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Increase in the Synthesis of Polyfructan in the Cultures of Chicory "Hairy Roots" with Plant Natural Growth Regulators

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Abstract

Experiments were conducted to study the benefit of using the new plant growth regulators (PGRs) Ivin, Emistim, Biolan and Charkor in nutrient medium ½ MS for intensification of biomass growth and the increased synthesis of polyfructan (PF) in the cultures of chicory "hairy roots" (Cichorium intybus L.), obtained by Agrobacterium rhizogenes-mediated transformation,. The best indexes of increased specific quantities of PF are observed after using Biolan at a concentration of 5.0 µL/L (up to 130 mg/g dry mass of roots) and Emistim at a concentration 2.5 µL/L (up to 220 mg/g dry mass of roots). The greatest stimulation of root growth activity was expressed on using the growth regulators Emistim, Ivin and Charkor, in concentrations between 2.5 and 10.0 µL/L, considerably raising the total quantity of PF: compared with the control the use of regulator Emistim showed a rise of up to 35 times, regulator Ivin showed an increase of up to 28 times and regulator Charkor showed an increase up to 7.0-7.5 times. The results thus obtained definitely prove the benefit of applying these regulators to increase the biomass growth and PF synthesis in the culture of chicory «hairy roots».

Keywords: *plant growth regulators, chicory "hairy roots", polyfructan.*

Introduction

In contemporary biotechnological practice some traditional synthetic growth regulators with auxin and cytokine activity, such as 2,4-D, NAA, BAP [1-4] are used widely in many countries across the world for the cultivation of isolated cells and tissues of ordinary plants as well as for producing transgenic medicinal plants *in vitro*. They are used for the increased synthesis of biologically active components in cultures – amino acids, peptides, and etc. Such substances are extensively used in medical, pharmaceutical, fragrance and food industries [5-7]. Innumerable new biologically active compounds of chemical origin capable of stimulating morphogenetic processes in isolated cells of plants important for agriculture, as well as to induce the synthesis of secondary metabolites in medicinal plant cultures have been developed and implemented into the plant biotechnological practice over the last few years. For example, in the cultures of isolated cells and tissues of different plant species (*Arachis hypogeae L., Pelargonium hortorum Bailey, Humulus lupulus L., Phaseolus radiatus L.*) in nutrient media that include the synthetic cytokinin substitutes TDS (thidiazuron, thiourea derivative) [8-11] and auxin BSAA (3 - [benzo] b]- selenienil acetic acid) [12], some results were obtained which revealed a significant induction of the morphogenetic processes by these regulators and a consequent increase in the levels of the endogenic phytohormones (auxins, cytokinins) in plant cells cultivated in these media.

Similar experiments were carried out earlier to study the possibilities of cultivating isolated plant cells: tobacco (*Nicotiana tabacum* L.), potato (*Solanum tuberosum* L.), and two species of tomatoes *(Lycopersicon esculentun* L. and L. *peruvianum*) in the nutrient medium with growth regulators synthesized at the Institute of Bioorganic Chemistry and Petrochemistry and the Institute

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of Organic Chemistry: Ivin (2,6-dimethylpyridine-N-oxide), Triamelon (tris-(2,2-trimethylammonium-methylphosphate iodide), D-107 regulator (1-acetylamino-1-acetylthio-2-oxo-2-phenylethane), and regulator N 2622 (derivative of tetrahydrothiophene dioxide) [13, 14]. In our experiments, we demonstrated that these regulators could be effective substitutes for the natural phytohormones auxin and cytokinins; we also studied the mechanisms of their action, by increasing the endogenic phytohormone pools (auxine and cytokinins) [14, 15].

At the Institute of Bioorganic Chemistry and Petrochemistry together with the National Enterprise Interdepartmental Science and Technology Center "Agrobiotech", several new natural growth regulators widely used in contemporary agriculture practice were developed. They included, Emistim, Poteitin, Zeastimulin, Charkor, Biolan, Biogen, Radostim, and etc. These regulators include a polycomponent content involving the metabolic products of a symbiotic fungus-endophyte of the ginseng root Panax ginseng M., cultivated *in vitro* (e.g., mixture of amino acids, carbohydrates, fatty acids, polysaccharides, phytohormones and microelements) that influences the plant growth processes. In particular, they stimulate seed germination and promote the productivity of a number of agricultural crops, as well as enhance their resistance to a wide range of pathogenic and parasitic organisms [16, 17]. The most effective among these regulators are the growth regulators Emistim, Biolan, and Charkor (stimulator of root formation in young plants of fruit and baccate cultures, decorative trees, flowers and medicinal plants) [18]. Therefore, the use of these regulators in biotechnology, more specifically for the increase in the synthesis of secondary metabolites in the *in vitro* cultures of transgenic plants stimulates great theoretical and practical interest.

Chicory (*Cichorium intybus* L.) is very important in agricultural and medicinal culture. Its medical properties are defined by its high content of bioactive polyfructans (PF), inulin polysaccharide being the most widespread among them. It has arelatively low molecular mass of ~5000-6000 Da [19-23]. Earlier, the scientists of the Institute of Cell Biology and Genetic Engineering (ICBGE) had obtained cultures of chicory "hairy roots" using *Agrobacterium rhizogenes*-mediated transformation with genes of the tubercular antigen ESAT6 (esxA), Ag85 (fbpB$^{\Delta TMD}$) and human leucocyte interferon *ifn-a2b*, and have thus determined the inulin content within the transformed roots [22]. The transgenic roots obtained demonstrated an increased PF content that may be explained as a probable result of the stress linked with genetic transformation.

The aim of this work was to study the possibility of inducing an increase in the amount of biomass by utilizing PGR Ivin, Emistim, Biolan, and Charkor in chicory "hairy roots" as well as to study the increase in the PF synthesis in them.

Material and Methods

In our experiments we have used the transgenic roots of chicory *Cichorium intybus* L. (*Palla Rossa* cultivar) from four lines (No. 2, 6, 14, and 21), obtained by the *Agrobacterium rhizogenes*-mediated transformation of the explants of the cotyledons with vector pCB 161 (selective gene *npt-II*, objective gene *ifn-alfa2b*), given by Matweeva N.A., PhD, the Head of Laboratory of Stress Resistance Biology and Biotechnology in the Institute of Cell Biology and Gene Engineering [22]. First, 10 mm sized terminal root explants were cultivated in agar and ½ MS liquid medium (Murashige and Skoog medium [24] with the concentration of macroelements diminished twice) at +24 °C for 30 days. Growth regulators Ivin, Emistim, Biolan, and Charkor were added sequentially to the nutrient medium in concentrations 0, 2.5, 5.0 and 10.0 μL/L, respectively.

For comparison we conducted experiments with traditional PGRs adding 0.5 mg/L of kinetin, IBA, BAP, and NAA separately to the ½ MS medium. Root cultivation was done under similar conditions.

We determined the initial root mass (m_0), root mass after 30 days of cultivation (m_i); increase in root mass (Δm), dry mass ($m_{i\text{-}dry}$), relative PF content (mg/g of dry root mass) and total PF content (mg/g of total dry mass of roots grown for 30 days).

To determine the total PF content, the roots were dried at 90 ºC for 10 min, and then at room temperature to constant mass. The PF content was determined using Selivanov's method based on the ability of keto-saccharose to become decolorized by 0.1 % ethanol solution of resorcinol in an acid medium [25]. For this purpose 5 mL of distilled water, 5 mL of 0.1 % ethanol solution of resorcinol, and 5 mL of concentrated hydrochloric acid were added to 100 mg of dry root material, and heated in a water bath at 80 ºC for 20 min. Then the solution was cooled and the color intensity was measured using a KPK-2 Photoelectric Colorimeter with a green color-filter (λ=550 nm). The PF concentration was determined by straight line calibration using fructose.

Each experiment was repeated four times. Calculation and processing of the data obtained were done with dispersion, correlation and regression statistical analyses [26, 27] and with the use of computer programs Statistica 6.0 and Microsoft Excel 2010.

Result and Discussion

A comparative study of the influence of PGRs (Ivin, Emistim, Biolan, and Charkor) on the increase in the growth of the chicory "hairy root" mass, as well as on the increase in the specific and total PF content have demonstrated that the PGRs Ivin, Emistim, and Charkor reveal high root growth activity (when applied in both the agar medium and the liquid medium).

On cultivation of four lines (No. 2, 6, 14, and 21) of transgenic chicory roots in the agar nutrient ½ MS medium with the addition of the growth regulators Ivin, Emistim, Biolan, and Charkor, the roots exhibited different degrees of sensitivity to the influence of these preparations. Although the presence of PGR in different concentrations (2.5-10.0 μL/L) in the agar medium resulted in a significant increase in the growth of the root mass in all four lines compared with the control, the best results were obtained for line No. 21 (Fig. 1).

The addition of the growth regulator Biolan to the nutrient ½ MS medium increased the total root mass much less (maximum in 7.6 times for 30 days for No. 21 line in a concentration of 5.0 μL/L) than in the control case (½ MS medium) as well as in the case of Charkor. For the lines No. 2, 6 and 14, growth inhibition was registered when tested at the maximum concentration (10.0 μL/L) of Biolan; for line No. 14 the increase in mass was 1.6 times less than in the control.

The addition of Charkor to the nutrient agar ½ MS medium activated root growth and significantly increased the chicory root mass (up to 11.5 times in comparison with the control) (Fig. 1). Maximal indices for root increments were obtained for line No. 21 in the nutrient ½ MS medium for this regulator. For this line (and for line No. 14 also) the root growth showed no reliable differences for different concentrations of the regulator (2.5 – 10.0 µL/L), although it was significantly higher (in 10.1 – 11.5 times) than in the control.

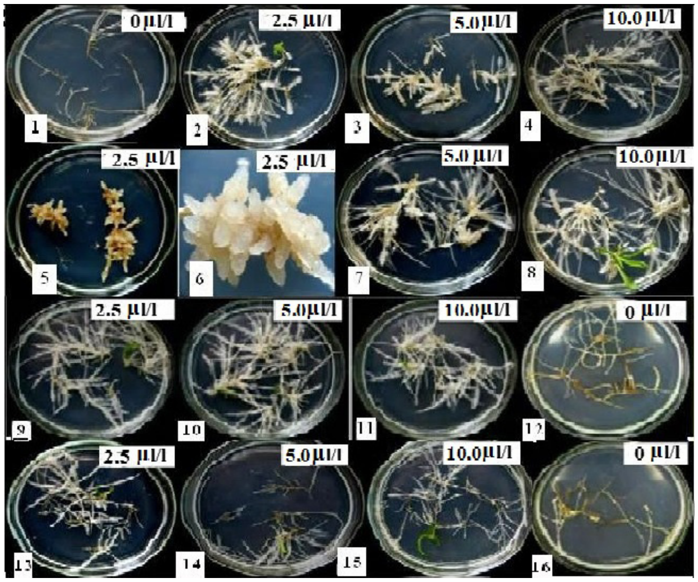

Figure 1.
Growth of the transgenic chicore roots (line No. 21), obtained by the Agrobacterium rhizogenes-mediated transformation (vector pCB 161) in the agar medium: 1) without regulator (control); 2-4) with the growth regulator Ivin (2.5-10.0 µL/L); 5-8) with the growth regulator Emistim (2.5-10.0 µL/L), 6) (macro photography) with the growth regulator Emistim (2.5 µL/L); 9-12) with the growth regulator Charkor (2.5-10.0 µL/L); 13-16) with the growth regulator Biolan (2.5-10.0 µL/L).

With the increased concentration of Charkor from 2.5 to 10.0 µL/L for line No. 6 and 2, an increase in the root mass, up to 9.6 times was recorded (10.0 µL/L for line No. 6). Under culture conditions (½ MS liquid medium), the addition of the Charkor growth regulator resulted in the increase in the root mass from 39 to 54 times (line No. 21) depending on the concentration (from 2.5 to 10.0 µL/L of medium). of the preparation. Although a minimal increase in the mass increment (for dry mass) have been recorded in the control case as well (medium ½ MS without the addition of any growth regulators).

A comparative analysis of the biological activities of Charkor and Biolan according to the index of increase in PF synthesis in the culture of chicory «hairy roots» demonstrated a significantly higher efficacy with Biolan, with respect to specific PF content (mg/g of root mass) (Fig. 2 B). The maximal increase in the specific PF concentration (up to 130 mg/g of dry root mass) compared with the control was obtained during the cultivation of the № 6 line roots in the presence of Biolan (5.0 µL/L). The increase in the concentration of the preparation, from 2.5 to 10.0 µL/L, during the cultivation of lines No. 21 and No. 2, resulted in the increase of the specific PF concentration. It must be mentioned that with the addition of Biolan in maximal concentration, the specific PF concentration was only 1.32-1.36 times higher than in the control. On the other hand, we have not registered statistically reliable differences in the influences of different concentrations of Charkor on the increase in the specific PF concentration on dry root mass, compared with the control (Fig. 2 B). However, during the cultivation of the transgene roots of line No. 6 in the nutrient medium with 5.0-10.0 µL/L Charkor, a decrease in the specific PF content was registered. This effect may be linked with the more intensive root growth under such conditions.

Nevertheless, the highest increase in the total amount of PF was obtained during the root cultivation using Charkor compared with the control or with the Biolan. As seen from the diagrams (Fig. 2 A, Fig. 2 C), this may be explained by the increase in the total root mass during the cultivation using Charkor (2.5 – 10.0 µL/L). The best indices were obtained for the transgene roots of line No. 21; these indices for total PF content increase were 7.0 – 7.5 times higher than in control (up to 48 - 130 mg/g of total dry mass).

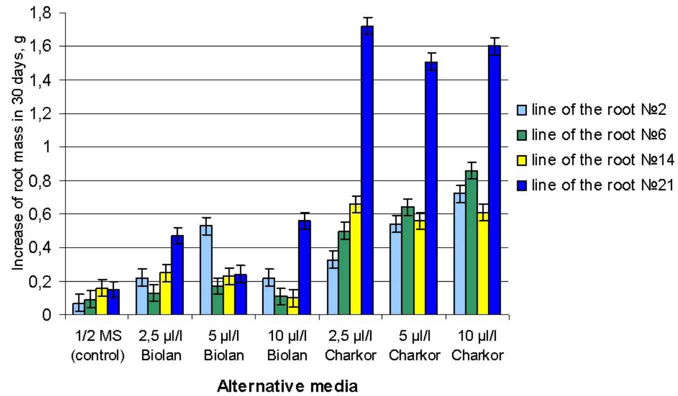

Fig. 2 A

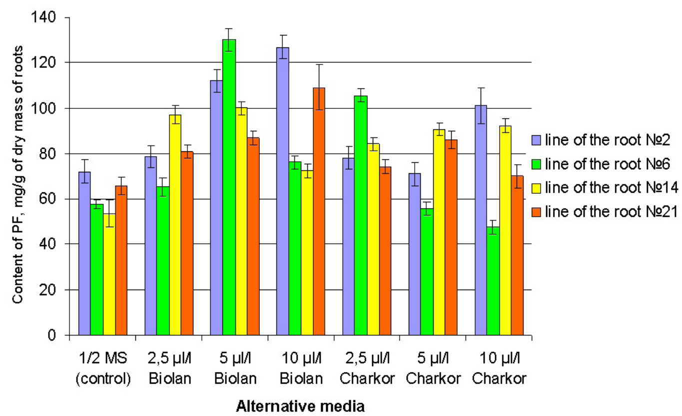

Fig. 2 B

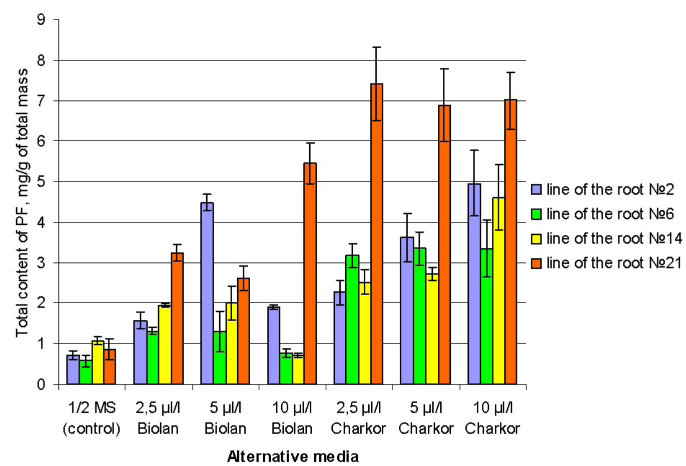

Fig. 2 C

Figure 2
Influence of growth regulators Biolan and Charkor on root mass increment (A), specific (B) and total (C) PF content in the cultures of chicory «hairy roots».

An increase in the Charkor concentration up to 10.0 μL/L caused an increase in the total PF concentration for the roots of lines No. 12 and 2 (Fig. 2 C). For the two other lines, No. 6 and 21, no statistically reliable differences regarding the influences of three concentrations of this regulator on the total PF content were registered.

Thus, the different lines of the transgene chicory roots revealed different sensitivity for both the regulators, Biolan and Charkor. As each line of the transgene roots is unique (separated transformation event), it can be assumed that such specific sensitivity is linked with the peculiarities of the endogenic phytohormone synthesis after the transmission of the alien genes into the plant genome and the integration of these transgenes into the different loci.

Therefore, the highest PF content was registered for the roots of No. 6 line (5.0 μL/L Biolan). At the same time under culture conditions with Charkor, the specific PF content in these roots was less compared with the roots of other lines under such conditions.

The addition of the regulators, Ivin and Emistim, caused a significant increase in the root masses (Fig. 3 A). The increase in mass depended upon the line and preparation, an increase from 2.4 (line No. 2) to 20.25 times (line No. 21) for Ivin and from 5.68 (line No. 2) to 26.75 (line No. 21) for Emistim.

Therefore, the growth regulator Emistim induced the most positive influence on the growth of the transgene chicory roots; the increase in mass in the best case was more than 26.75 times higher than in the control.

A significant increase in the total amount of PF compared with the control was registered also during the cultivation of the transgene chicory roots in the agar ½ MS medium, in the presence of Ivin and Emistim. The effect was due to the increase in the amount of specific PF as well as because of the greater increase in the root masses capable of synthesizing PF (Fig. 3 B, Fig. 3 C).The total PF content was dependent upon root line and the presence of the growth regulator.

For example, the addition of Ivin increased the total PF, which was higher than in the control (2.6 times for line No. 2 and 28.9 times for line No. 21). In experiments with the addition of Emistim, this increase was 5.27 and 35 times higher than in the control (for lines No. 2 and 21, respectively).

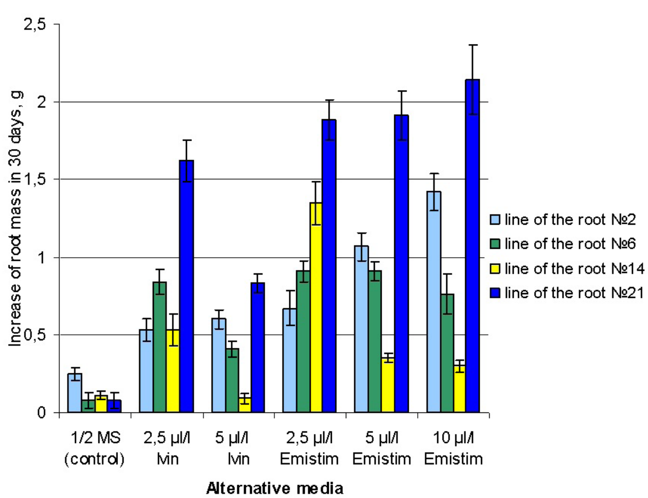

Fig. 3 A

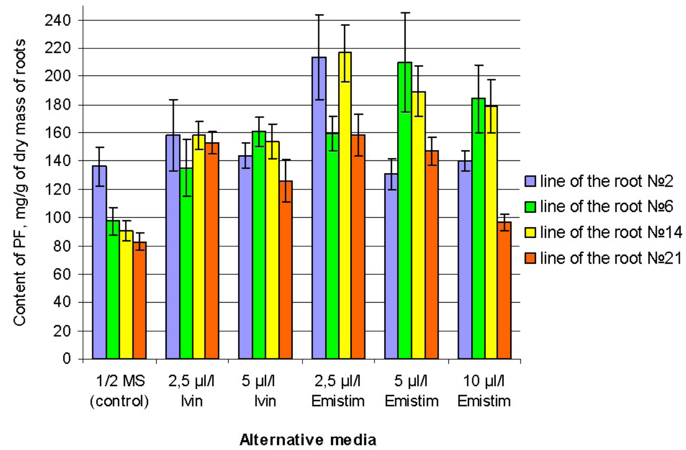

Fig. 3 B

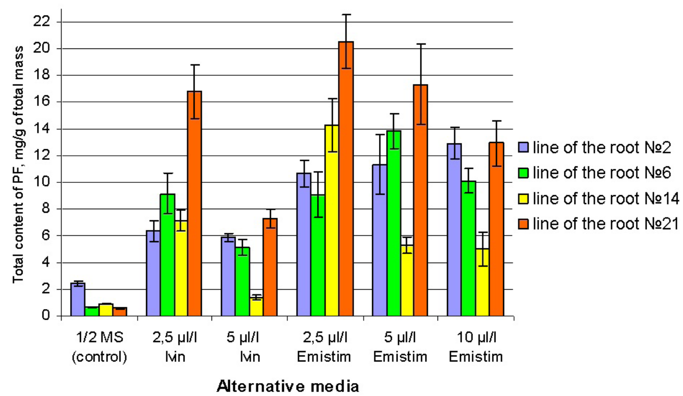

Fig. 3 C

Figure 3.
Increase in the root mass (A), specific (B) and total (C) PF content in the cultures of chicory «hairy roots», cultivated in the presence of growth regulators like Ivin and Emistim for 30 days.

In our experiments, we also studied the influence of the uniqueness of Charkor compared with that of the traditional growth regulators (BAP, IBA, NAA, kinetin) for two lines (No. 6 and 21) of chicory «hairy roots» cultures. The results gave evidence of the visible increase of the intensity of the transgene root growth in the presence of auxins (IBA, NAA), which consequently promoted the increase of the total PF (Fig. 4 A, Fig. 4 B). Maximal increment of root masses was registered after the addition of 0.5 mg/L IBA to the nutrient medium; a similar effect was also registered in presence of 5.0 μL/L of Charkor. Insignificant increase of the same indices was registered after the addition of kinetin and BAP as well, into the medium (each of 0.5 mg/L).

The results obtained with changes in the phenotype characteristics (biomass growth, root disintegrations) and efficacy indices (total PF content) between the control and the experimental roots gave evidence regarding the processes of partial reprogramming in the genome lines studied under the influence of Ivin, Emistim, Biolan, and Charkor. “Switching on” happened to the cascades of genes which were previously non-active but closely related in their functions in multigene families or gene superfamilies of endogenic phytohormone biosynthesis (which accelerated the growth of the biomass and increased the efficacy). In these cascades each element of the family differs slightly by its nucleotide consequence in its regulatory, coding, and non-coding segment structures, and may be regulated by different factors, for example, by plant growth factors. We demonstrated this earlier in our prior works [15, 16, 28-30]; also works of other authors revealed the same [8, 9, 10 31-34]. Probably, under *in vitro* conditions the contrast growth acceleration of the «hairy root» cells may be explained to be a result of the significant enhancement of the synthesis of the endogenic phytohormone pool due to the influence of the growth regulators in our study.

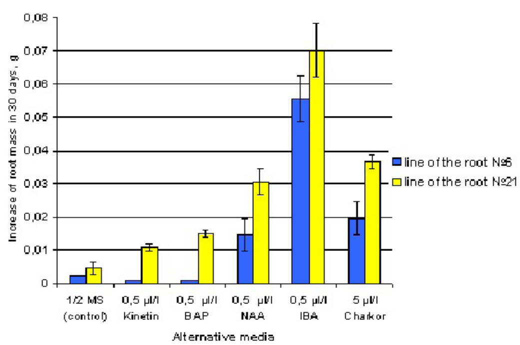

Fig. 4 A

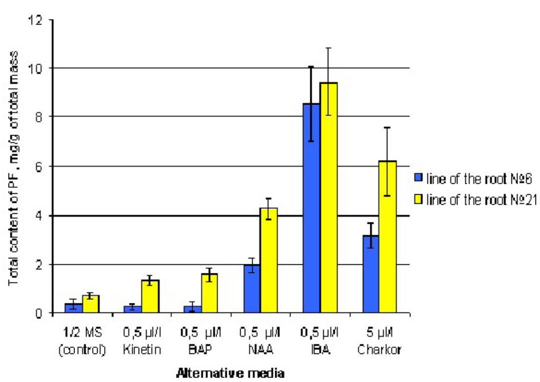

Fig. 4 B

Figure 4
Increase in the root mass (A) and total (B) PF content in the cultures of chicory «hairy roots», cultivated for two lines in the presence of growth regulators like kinetin, BAP, NAA, IBA, Charkor.

Conclusion

The experimental tests on the biological activity of the growth regulators Emistim, Ivin, Charkor, and Biolan on cultivated chicory root cells (*C. intybus* L.) transformed by *A. rhizogenes* have demonstrated that these regulators significantly increase the growth of the total root biomasses: up to 26.75 times with Emistim, up to 20.25 times with Ivin, up to 9-11.5 times in the agar medium ½ MS, and up to 39-54 times in the liquid medium ½ MS with Charkor, and up to 7.6 times with the Biolan. The influences of the regulator on the indices of the PF specific content in different root lines were found to be different.

It was possible to register high indices of PF specific content for line No. 6 – up to 130 mg/g of dry root mass (Biolan – 5.0 µL/L), and for line No. 2 – up to 220 mg/g of dry root mass (Emistim – 2.5 µL/L).

The highest root stimulating activity was demonstrated by using growth regulators such as Emistim, Ivin and Charkor. During the cultivation of the roots of line No. 21 in the nutrient medium with these regulators in concentrations from 2.5 to 10 µL/L the specific PF content was enhanced significantly, up to 130 - 216 mg/g of dry root masses, as well as the total PF content, which went up to 35 times with 2.5 µL/L Emistim vs the control, up to 28 times with 2.5 µL/L Ivin, and up to 7.0 – 7.5 times with 2.5 µL/L Charkor.

A comparative study with similar experiments using traditional synthetic phytohormone substitutes (BAP, IBA, NAA, kinetin) demonstrated that the addition of the growth regulator Charkor to the nutrient medium caused an increase in the total PF content due to the significant increase in the root biomasses. The influence of the growth regulator Charkor on the increase in root mass was similar to the effect induced by auxins, indolebutyric acid in particular.

The results obtained support the benefits of using growth regulators such as Ivin, Emistim, Biolan, and Charkor for the stimulation of the increase of biomass and synthesis of PF in the cultures of chicory "hairy roots".

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