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OBTAINING BIOMASS OF ECHINACEA PURPUREA IN VITRO

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This work investigated the possibility of introducing Echinacea purpurea into in vitro culture in order to obtain callus biomass. The method of seed sterilization and seedling cultivation on Murashige-Skoog medium was described. The effect of various combinations of growth regulators (IAA, BAP, NAA, 2,4-D) on callus formation from leaf, stem and root explants was experimentally determined. It was found that the formation of callus biomass occurs better on a medium with the addition of growth regulators IAA and BAP in a ratio of 2:1 mg/l than NAA, 2,4-D in a ratio of 1:1 mg/l. Callus of yellow-green and light brown color was formed on leaf and stem explants. Root explants did not show callusogenesis.

Keywords: plant raw materials, cultivation, callus biomass, *Echinacea purpurea*.

Introduction

In modern conditions, the cultivation of plants under *in vitro* conditions makes it possible to produce a large amount of homogeneous plant seedlings in a short time, allows for genetic modification, opens wide prospects for increasing yield, resistance to stress conditions and protection against diseases and pests, allows for the preservation of the gene pool of rare, vulnerable, or agriculturally important plants, which is important for the preservation of biodiversity and food security. Also, tissue culture can be used to study the influence of various stress factors on plants, for example, drought, soil and air pollution, and to develop methods of adaptation. The cultivation of plants *in vitro* makes it possible to grow plants at any time of the year, regardless of weather conditions, without occupying large areas [1–3].

It is known that medicinal plants are a source of metabolites that are used in pharmaceutical preparations, cosmetic products, and food items. The use of medicinal plants originates from ancient times and is considered the foundation of modern medicine. Currently, plant extracts are widely used for the prevention of various diseases and account for about 30 % of the medicine market. Plant metabolites are quite well studied; the mechanisms of their action and applications are known according to the standards of evidence-based medicine. Plants contain various metabolites (primary metabolites: amino acids, carbohydrates, lipids, vitamins; secondary metabolites: antibiotics, alkaloids, flavonoids, plant growth hormones, toxins, etc.). Therefore, as raw materials, they are used in various branches of industry, including pharmaceutical, cosmetic, food, and agrochemical. To obtain valuable compounds from plants in maximum quantities, it is necessary to optimize cultivation conditions. This is not always possible when growing in natural conditions, so cultivating plants *in vitro* is a good alternative.

Plants of the genus *Echinacea* occupy an important place among medicinal plants. *Echinacea purpurea* is one of the nine species of *Echinacea* and one of three used as a medicinal plant with wide therapeutic application. The metabolites of the plant are alkylamides, polysaccharides, glycoproteins, flavonoids, and phenolic compounds, which include derivatives of caffeic acid such as caffeic acid, chicoric acid, caftaric acid, chlorogenic acid, and echinacoside, the amount of which changes depending on the organ of the plant. Also found in plant extracts are fatty acids, aldehydes, terpenoids, phylloxanthobilins, β-phellandrene, acetaldehyde, dimethyl sulfide, camphene, hexanal, α-pinene, and limonene, which are present in all plant tissues regardless of the species of the plant. It is also known that extracts of *Echinacea purpurea* have the following properties: immunomodulatory, antiviral, antibacterial, anti-inflammatory, antioxidant, antitumor, and others. Most often, this plant is used as a component of medicinal products and vitamin complexes with immunomodulatory properties [4, 5].

When analyzing publications on the *Echinacea genus*, it can be noted that until 2009 there was an increase in research, then a slight decrease until 2015, and again an increase in the number of publications until today [4].

The aim of our study is to introduce *Echinacea purpurea* into *in vitro* culture and optimize the cultivation conditions for maximum production of callus biomass.

To achieve the goal, the following tasks were performed:

- analysis of the literature on the cultivation of the medicinal plant *Echinacea purpurea* in *vitro* and on methods for optimizing cultivation conditions;
- obtaining of callus biomass of *Echinacea purpurea* plant *in vitro*;
- optimization of cultivation conditions for *Echinacea purpurea* .

Materials and research methods

Plant material. The raw material used for the research was seeds of *Echinacea purpurea* from two different producers – "Nasinnia Ukrainy" (*Seeds of Ukraine*) and "Helios". The understudied raw material was introduced into Murashige and Skoog (MS) medium under *in vitro* conditions.

Stage I of cultivation:

To introduce *Echinacea purpurea* seeds into *in vitro*, MS medium of the following composition was prepared: *macrosalts* : NH_4NO_3 (1650 mg/l), $CaCl_2{\cdot}2H_2O$ (440 mg/l), $MgSO_4{\cdot}7H_2O$ (370 mg/l), KH_2PO_4 (170 mg/l), KHO_3 (1900 mg/l); *microsalts:* H_3BO_3 (6.2 mg/l), $CoCl_2{\cdot}6H_2O$ (0.025 mg/l), $CuSO_4{\cdot}5H_2O$ (0.025 mg/l), $MnSO_4{\cdot}5H_2O$ (22.3 mg/l), KI (0.83 mg/l), $Na_2MoO_4{\cdot}2H_2O$ (0.25 mg/l), $ZnSO_4{\cdot}7H_2O$ (8.6 mg/l); *chelates:* Na_2EDTA (Trilon B) (37.23 mg/l), $FeSO_4{\cdot}7H_2O$ (27.95 mg/l); *vitamins* : thiamine (B1) (0.1 mg/l), nicotinic acid (B5) (0.5 mg/l), pyridoxine (B6) (0.5 mg/l), mesoinoside (100 mg/l); *auxiliary components:* glycine (2 mg/l), sucrose (30,000 mg/l), agar (5,000 mg/l).

The seeds were soaked in water 18 hours before planting. The medium was sterilized in an autoclave and poured into Petri dishes. The seeds were sterilized by immersion in 70 % ethyl alcohol for 30 seconds, then in 30 % hydrogen peroxide for 5 minutes, and rinsed three times with sterile distilled water. In addition, to test seed germination, they were planted in different soil types: natural soil and universal commercial soil bought from a store. All laboratory glassware was sterilized in a drying oven at 170 °C for 3 hours. The sterile seeds were germinated in a chamber under 24-hour darkness at 23 °C. After 42 days, *Echinacea purpurea* seedlings were obtained, which were used as explants for Stage II of cultivation. The seedlings were divided with a scalpel into fragments – separately leaves, stems, and roots.

Stage II of cultivation:

Sterile explants were introduced into modified MS medium supplemented with different combinations of growth regulators. Indole-3-acetic acid (IAA) and benzylaminopurine (BAP) were used in a ratio of 2:1 mg/L, α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in a ratio of 1:1 mg/L of medium. The experiment was carried out in 10 Petri dishes, with five explants of leaves, stems, and roots in each dish. Cultivation was performed in a laminar flow cabinet at a temperature of (23±2) °C, photoperiod of 16/8 hours (light/dark), relative humidity of 60–70 %, illumination of 2000 lux with cool white light. Callus formation and biomass growth were quantitatively evaluated on the 65th day of cultivation [2, 11].

Results and discussion

Based on the analysis of scientific literature, it was found that *Echinacea* is an interesting object of study due to the presence of a number of polyphenolic compounds and flavonoids, which exhibit immunomodulatory, antioxidant, neuroprotective, antimicrobial, and several other therapeutic properties [6–8].

The plant is used in official medicine as medicinal plant raw material obtained from cultivated plants grown in nature [7]. There is also information about the cultivation of the plant under *in vitro* conditions, which is promising for the further use of plant biomass [9–14].

During *in vitro* cultivation, the pH of MS medium was adjusted to 5.6 using 1N KOH or 1 % HCl, then sterilized in an autoclave at 120 °C and a pressure of 0.8 atm for 20 minutes. The seeds were washed three times with soap and water, then sterilized in 30% hydrogen peroxide for 20 minutes and aseptically transferred into Petri dishes. The

Echinacea seeds from "Nasinnia Ukrainy" germinated on the 35th day in darkness at 23–25 °C (Fig. 1). In soil, germination occurred faster – on the 8th day (Fig. 2). The seeds from "Helios" did not germinate either in the soil or in the MS medium.

The plants obtained from seeds were cut using a sterile scalpel and aseptically transferred into Petri dishes with modified medium for callus induction.

The explants used for callusogenesis initiation were leaves, hypocotyl, and roots of the plant obtained during the first stage of cultivation (Fig. 3).

Various conditions were experimentally selected (temperature, lighting), the type of explant, and different concentrations of growth regulators. Cultivation was carried out at temperatures of 15 °C and 23 °C under both light and dark conditions using two modifications of MS nutrient medium. Cultivation was conducted with a photoperiod of 18/6 (light/dark), illumination of 2000 lux. Photos of Petri dishes with leaves, stems, and roots are shown in Fig. 3.

Explants were visually analyzed every three days. On Some explants, the growth of callus mass was observed as early as two to three weeks after inoculation. Thickening occurred, especially at the ends of the segments, gradually expanding until fully covered with primary callus mass. No infected explants were detected, indicating that the sterilizing agents were successfully selected.

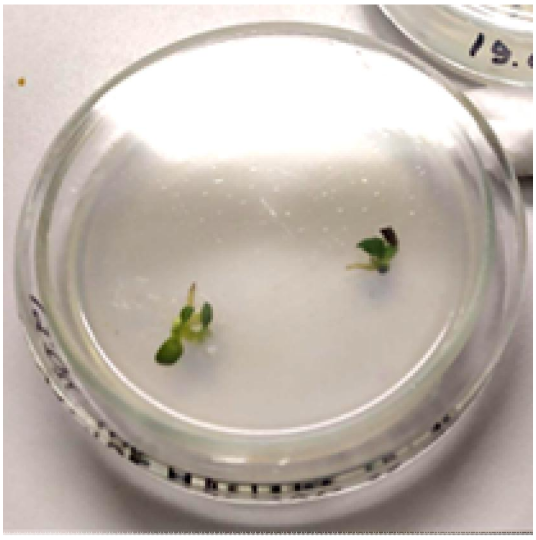

Fig. 1. Germinated seeds of Echinacea purpurea in MS medium for 5 weeks

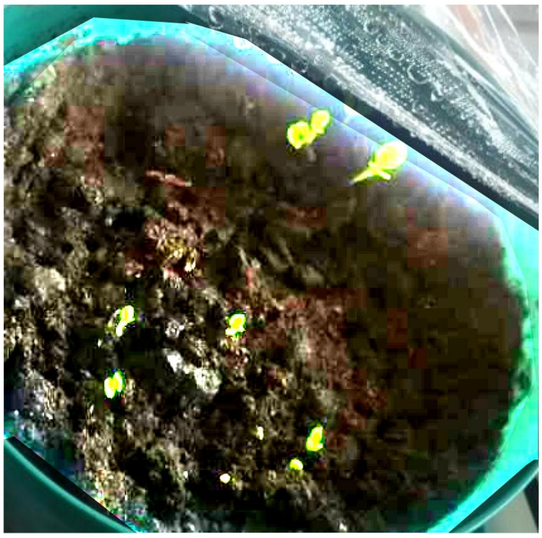

Fig. 2. Germinated seeds of Echinacea purpurea in soil for 4 weeks

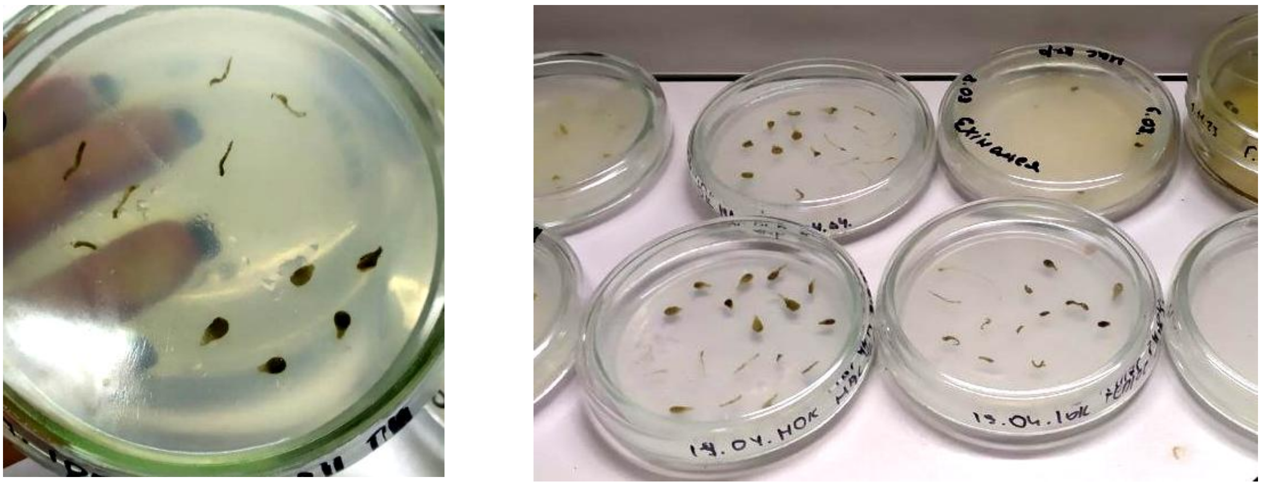

Fig. 3. Explants of Echinacea purpurea (leaves, stems, and roots of the plant) on modified MS medium

Over the course of 65 days following the placement of explants on modified MS media, biomass of yellow-green and light-brown color with a soft texture and uneven edges was obtained (Fig. 4). The results of the influence of growth regulators on the growth of callus biomass of *Echinacea purpurea* are presented in Fig. 5. On MS medium with IAA:BAP in a ratio of 2:1 mg/L, better growth results of leaves and stems were obtained compared to the MS medium with NAA:2,4-D in a ratio of 1:1 mg/L. On roots, no callus biomass was obtained on any of the media. At a temperature of 15 °C and in darkness, no positive results of callus biomass formation were observed either. The biomass obtained from leaf explants on MS medium with IAA:BAP (2:1) was taken as 1. Accordingly, the biomass from leaf explants on MS medium with NAA:2,4-D (1:1) was evaluated as 0.9. The biomass from stem explants on MS medium with IAA:BAP (2:1) – 0.6, and the biomass from stem explants on MS medium with NAA:2,4-D (1:1) – 0.45.

Fig. 4. Biomass of Echinacea purpurea on 65th day of cultivation

The obtained biomass was removed from the MS medium and weighed. The amount of biomass obtained as a result of the experiment was 120 g.

This study clearly demonstrates that the proposed sterilization technique – 70 % ethyl alcohol for 30 seconds followed by 30 % hydrogen peroxide for 5 minutes – provides 100 % sterilization efficiency.

Also, on both modified MS media used, with IAA:BAP (2:1) and NAA:2,4-D (1:1), callus biomass was obtained from leaf and stem explants within 56 days.

It was established that the most optimal medium is modified MS with the addition of IAA:BAP (2:1), at a temperature of 23 °C, illumination of 2000 lux, and a photoperiod of 16/8.

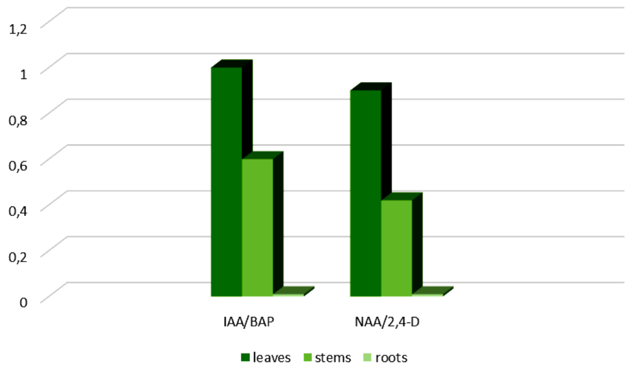

Fig. 5. Influence of growth regulators on biomass production from different explants (leaves, stems, roots) on two different modified MS media

The obtained biomass was removed from the MS medium and weighed. The amount of biomass obtained as a result of the experiment was 120 g. This study clearly demonstrates that the proposed sterilization technique – 70 % ethyl alcohol for 30 seconds followed by 30 % hydrogen peroxide for 5 minutes – provides 100 % sterilization efficiency. Also, on both modified MS media used, with IAA:BAP (2:1) and NAA:2,4-D (1:1), callus biomass was obtained from leaf and stem explants within 56 days.

It was established that the most optimal medium is modified MS with the addition of IAA:BAP (2:1), at a temperature of 23 °C, illumination of 2000 lux, and a photoperiod of 16/8.

Conclusions

Callus biomass of *Echinacea purpurea* was obtained *in vitro* on Murashige and Skoog nutrient medium with growth regulators indole-3-acetic acid (IAA) and benzylaminopurine (BAP) in a ratio of 2:1, and α-naphthaleneacetic acid (NAA) with 2,4-dichlorophenoxyacetic acid (2,4-D) in a ratio of 1:1 mg/L of medium. It was determined that the use of IAA and BAP (2:1) provides greater biomass increase of *Echinacea purpurea,* than described in previous studies.

Cultivation conditions for maximum callus biomass production of *Echinacea purpurea* were determined: temperature (23±2) °C, photoperiod 16/8 (light/dark), relative humidity 60–70 %, illumination 2000 lux, cool white light.

The obtained plant biomass can be used to create cosmetics and medicines with immunomodulatory, anti-inflammatory, antioxidant and antiviral properties. Therefore, further studies are planned on the influence of growth regulators and cultivation conditions on the increase of callus biomass of *Echinacea purpurea*, determining the qualitative and quantitative composition of the obtained biomass, and compare it with the plant from nature.

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ОТРИМАННЯ БІОМАСИ ECHINACEA PURPUREA IN VITRO

У роботі досліджено можливість введення *Echinacea purpurea* в культуру *in vitro* з метою отримання калусної біомаси. Описано методику стерилізації насіння та вирощування проростків на середовищі Мурасіге-Скуга. Експериментально визначено вплив різних комбінацій регуляторів росту (ІОК, БАП, НОК, 2,4-Д) на утворення калусу з листкових, стеблових і кореневих експлантів. Встановлено, що вищі ростові характеристики калусної біомаси на середовищі з додаванням регуляторів росту ІОК та БАП у співвідношенні 2:1 мг/л, ніж НОК, 2,4-Д у співвідношенні 1:1 мг/л. На листкових і стеблових експлантах утворювався калус жовто-зеленого і світло-коричневого кольору. Кореневі експланти калюсогенезу не проявляли.

Ключові слова: рослинна сировина, культивування, калусна біомаса, *Echinacea purpurea.*