

Short communication

Galanthamine production by *Leucojum aestivum* in vitro systems

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Abstract

The callus induction from young fruits of *Leucojum aestivum* was performed on Murashige–Skoog nutrient medium supplemented with 4 mg/L 2,4 dichlorophenoxyacetic and 2 mg/L 6-benzylaminopurine. Further, by planting the obtained calluses on the same nutrient medium supplemented with 1.15 mg/L α -naphthylacetic acid and 2.0 mg/L 6-benzylaminopurine shoot cultures were established. The growth and galanthamine accumulation of obtained *L. aestivum* in vitro systems were studied. It was established that the amount of accumulated galanthamine strongly depended on the level of the differentiation. The maximum yield of biomass (17.8 g/L) and the maximum amount of accumulated galanthamine (2.5 mg/L) were achieved after day 35 of submerged cultivation of *L. aestivum* 80 shoot culture, performed under illumination. Data concerning the time courses of the utilization of the main nutrient components of the medium during cultivation of *L. aestivum* shoot culture are presented as well. © 2007 Elsevier Ltd. All rights reserved.

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1. Introduction

Amaryllidaceae-type alkaloids possess antiviral [1] and anti-tumor properties [2], as well as an anticholinesterase activity [3]. The most studied compound in this area is galanthamine, which is widely used in medicine as a strong reversible inhibitor of acetylcholinesterase [4–6] for the treatment of Alzheimer's disease, as well as for the treatment of poliomyelitis and other neurological diseases [7–9]. Currently, the list of plants from which galanthamine has been isolated includes more than 20 species and the search for new sources is still continuing [10]. Although chemical synthesis of galanthamine has been successfully performed [11], the main commercial source in Bulgaria for the production of the galanthamine-based medicine under trade name Nivalin[®] is *Leucojum aestivum*, a

Euro-Mediterranean species, also called summer snowflake. Since 1989, prescription regime of the utilization of this plant species has been imposed and, from these perspectives, galanthamine production from in vitro cultures is considered an attractive alternative.

The callus induction is a key point both for micropropagation and for obtaining of in vitro systems for alkaloid production. To date, there have been only a few reports on callus induction from different explants of Amaryllidaceae plants as a step of micropropagation [12–14]. The accumulation of galanthamine has been detected in callus [12] and in shoot-clump cultures [11] of *Narcissus confusus*, but to our knowledge there is not enough data available on galanthamine production by *L. aestivum* in vitro systems, or on the relationships between in vitro growth, nutrition and galanthamine biosynthesis in these species.

In this study, callus induction and further shoot formation, as well as the relationships between growth and galanthamine production by *L. aestivum* in vitro systems, in parallel to the

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utilization of the main nutritive compounds from the medium, are presented.

2. Materials and methods

2.1. Plant material

The young fruits of *L. aestivum* L. plants (at the beginning stage of seed formation) were collected from the experimental field of the Institute of Botany–Bulgarian Academy of Sciences. They were carefully washed with tap water, surface sterilized by treatment with 70% EtOH for 10 s and then with 7% Ca-hypochloride (Sigma, USA) for 15 min. Further, they were thoroughly rinsed with sterile distilled water, dried on sterile tissue paper and used as explants in the experiments.

2.2. Culture conditions

2.2.1. Nutrient medium

For all experiments Murashige–Skoog (MS) nutrient medium (Dushefa, The Netherlands) was used, supplemented with 30 g/L sucrose and different amounts of growth regulators and agar according to the experiments. The sucrose and growth regulators were sterilized by filtration (Millipore filters, type GTTP, 0.2 μ m) and add to the MS nutrient medium after its autoclaving (121 °C).

2.2.2. Callus induction

The explants were cultivated on MS medium supplemented with 30 g/L sucrose, 5.5 g/L “Plant agar” (Duchefa, The Netherlands) and different concentrations and combinations of auxins (2,4 dichlorophenoxyacetic acid (2,4D) (Sigma, USA) – 0.2; 1.0; 2.0; 3.0; 4.0; 5.0 mg/L, α -naphthylacetic acid (NAA) (Duchefa, The Netherlands) – 1.0; 2.0; 3.0; 4.0; 5.0 mg/L, Picloram (Duchefa) – 1.0; 2.0; 3.0 mg/L) and cytokines (kinetin (Sigma) – 0.5; 1.0; 2.0; 3.0; 4.0 mg/L, 6-benzylaminopurine (BAP) (Duchefa) – 0.5; 1.0; 2.0; 3.0; 4.0 mg/L and Zeatin (Duchefa) – 0.5; 1.0; 2.0 mg/L). The cultivation was carried out at 26 °C in darkness.

2.2.3. Shoot formation

The shoot cultures were established by planting the obtained calluses on MS nutrient medium supplemented with 30 g/L sucrose, 5.5 g/L “Plant agar” and three combinations of growth regulators as follows: 0.2 mg/L 2,4D and 1.0 mg/L BAP; 1.15 mg/L NAA and 2.0 mg/L BAP; 5.0 mg/L NAA and 2.0 mg/L isopentenyladenosine (iPA) (Duchefa). The cultivation was carried out at 26 °C in darkness. The further subcultivations of obtained shoot cultures were performed every 28 days under the same conditions.

2.3. Submerged cultivation of *Leucojum aestivum* 80 shoot culture

After the establishment, the *L. aestivum* 80 shoot culture was maintained on a MS medium supplemented with 5.5 g/L “Plant agar”, 30 g/L sucrose, 1.15 mg/L NAA and 2 mg/L BAP, in darkness, at 26 °C, with subcultivation period of 28 days. For the experiment, the shoot culture was transferred into 100 ml conical flasks containing 20 ml of liquid MS medium, at the same combination of growth regulators. The cultivations were performed on a shaker (110 rpm) at 26 °C both under illumination (16 h light/8 h dark per day) and in darkness. The inoculation was performed with about 1 g shoots per flask cultivated on agar medium under the above-mentioned conditions.

2.4. Analysis

2.4.1. HPLC analysis of alkaloids

The samples (about 50 mg dry tissue) were extracted three times with methanol (hydro module = 10) in an ultrasonic bath for 30 min. After filtration, centrifugation and concentration, the methanol extract was analyzed on an HPLC system (WATERS, Fluorescence detector 414) in isocratic regime with scanning fluorescence detector; RP-C8 column (150 mm $\times$ 4.6 mm, 5 μ m);

mobile phase: acetonitril/methanol/water (containing 7.5 mM triethanolamine, pH 6.9) (20/15/65); column temperature: 35 °C; flow rate: 1.0 ml/min; injection volume: 20 μ l.

2.4.2. Gas chromatography–mass spectroscopy (GC/MS) analyses of alkaloids

Dry biomass of in vitro cultures (0.1–0.5 g dry weight) was extracted three times with 5 mL of methanol in an ultrasonic bath for 45 min. The extracts obtained were concentrated and purified according to Berkov et al. [15] and were subjected to GC/MS investigation. The GC/MS analyses were conducted using Hewlett Packard (HP; Palo Alto, CA, USA) 6890 + M.S.D. 5973 equipment operating in the EI mode at 70 eV. An HP-5 MS column (30 m $\times$ 0.25 mm i.d.; 0.25 μ m) was employed with a 30 min temperature program of 80–280 °C at 10 °C/min followed by a 10 min hold at 280 °C. The injector temperature was 280 °C; the flow rate of the carrier gas (helium) was 0.8 mL/min; the split ratio was 1:20. The identification of the alkaloids was performed by comparing the measured data with those of the standards, of authentic compounds according to the NIST 98 database (HP Mass Spectral Library, Palo Alto, CA, USA), or with literature data as specified.

2.4.3. The growth of the shoots

The growth of the shoots was monitored by measurement of the dry biomass and the accumulated dry biomass (ADB) calculated on this base, respectively [16,17].

2.4.4. Conductivity

Conductivity of the medium was measured by a pH/cond. meter (INOLAB, WTW, Germany).

2.4.5. Sucrose, glucose and fructose

Sucrose, glucose and fructose contents in the culture medium were determined by means of an enzyme test combination (R-Biopharma, Germany).

2.4.6. Nitrate, ammonium and phosphate

Nitrate, ammonium and phosphate ions in the culture medium were determined by chemical test combinations Spectroquant® (Merck, Germany).

The data presented are the averages from three independent experiments, which were repeated twice, and expressed as the means with standard deviations ($\pm$ S.D.). The results for Gal production were analyzed by one-way ANOVA and Turkey HSD test with $\alpha = 0.05$ and $\alpha = 0.1$.

3. Results and discussion

3.1. Callus induction from *leucojum aestivum* fruits

The best results concerning callus formation were observed when the explants were planted on MS nutrient medium supplemented with 3 or 4 mg/L 2,4D and 2 mg/L BAP. It has been reported [13,18] that the best potential for callus formation in *Narcissus* comes from out fruits, but there have been no reports on callus formation from fruits of *L. aestivum*. It has to be noted, however, that the developmental stage of used fruits was an important factor in callus induction. While young fruits (at the beginning stage of seed formation) were good primary material for callus formation, calluses were not developed from older fruits. Additionally, an advantage of using young fruits as a primary material for obtaining in vitro cultures of *L. aestivum* was that their sterilization was not a problem, while when bulbs as a primary plant material were used, the disinfection procedures were defined as a critical point [19].

The callus formation started 35 days after the onset of explants. Obtained calli on the first stage were white and small

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