

EFFECT OF PLANT GROWTH REGULATORS ON THE PRODUCTION OF FLAVONOIDS BY CELL SUSPENSION CULTURES OF *DIGITALIS LANATA*

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Abstract

In this paper we investigated the effect of some phytohormones on the production of flavonoids in cell suspension cultures of *Digitalis lanata*. There were used two cell lines and different combinations and concentrations of phytohormones. The highest flavonoids amounts were obtained after 14 days of treatment. Among the tested combinations, the highest yield of flavonoids, 7.58 mg/g d.w. (dry weight) was obtained, for line 13C-100, with 0.1 mg/L IAA (β -indoleacetic acid) + 1 mg/L BA (6-benzylaminopurine). For line 11, the most significant increase in flavonoid amount was achieved with 0.1 mg/L NAA (α -naphthaleneacetic acid) + 1 mg/L BA.

Rezumat

Lucrarea de față prezintă rezultatele testării efectului unor fitohormoni asupra producției de flavone în culturi celulare de *Digitalis lanata*. Au fost efectuate mai multe experimente, folosind diferite combinații și concentrații de fitohormoni și două linii celulare de *Digitalis lanata*: linia 11 și linia 13C-100. Cea mai mare producție de flavone a fost obținută după 14 zile de tratament, pentru ambele linii celulare. Dintre combinațiile testate, cantitatea cea mai mare de flavone, 7,58 mg/g s.u. (substanță uscată) a fost obținută, pentru linia 13C-100, cu 0,1 mg/L AIA (acid β -indolilacetic) + 1 mg/L BA (6-benzilaminopurină). Pentru linia 11, cea mai semnificativă creștere a cantității de flavone 5,125 mg/g s.u., s-a obținut folosind combinațiile: 0,1 mg/L ANA (acid α -naftilacetic) + 1 mg/L BA și 0,1 mg/L AIA + 1 mg/L BA.

Keywords: auxins, cytokinins, flavonoids

Introduction

In vitro cultivated plants such as: plant tissue cultures, plants cell cultures, hairy root cultures represent valuable alternative sources for the secondary metabolites production of pharmaceutical importance. *In vitro* cultures have several advantages: the desired product (secondary metabolite) is obtained under controlled environmental conditions (temperature, humidity, photoperiod); lack of microbes and insects (which can affect a plant in natural conditions); continuous production, no matter of season, etc. [2, 12]. Furthermore, if the experimental conditions are well established and standardized, the concentration/production of desired compounds is constant, while in the living plant the concentration depends on many factors such as: climatic conditions, season, etc. In plant cell cultures it is necessary to determine the optimum culture conditions to improve the desired product yields.

Besides elicitors, various strategies including concentration and carbon source, basal medium components, concentration and type of plant growth

regulators (PGR) have been used to enhance the metabolites content in plant cultures [11]. Plant growth regulators have been assessed in order to stimulate secondary metabolites biosynthesis in different plant cell cultures [1, 10, 11, 13, 15, 18]. The present paper describes the study on the influence of different auxins and cytokinins with various concentrations, on the biosynthesis of flavones (which belong to secondary metabolism) in *Digitalis lanata* cell suspension cultures. Earlier reports describe the influence of auxins on cardiac glycosides (important secondary metabolites) biosynthesis in *Digitalis lanata* callus cultures [9].

Materials and Methods

Plant material: The experiments were performed with two cell lines of *Digitalis lanata*: line 11 and line 13C-100, obtained as reported previously [3]. Cell suspension cultures have been maintained on Murashige-Skoog medium [7] supplemented with 3% sucrose, 0.1% inositol, 1mg/L 2,4-dichlorophenoxyacetic acid and 2 mg/L 6-benzylaminopurine in 300 mL Erlenmeyer flasks containing 50 mL medium. They

were subcultivated every 14 days, the ratio inoculum to fresh medium was 1:4. The flasks were kept on a gyratory shaker at 100 rpm for a photoperiod of 16 hours, with illumination of $35 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a temperature of 25°C .

In our experiments there were used the following auxins and cytokinins: 2,4-dichlorophenoxyacetic

acid (2,4-D), α -naphthaleneacetic acid (NAA), β -indoleacetic acid (IAA) and 6-benzylaminopurine (BA). To test the plant growth regulators effect, the plant cell suspensions were transferred from initial culture medium to another culture medium containing the following combinations:

2,4-D (1 mg/L) + BA (2 mg/L) - control;

NAA (0.1 mg/L) + BA (1 mg/L) - named 1B₁ (line 11) and 1B₃ (line 13C-100);

IAA (0.1 mg/L) + BA (1 mg/L) - named 1Bi₁ and 1Bi₃;

NAA (1 mg/L) + BA (2 mg/L) - named 5B₁ (line 11) and 5B₃ (line 13C-100);

IAA (1 mg/L) + BA (2 mg/L) - named 5Bi₁ and 5Bi₃.

For all the experiments, the plant growth regulators were added before sterilisation. The pH of the medium was adjusted to 5.7. The samples were harvested after 6, 12 and 14 days. Cells were separated from the culture medium by filtration under reduced pressure using a Buchner funnel with filter paper. Filtered cells were dried and then were ground to obtain a powder.

Flavonoids quantification was performed in accordance with the Romanian Pharmacopoeia, by a spectrophotometric method (*Cynarae folium* monography) using aluminium chloride as reagent and a calibration curve constructed with rutoside [4, 5, 14]. The qualitative analysis of flavonoids was performed in accordance with the Romanian Pharmacopoeia by a thin layer chromatography (TLC) method (*Calendulae flos* monography) [5], using rutoside (R) as standard. Samples were prepared as solutions in methanol (10 mL) using 0.6 g powder obtained previously and were applied on the TLC silica gel 60 plates (Merck) $10 \mu\text{L/spot}$. After development, the plates were dried at room temperature and were evaluated in 366 nm UV light. The flavonoids detection was also performed with Neu reagent [17], in 366 nm UV light.

Results and Discussion

Among other factors, plant growth regulators can influence the secondary metabolism. There are many earlier reports which describe the essential role of phytohormones on the secondary metabolites biosynthesis. Still, the way they act on biosynthetic pathways it's less known. Furthermore, there are many other unknown aspects since the same plant growth regulators can stimulate or inhibit the biosynthesis of certain secondary compounds depending on plant growth regulation (PGR) concentration and the species which were cultivated *in vitro* [8].

Thus, for best results, it's important to determine the optimum concentrations of auxins and cytokinins, for each experiment. Therefore, in our

experiments three auxins (2,4-D; IAA; NAA) and one cytokinin (BA) on different combinations and concentrations were tested. The results are shown in Figures 1, 2, 3 and 4.

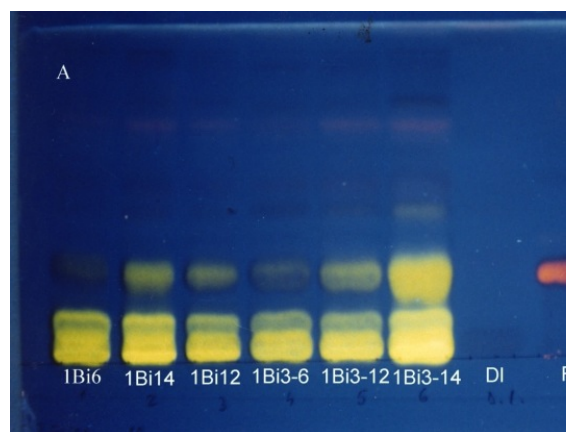


Figure 1.

TLC- variant 1B₁ and 1B₃, detection in UV light with Neu reagent

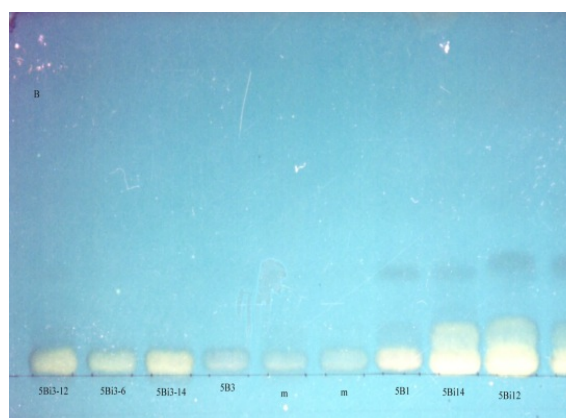


Figure 2.

TLC-variant 5B₁ and 5B₃, detection in UV light with oxalic acid + boric acid

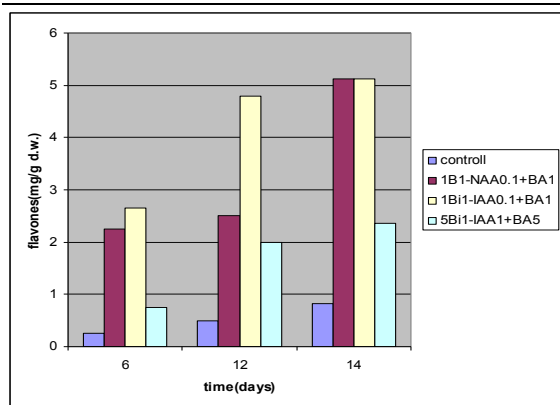


Figure 3.

Flavonoids biosynthesis, line 11

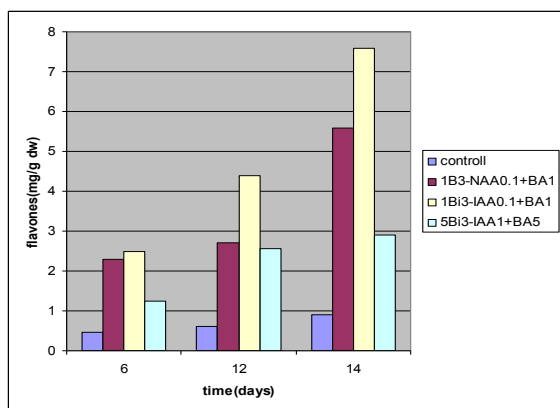


Figure 4.

Flavonoids biosynthesis, line 13C-100

Variants 5B₁ and 5B₃, after 7 days of treatment, were infected and the experiments in these cases were stopped.

For all variants we noticed that the flavonoids amount increased with the age of suspension cultures. Thus, the highest flavonoids levels were recorded for all samples harvested after 14 days. These results corroborate with earlier reports. Thus, the highest flavonoids production was reached after 15 days in cell suspension cultures of *Saussurea medusa*, using a combination of 0.5 mg/L BA and 2 mg/L NAA [6]. The auxins NAA and IAA stimulated stronger the flavonoids biosynthesis than 2, 4-D. The best results were recorded generally for line 13C-100 which responded better to hormones combinations action. The increasing concentrations of IAA and BA decreased significantly (up to three times) the flavonoids amounts. Thus, line 1B₁ and 1B₃ accumulated higher levels of flavonoids than lines 5B₁ and 5B₃ for all experiments. For example, variant 1B₁ accumulated 2.65 mg/g d.w. flavonoids after 6 days and variant 5B₁ only 0.75 mg/g d.w. flavonoids after 6 days about three times less. Flavonoids amount was 4.4 mg/g d.w. for variant 1B₃ and 2.57 mg/g d.w. for variant 5B₃, after 12 days, and the examples can continue.

In contrast, in cell suspension cultures of *Angelica archangelica* L., increasing concentrations of IAA and NAA enhanced scopoletin accumulation. The highest scopoletin levels were obtained with 10 mg/L NAA and 20 mg/L IAA [12]. IAA (variants 1B₁ and 1B₃) determined better responses than NAA (variants 1B₁ and 1B₃) for lower concentrations. For example, variant 1B₁ accumulated 4.8 mg/g d.w. flavonoids after 12 days, while variant 1B₁ accumulated only 2.5 mg/g d.w. flavonoids in the same conditions. The same, variant 1B₃ accumulated 4.4 mg/g d.w. flavonoids and variant 1B₃ only 2.7 mg/g d.w. flavonoids after 12 days.

The most significant increases in flavonoids production of about 3 times and the highest flavonoids amount were achieved for line 13C-100 from 2.5 mg/g d.w. after 6 days to 7.58 mg/g d.w. after 14 days, for variant 1B₃. For line 11 the most significant increase in flavonoids amount was recorded for variant 5B₁ from 0.75 mg/g d.w. after 6 days to 2.36 mg/g d.w. (about 3 times), after 14 days. The highest flavonoid accumulation was recorded for variants 1B₁ and 1B₁, 5.125 mg/g d.w., after 14 days. In cell suspension culture of *Saussurea involucreata*, the most suitable combination for flavonoids production was 2 mg/L of NAA + 0.2 mg/L BA [16].

Conclusions

Different combinations and concentrations of auxins and cytokinins strongly stimulated the flavonoids production compared with control in cell suspension cultures of *Digitalis lanata*. The best results were obtained after 14 days of treatment. 0.1 mg/L IAA + 1 mg/L BA was the most suitable combination for enhancing the flavonoids production in our experiments.

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