was heated to 60°C for 15-20 min. After cooling, the culture was shaken.

Viability was determined with the aid of a 0.1% solution of Evans' blue dye. Dead cells were stained.

**Chemical Analysis.** Microcolumn HPLC was conducted on a Milikhrom instrument from Nauchpribor PO (Opel), using KAKh-2 microcolumns with Sepaharon $C_{18}$ (reversed stationary phase) and KAKh-1-64-3 microcolumns with Silasorb C600 (normal phase). Eluents: 1) 25% aqueous acetonitrile; 2) dichloromethane–methanol (7:1). The eluents were prepared from purified solvents [3], and before analysis they were degassed by the passage of helium. Samples with a volume of 2 μl

were introduced into the column through a Milikhrom injector. The rate of elution was 100 μl/min. Detection was effected at λ = 246 nm. Quantitative calculation was based on peak heights. TLC was conducted on Silufol UV-254 plates in system 3) chloroform–methanol (4:1).

**Analysis of Samples of Calluses Grown in the Dark (D) and in the Light (L).** Samples (L — 0.5033 g; D — 0.5088 g) were extracted with methanol (20 ml each) by stirring on a magnetic stirrer with gentle heating (for 2 h, three times). In each case, the combined extracts were concentrated in a rotary evaporator to 5 ml and were treated with hexane (2 × 2 ml) to free them from chlorophyll. Then they were evaporated to dryness and the residue was dissolved in 7 ml of water and exhaustively extracted with butan-1-ol. The process was monitored by TLC in system 3.

The butanolic extracts were washed with water (2 × 1/4 volume) and evaporated to dryness. The residue was dissolved in 1 ml of methanol, 9 ml of water was added, and the solution was passed through a column (d = 1.7 cm) of Silasorb-$C_{18}$ (layer height 3 cm). The column was eluted first (10 ml) with 15% and then (5.5 ml) with 85% aqueous methanol, 2-ml fractions being collected. Monitoring by TLC in system 3 and by HPLC (system 1, KAKh-2 column). Fractions containing ecdysteroids were combined, dissolved in 1 ml of methanol, and their ecdysterone content was determined by HPLC, using ecdysterone as external standard.

In the sample grown in the light it was impossible to calculate the ecdysterone content because of its minuteness. The sample grown in the dark contained 0.35 mg/ml of ecdysterone, which constituted 0.069% of its weight.

**Analysis of the Suspension Cultures N-0, N-1, and N-2.5.** Samples: N-0 - 0.81540 g; N-1 - 0.80075 g; N-2.5 - 0.80140 g, the NAA contents of which were, respectively: none, 1 mg/l and 2.5 mg/l. Each, separately was extracted with methanol (4 × 15 ml) once at room temperature and 3 times with heating on the water bath for 2 h each time.

In each case the combined extracts were evaporated to dryness and the residue was dissolved in 9 ml of water and extracted with butan-1-ol (6 × 5 ml). The butanolic extract was washed with water (2 × 4 ml) and evaporated to dryness, and the residue was dissolved in 2 ml of system 3 and passed through a column (d = 0.9 cm) of $Al_2O_3$ (V = 1 $cm^3$) with elution by the same system and the collection of 1-ml fractions (monitoring by TLC). Fractions 1 and 10 were discarded as barren and the remainder were combined, evaporated to dryness, dissolved in 0.2 ml of methanol and analyzed for their ecdysterone content by HPLC (system 2, KAKh-1 column). Sample N-0 contained 0.0057% of ecdysterone, H-1 — 0.0051%, and H-2.5 — 0.0042%.

**Analysis of Samples of the Suspension Culture after Growth for 14, 20, and 25 Days.** The extraction of samples after 14, 20, and 25 days' growth (1.7, 1.55, and 0.50 g, respectively) was carried out as described for the callus tissue grown in the dark and in the light. The working up of the extracts and their purification were performed similarly, avoiding the use of hexane.

HPLC analysis (system 2, KAKh-1) and calculations showed that the 14-day sample contained 0.021% of ecdysterone, the 20-day sample 0.029%, and the 25-day sample 0.024%.

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