Optimized Nutrient Medium for Galanthamine Production in *Leucojum aestivum* L. *in vitro* Shoot System

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The common effect of NH_4^+, NO_3^-, KH_2PO_4 and sucrose on the biosynthesis of galanthamine by a *Leucojum aestivum* shoot culture was studied. Polynominal regression models were elaborated for the description of the galanthamine biosynthesis as a consequence of variation of the investigated variables (NH_4^+ between 0.20 and 0.54 g/L; NO_3^- between 1.44 and 3.44 g/L; KH_2PO_4 between 0.10 and 0.24 g/L, and sucrose between 30.00 and 60.00 g/L). Optimization procedures allowed us to establish the optimal concentrations of the investigated variables and to propose the modified MS nutrient medium, with 4.50 g/L KNO_3, 0.89 g/L NH_4NO_3, 1.25 g/L $(NH_4)_2SO_4$, 0.10 g/L KH_2PO_4 and 60 g/L sucrose, for the galanthamine production by a *Leucojum aestivum* shoot culture. The proposed modified MS medium provided considerable increase of both the production yield and the relative content of the target alkaloid in the alkaloid mixture.

Key words: Galanthamine, *Leucojum aestivum* Shoot Culture, Medium Optimization

Introduction

Galanthamine, an isoquinoline alkaloid acetylcholinesterase inhibitor, is an important agent used all around the world for the symptomatic treatment of senile dementia of the Alzheimer's type (Diop *et al.*, 2007), as well as for the treatment of poliomyelitis and other neurological diseases (Radicheva *et al.*, 1996; Novikova and Tulaganov, 2002; Heinrich and Teoh, 2004). Although the chemical synthesis of galanthamine has been successfully performed (Barton and Kirby, 1960; Heinrich and Teoh, 2004; Marco and do Carmo Carreiras, 2006), the main commercial sources for the production of galanthamine-based medicines are Amaryllidaceae plants (Cherkasov and Tolkachev, 2002; Ingkaninan *et al.*, 2002; Kreh, 2002; Pavlov *et al.*, 2007). Nowadays, a prescription regime of the utilization of most of these plant species has been imposed and, from these perspectives, the galanthamine production from *in vitro* cultures is considered as an attractive alternative (Bergoñón *et al.*, 1996; Colque *et al.*, 2004; Diop *et al.*, 2007; Georgieva *et al.*, 2007; Pavlov *et al.*, 2007). Till now the research on galanthamine biosynthesis by different plant *in vitro* systems has been focused on the relationship between the degree of differentiation and alkaloid yields (Sellés *et al.*, 1999; Berkov *et al.*, 2005; Diop *et al.*, 2007), on physiological characteristics of a liquid shoot culture and assessment of the relationships in this biological system (Pavlov *et al.*, 2007), on the mono-factor effect of sucrose on galanthamine yields (Sellés *et al.*, 1997), as well as on the influence of precursors (Bergoñón *et al.*, 1996) and elicitors on its biosynthesis (Colque *et al.*, 2004). However, investigations concerning the nutrient medium optimization for maximal galanthamine production by plant *in vitro* systems have not been reported.

The aim of the present study was to improve the basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for *Leucojum aestivum* L. shoot culture cultivation in order to achieve maximal galanthamine yields.

Material and Methods

Shoot culture

The shoot cultures were established by planting formerly obtained *Leucojum aestivum* L. calli on MS nutrient medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 5.5 g/L "plant agar", 1.15 mg/L α-naphthaleneacetic acid (NAA), and 2.0 mg/L benzylaminopurine (BAP) (Pavlov *et al.*, 2007). The obtained *L. aestivum* line 80 shoot culture was maintained under illumination (16 h light/8 h darkness per day), at 26 ºC, for more than two years with a subculturing period of 28 d. This shoot line showed stable growth and morphological characteristics, as well as stable and high amounts of accumulated galanthamine, confirmed by GC/MS (Pavlov *et al.*, 2007).

Medium optimization

For the experiment, the shoot culture was transferred into 200-mL conical flasks containing 40 mL of liquid MS medium added with the same combination of growth regulators mentioned above, as well as with different amounts of sucrose, NH_4NO_3, KH_2PO_4, KNO_3 and $(NH_4)_2SO_4$, according to the experimental design shown in Table I. The culture cultivations were performed on a shaker (110 rpm) at 26 ºC under light conditions (16 h light/8 h darkness) for 35 d. The inoculation was performed with about 1 g of fresh shoots per flask cultivated on agar medium for 21 d under the above-mentioned conditions.

Extraction of galanthamine from tissues

300 mg of lyophilized shoot biomass were extracted with 5 mL MeOH for 16 h. After filtration, the separated biomass was re-extracted twice with 5 mL MeOH for 30 min, and the combined methanol extracts were evaporated under reduced pressure. The residue was dissolved in 2 × 2 mL 3% H_2SO_4 (pH ~ 2.0). The nonpolar compounds were removed by extracting three times with 5 mL diethyl ether for 30 min. Then, the aqueous fraction was basified (pH ~ 11) by adding 1 mL of 25% NH_3 in water. The alkaloids were extracted three times with 5 mL chloroform for 30 min. The combined chloroform extracts were dried by passing through a column packed with Na_2SO_4, and evaporated to dryness under reduced pressure. The residue was dissolved in 3 × 400 μL MeOH and transferred to an Eppendorf tube. After evaporation of the solvent, the obtained dry alkaloids extract was used for alkaloids determination.

Extraction of galanthamine from the liquid medium

After filtration the culture medium from each flask was evaporated to dryness under vacuum and the residue was dissolved in MeOH up to a final volume of 10 mL. The MeOH extracts were deposited at –20 ºC for 24 h. Then the supernatants were carefully collected from the precipitate. 5 mL of the collected supernatants were evaporated to dryness under vacuum. The next steps of the alkaloids extraction followed the procedure described above.

Accumulated biomass

The growth of the shoots was monitored by accumulation of the dry biomass, according to the equation: ADB = FDW – IDW, where ADB is the accumulated dry biomass, FDW the final dry weight of the shoot culture and IDW the initial dry weight of the shoot culture used for inoculation. The dry biomass was measured according to Dixon (1985).

Galanthamine quantification

The dry alkaloids extracts were dissolved in 100 μL MeOH. 20 μL of each sample and the galanthamine standard (2.0, 5.0, 10.0 and 20.0 μg/spot) were spotted on the same TLC plate (ALUGRAM® SIL G Silica gel 60, Macherey-Nagel, Germany). The mobile phase was chloroform/methanol/25% ammonia (12:2:0.1). The spots were visualized by spraying with Dragendorff's reagent. Quantification of galanthamine in the samples was carried out by using the densitometric software QuantiScan® (BioSoft, Cambridge, UK).

GC/MS analysis

GC/MS analyses were performed on a Hewlett Packard 6890$^+$/MSD 5975 instrument (Hewlett Packard, Palo Alto, CA, USA) operating in the EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180 ºC at 15 ºC/min, 180–300 at 5 ºC/min, and 10 min hold at 300 ºC. The injector temperature was 250 ºC. The flow rate of

the carrier gas (helium) was 0.8 mL/min. The split ratio was 1:20. 1 μL of the solution was injected.

The alkaloids were identified by comparing their MS spectra and RI (Kovats retention index) with those of previously isolated standards identified by other spectroscopic methods (NMR, UV, CD) and comparing the mass spectral fragmentation of the compounds with those of standard reference spectra from the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0–2005, National Institute of Standardization and Technology, Gaithersburg, MD, USA) as indicated in Table III. The MS spectra were deconvoluted by AMDIS 2.64 software (NIST). The RI of the compounds were recorded with a standard calibration *n*-hydrocarbons mixture (C_9–C_{36}) using AMDIS 2.64 software (NIST).

Experimental design and statistical analysis

The software MINITAB 14 was used for the regression analysis of the process and assessment of the obtained experimental results, and for models development using the response surface methodology. It was also used for the optimization of the regression models.

The results of the present study have been summarized from two independent experiments, repeated twice. A total of 17 experiments were performed in random order.

Results and Discussion

L. aestivum shoots can be considered as a prospective alternative approach for galanthamine production (Pavlov *et al.*, 2007). The *L. aestivum* line 80 shoot culture was selected for further investigation under submerged conditions, because of its stable growth and morphological characteristics, as well as due to the stable amounts of accumulated galanthamine. Its alkaloids profile was checked by GC/MS, and it was confirmed that the main alkaloid biosynthesized was galanthamine. Several relationships in *L. aestivum in vitro* systems with respect to the galanthamine biosynthesis were described. The results obtained from our study of time courses of the growth of the *L. aestivum* line 80 shoot culture, the galanthamine accumulation and the utilization of the main nutrient compounds outlined that the concentrations of ammonium, nitrate and phosphate ions, as well as sucrose related to the biosynthesis of galanthamine. Sellés *et al.* (1997) reported that an increase of the sucrose concentration has influence upon the enhancement of galanthamine yields.

On that basis a full factor experiment plan (FFE) 2^4 experimental scheme for the achievement of maximal yields of galanthamine by modifying the content of MS basal medium was developed (Table I). The obtained statistical regression models, taking into account the effect of the four investigated factors X_1 (ammonium ions), X_2 (nitrate ions), X_3 (potassium hydrogen phosphate) and X_4 (sucrose) on the values Y_1 (accumulated dry biomass), Y_2 (intracellular galanthamine, EndoGal), Y_3 (released galanthamine, ExoGal) and Y_4 (total amount of produced galanthamine, TotalGal), had high coefficients of determination (R^2 = 88.6, R^2 = 93.4, R^2 = 88.4 and R^2 = 93.6 for Y_1, Y_2, Y_3 and Y_4, respectively).

The optimal values of variables (X_1 = 0.54 g/L, X_2 = 3.44 g/L, X_3 = 0.10 g/L and X_4 = 60 g/L) were obtained using "response optimizer" of MINITAB 14 software to provide the maximum biosynthesis of galanthamine by a *Leucojum aestivum* L. shoot culture (Y_4 = 86.94 μg/flask). The deviation between the theoretically studied maximum amount of galanthamine and the experimentally obtained one was 9.49 μg/flask, which is less than 10%. This fact is an evidence of adequacy of the proposed regression model. On this basis, we propose the modification of the concentrations of macro-salts and carbon sources of the basal MS medium as shown in Table II to receive the maximal yield of galanthamine by *L. aestivum* L. shoot cultures.

The modified MS medium used in this work differed from the standard one by the concentration of KNO_3, NH_4NO_3, $(NH_4)_2SO_4$ and sucrose. The maximal galanthamine yield was achieved when the C/N ratio was increased from 15 in the standard medium to 21 in the modified one. At the same time, nevertheless the concentrations of both ammonium and nitrate ions were higher in the modified medium, the NO_3^-/NH_4^+ ratio remained constant – 6.6 and 6.4 in the standard and modified medium, respectively. The higher concentrations of carbon and nitrogen were expected as far as the relationship between the concentration of the carbon source and the biosynthesis of secondary metabolites is proportional (Ilieva and Pavlov, 1997), and the galanthamine molecule itself contains nitrogen.

Table I. Experimental design of the full factor experiment 2^4 and output of the process.

Run	Independent variables				Outcome of the process			
	X_1 NH_4^+ [g/L]	X_2 NO_3^- [g/L]	X_3 KH_2PO_4 [g/L]	X_4 Sucrose [g/L]	Y_1 Biomass [g/flask]	Y_2 EndoGal [μg/flask]	Y_3 ExoGal [μg/flask]	Y_4 TotalGal [μg/flask]
1	0.20	1.44	0.10	30.00	0.35	5.82	5.07	10.89
2	0.20	1.44	0.24	30.00	0.49	12.92	21.17	34.09
3	0.20	3.44	0.10	30.00	0.41	15.93	24.23	40.16
4	0.54	1.44	0.10	30.00	0.33	1.11	6.55	7.66
5	0.54	3.44	0.24	30.00	0.52	4.88	16.03	20.91
6	0.54	3.44	0.10	30.00	0.41	3.26	9.34	12.60
7	0.54	1.44	0.24	30.00	0.21	0.10	1.17	1.27
8	0.20	3.44	0.24	30.00	0.56	23.31	24.95	48.26
9	0.20	1.44	0.10	60.00	0.59	4.21	14.25	18.46
10	0.20	1.44	0.24	60.00	0.49	0.10	0.95	1.05
11	0.20	3.44	0.10	60.00	0.53	8.93	12.64	21.57
12	0.54	1.44	0.10	60.00	0.70	28.30	21.35	49.65
13	0.54	3.44	0.24	60.00	0.88	23.08	32.97	56.05
14	0.54	3.44	0.10	60.00	0.63	52.97	43.46	96.43
15	0.54	1.44	0.24	60.00	0.56	9.37	15.44	24.81
16	0.20	3.44	0.24	60.00	0.40	11.32	6.86	18.18
17	0.37	2.44	0.17	45.00	0.16	20.92	20.38	41.30

Table II. Modified MS medium for the production of galanthamine from an *L. aestivum* shoot culture.

Component	Concentration [g/L]	
	Modified MS medium	Standard MS medium
NH_4NO_3	0.89	1.65
KNO_3	4.50	1.90
KH_2PO_4	0.10	0.17
$(NH_4)_2SO_4$	1.25	0.00
$CaCl_2$	0.44	0.44
Sucrose	60.00	30.00

Table III. Alkaloids found both in biomasses and in culture liquids of an *L. aestivum* line 80 shoot culture, cultivated in standard and modified MS nutrient media. The results are presented as % of total ion current.

Alkaloid	$[M]^+$	Optimized medium		Standard medium		Standard reference
		Medium	Shoots	Medium	Shoots	
Trisphaeridine (**1**)[a]	223	1.87 ± 0.97	2.23 ± 0.54	1.16 ± 0.18	3.5 ± 1.59	Brine *et al.* (2002)
Galanthamine (**2**)[a]	287	89.91 ± 5.72	97.5 ± 0.59	87.75 ± 2.37	85.45 ± 7.53	Berkov *et al.* (2008)
Buphanisine (**3**)[a]	285				8.26[b]	NIST 05
epi-Galanthamine (**4**)[a]	287	2.42 ± 1.99	0.25 ± 0.01	2.69 ± 0.55	0.21 ± 0.07	Berkov *et al.* (2008)
Vittatine (**5**)[a]	271	0.47[b]		0.32 ± 0.22	1.35 ± 0.63	Cabezas *et al.* (2003)
Narwedine (**6**)[a]	284	4.71 ± 3.33		4.40 ± 0.71		Berkov *et al.* (2008)
A-315 (**7**)	315			1.41 ± 0.5		–
8-*O*-Demethyl-marithidine (**8**)[a]	273				2.59 ± 1.93	Cabezas *et al.* (2003)
Buphanidrine (**9**)[a]	315				2.85[b]	NIST 05
Haemanthamine (**10**)[a]	301			0.73 ± 0.43		Cabezas *et al.* (2003)
Lycorine (**11**)[a]	287	2.53[b]	0.34[b]	1.94 ± 2.37	4.12 ± 1.74	Berkov *et al.* (2007)
Homolycorine (**12**)[a]	301			0.29 ± 0.20		Bastida *et al.* (1992)

[a] Identification by co-chromatography with previously isolated standard compounds.
[b] Found in one sample.

Twelve Amaryllidaceae alkaloids of narciclasine-, **1**, galanthamine-, **2**, **4**, and **6**, haemanthamine-, **3**, **5**, **8**–**10**, lycorine-, **11**, and homolycorine-types, **12**, as well as an unidentified alkaloid, **7**, were found in the alkaloids mixtures of the studied samples. The alkaloids pattern of the *L. aestivum* line 80 shoot culture was dominated by the galanthamine-type compounds, representing more than 85% of all alkaloids. Optimization of the nutrient composition, besides improvement of the production yield, shifted the alkaloid biosynthesis toward galanthamine and other galanthamine-type compounds. Thus, the percentage contribution of both haemanthamine- and lycorine-type compounds was significantly decreased in alkaloid mixtures of cultures grown with the optimized medium. The percentage of galanthamine in the cultures grown with the optimized MS nutrient medium reached 98% of TIC (total ion current) in the shoots and 90% in the liquid medium.

It should be mentioned that, on the one hand, the alkaloids patterns of samples obtained from the culture media consisted of more compounds than those obtained from the shoots and, on the other hand, the alkaloids patterns of samples from cultures grown with the optimized MS medium showed fewer compounds. These results, definitively, have a great practical significance because such a high level of galanthamine in alkaloids mixtures with a few accompanying compounds means that the target compound can be easier isolated and purified.

In conclusion, as a result of previous physiological studies on *L. aestivum* liquid shoot cultures (Pavlov *et al.*, 2007) and the consequent investigation of the overall effect of concentrations of the main nutrients in the medium, a modified composition of the MS culture medium could be proposed for maximal production of galanthamine by an *L. aestivum* L. shoot culture. The optimization of the standard MS nutrient medium led to a balance in its composition, which led to a considerable increase of the production yield as well as of the relative content of the target alkaloid in the alkaloids mixture. This is a good basis for the following scale-up of the production process of galanthamine by an *L. aestivum* L. shoot culture.

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