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Galanthamine and Related Alkaloids Production by *Leucojum aestivum* L. Shoot Culture using a Temporary Immersion Technology

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Abstract The process of galanthamine and related alkaloids production by *Leucojum aestivum* shoot culture in a temporary immersion system was studied. It was established that temporary immersion approach is prospective for development of a biosynthetic process for obtaining valuable Amaryllidaceae alkaloids. Both immersion frequency and temperature had significant effect on biomass accumulation and the yields of galanthamine and related alkaloids. The maximal yield of galanthamine was achieved at the cultivation of *L. aestivum* shoot culture in temporary immersion RITA® system at immersion frequency 15 min flooding and 8 h stand-by periods, at 26 °C. Data on the relationships in the biological system "Nutrient medium–*L. aestivum* shoot culture–galanthamine" are presented as well.

Keywords Galanthamine · Lycorine · Norgalanthamine · *Leucojum aestivum* · Shoots · Amaryllidaceae · Temporary immersion system

Introduction

Amaryllidaceae-type alkaloids is a well-known group of bioactive substances possessing antiviral [1] and antitumor properties [2], as well as an anticholinesterase activity [3]. The best studied galanthamine is widely used in medicine for the treatment of Alzheimer's disease [4–7], as well as for the treatment of poliomyelitis and other neurological diseases [8–11]. Another valuable member of Amaryllidaceae alkaloids family is lycorine, a pyrrolophenanthridine alkaloid. It possesses a strong antiviral effect against poliovirus, measles, and Herpes simplex type 1 viruses [12], as well as high antiretroviral [13], strong antimitotic [14], and cytotoxic activities [15]. Nowadays, the Sanochemia Pharmazeutika AG produces galanthamine through chemical synthesis; however, medicinal doctors prefer

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using the naturally isolated. The main commercial sources for the production of the galanthamine-based medicines are *Narcissus confusus* (for production of Razadyne®-formely Reminyl®) and *Leucojum aestivum* (for production of Nivalin®) [16]. *L. aestivum* is an 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 as an attractive alternative [17]. Nevertheless, galanthamine and related alkaloid have been found in many *Amaryllidaceae* plants, only two species have been studied for their in vitro galanthamine production—*Narcissus confusus* and *L. aestivum* [16]. Up to now the investigations have been performed at the shaken-flask stage [17–20] and there is not information available about biotechnological process for galanthamine and related alkaloids production based on bioreactor cultivation of plant in vitro systems. This is probably due to the fact that the level of the Amaryllidaceae alkaloids biosynthesis is strongly influenced from the level of cell differentiation and shoot cultures were found to be the best producers [17, 18]. Currently, little is known about shoot in vitro systems as producers of bioactive plant secondary metabolites as the main limitation is development of bioreactor system with suitable design. The temporary immersion technology seems to be the most prospective approach not only for micropropagation [21] but also for development of biosynthetic processes based on differentiated plant in vitro systems [22].

In the present study, we describe the process of galanthamine and related alkaloids biosynthesis by *L. aestivum* shoot culture in a temporary immersion RITA® system.

Materials and Methods

L. aestivum Shoot Culture

The shoot cultures were established by planting the previously obtained calli [17] on Murashige and Skoog (MS) nutrient medium, supplemented with 30 g/L sucrose, 1.15 mg/L 1-naphthylacetic acid (NAA, Duchefa—The Netherlands), 2.0 mg/L 6-benzylaminopurine (BAP, Duchefa—The Netherlands), and 5.5 g/L "Plant agar" (Duchefa—The Netherlands). They were maintained for more than 5 years at 26 °C under illumination (16 h light/8 h dark per day). The subcultivation period was 28 days.

Conditions of the Temporary Immersion Cultivation

The *L. aestivum* 80 shoot culture was cultivated for 35 days in RITA® apparatus (CIRAD ltd., France, http://pagesperso-orange.fr/vitropic/rita/en/accueil.htm) with 200-ml optimized MS medium [20] under illumination 16:8 hours (light:dark) at 18 °C, 22 °C, 26 °C, and 30 °C with immersion frequencies with 15 min flooding and 6, 8, 10, and 12 h stand-by periods in agreement to the experimental design. The flow rate of the inlet air was 60 l/h for each RITA® apparatus.

Extraction of Alkaloids

Intracellular Alkaloids Dry biomass (0.2–0.3 g) was extracted three times with 5 ml of methanol in an ultrasonic bath for 15 min. The combined extracts were concentrated under vacuum and dissolved in 2×2 ml of 3% sulfuric acid. The neutral compounds were removed by extraction (three times) with diethyl ether. The alkaloids were fractionated after

basification of the extracts with 1 ml of 25% ammonia and extraction with chloroform (3× 3 ml). The chloroform extracts were then dried over anhydrous sodium sulfate and evaporated to dryness.

Extracellular Alkaloids Fifty milliliters culture liquids were evaporated to dryness and dissolved in 10 ml methanol. After centrifugation and separation of pellets, 8 ml of supernatants were evaporated to dryness and residuals were dissolved in 2×2 ml of 3% H_2SO_4 and then processed as described above.

Analyses

Dry Biomass

The growth of *L. aestivum* 80 shoot culture was monitored by accumulated dry biomass (ADB) and growth index (GI) according to [20].

Alkaloids Content

For alkaloids determination the Waters HPLC system was used equipped with a Dual λ absorbance detector (Waters 2487, Milford, USA) and binary pump (Waters 1525, Milford, USA). The chromatographic column was Symmetry® C18 reversed phase (150×4.6 mm, 5 μm, Waters Milford, USA). The elution was carried out with acetonitrile as the organic phase (solvent A) and 1% (*w*/*v*) ammonium acetate buffer adjusted to pH 6.6 with acetic acid (solvent B) in gradient regime (Table 1). The alkaloids were detected at wavelength 287 nm. The volume of injection was 20 μl.

Sugars

Sucrose, glucose, and fructose in the culture medium were determined by means of enzyme test combination (Megazyme, Ireland, Cat. No.: K-SUFRG).

Inorganic Ions

Nitrate, ammonium, and phosphate ions were determined by chemical test combinations (MERCK,Germany, Cat. No. 1.14773.0001, 1.14752.0001, 1.14842.0001)

The utilization of the main components of the nutrient medium was express as degree of utilization using the equation: *Degree of utilization*=(*Final concentration*/*Initial concentration*)× 100. In the case of sugars, the "Initial concentration" is the concentration of added to the culture medium sucrose, and the "Final concentration" is a sum of the final concentrations of sucrose, glucose, and fructose.

Table 1 Gradient regime for the HPLC analysis of alkaloids.

Time (min)	0	11	15	16	18	20	22	31
Solvent A%	10	31	70	90	90	31	10	10
Solvent B%	90	69	30	10	10	69	90	90
Flow rate (ml/min)	0.4	0.3	0.5	0.5	0.5	0.4	0.4	0.4

Statistical Analyses

The software package 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. The MINITAB 14 software also was used for the optimization of the regression model for galanthamine biosynthesis.

The results presented in the study have been summarized from two independent experiments, repeated twice.

Results and Discussion

The shoot culture of *L. aestivum* was obtained via callus 5 years ago [17]. This approach has been chosen because of the possibility to obtain more high-alkaloid synthesizing in vitro lines because of the somaclonal variations typical for the calli. Further we found that the best line obtained was shoot line 80 (with stable growth and biosynthetic characteristics) and therefore it was used in the next experiments. Additionally, the nutrient medium for maximal galanthamine yields has been optimized [20] and the profiles of the both extra- and intracellular alkaloid fractions of *L. aestivum* shoot culture have been investigated as well [23, 24].

Temporary immersion RITA systems have been widely used for micropropagation of plant in vitro cultures but there are scanty data concerning their application for secondary metabolite biosynthesis so far [22, 25, 26]. As far as we investigated temporary immersion technology it was clear that immersion frequency is the most important factor that should be analyzed. *L. aestrivum* 80 shoot culture showed balanced growth at everyone of the tested immersion frequencies. During the cultivation shoots increased significantly their linear dimensions and at the same time a number of meristem cells differentiated to new shoots. This is one of the main advantages of the temporary immersion cultivation of shoot clumps, because it is clear that other type bioreactor systems could not be appropriate for their cultivation. As the highest amount of dry biomass (92.40 g/RITA) *L. aestivum* 80 shoot culture accumulated during its cultivation in temporary immersion RITA system, operating at 15 min flooding and 8 h stand-by periods (Table 2). At the same regime the highest growth index (2.98) and ADB (2.40) were achieved as well.

Biomass production was also found to be affected by the temperature in parallel with immersion frequency. To investigate the influence of the temperature on biomass accumulation, the *L. aestivum* 80 shoot culture was cultivated in RITA apparatus with 15 min flooding and 8 h stand-by periods at 18 °C, 22 °C, 26 °C, and 30 °C. The achieved results (Table 2) showed that the temperature had comparable effects to the immersion frequency, on biomass accumulation from *L. aestivum* 80 shoot culture. The best conditions for the growth of *L. aestivum* 80 shoot culture in temporary immersion RITA system were 15 min flooding, 8 h stand-by periods and 26 °C (Table 2). It should be underlined that the growth index achieved at these conditions (2.98) is higher than the growth index (2.5) achieved during the cultivation of *L. aestivum* 80 shoot culture in liquid medium in flasks [17, 20]. This fact once again proved higher efficiency of the temporary immersion technology compared to the submerged cultivation of differentiated plant in vitro systems. This is probably due to the composition of the internal atmosphere in the culture vessel and an expression of hyperhydricity [27]. Perez-Alonso and co-workers [26] described the

Table 2 Maximal ADB and GI, achieved during the cultivation of *L. aestivum* 80 shoot culture in temporary immersion RITA® system.

Immersion frequency, temperature (flooding, min/stand-by, hours/temperature, °C)	ADB[a], g/RITA	GI[a]
15/4/26	2.28±0.49	2.06±0.15
15/6/26	1.55±0.43	1.39±0.36
15/8/26	2.40±0.54	2.98±0.64
15/10/26	2.17±0.05	2.10±0.18
15/12/26	1.46±0.37	1.83±0.35
15/8/18	1.17±0.38	1.12±0.40
15/8/22	1.49±0.25	1.32±0.32
15/8/30	2.13±0.04	1.34±0.31

[a] Accumulated dry biomass (ADB) and growth index (GI) were calculated on a base of inoculums and final dry weights

immersion frequency as the most critical parameter that affect on biomass accumulation in temporary immersion systems. However, our results clearly underlined that the assessment of the cultivation systems potential should be done on the base of multi-parameter analyses of different factors.

Nutrient availability is one of the major factors involved in the cultivation of plant in vitro systems, which affect on the biomass accumulation and metabolite biosynthesis [22]. The relationships between immersion frequencies, temperatures of cultivation and sugar metabolism of *L. aestivum* 80 shoot culture are clearly outlined (Fig. 1). The highest degree of utilization of sugars was achieved when *L. aestivum* 80 shoot culture was cultivated at 26 °C and at regime with 15 min flooding and 8 h stand-by periods. Although final concentrations of fructose and glucose at the other investigated regimes and temperatures of cultivation were comparable, the reminders of unhydrolyzed sucrose were significantly higher. Hence, sucrose influenced both growth and metabolism of the culture not only as a carbon source but through changes in the osmotic environment of biological system [28, 29]. The immersion frequencies and temperature of cultivation did

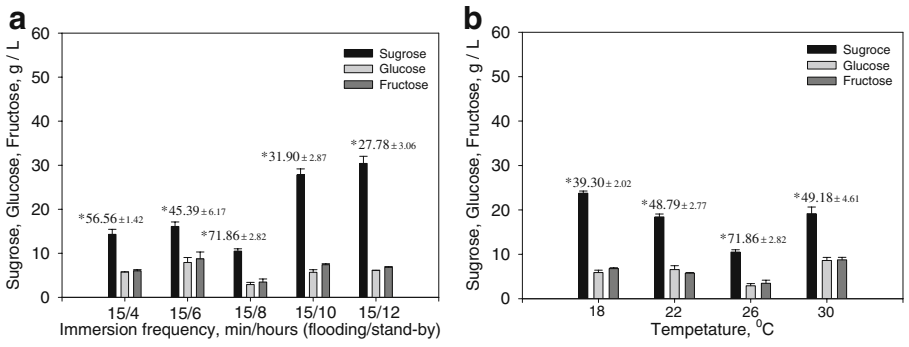

Fig. 1 Final amounts of sucrose, glucose, and fructose and degree of utilization (*asterisks*) of total sugars from *L. aestivum* 80 shoot culture during its cultivation at different immersion frequencies (**a**) and temperatures (**b**). Initial concentration of sucrose was 60 g/L

not influence utilization of nitrogen ions (nitrate and ammonium) by *L. aestivum* 80 shoot culture (Fig. 2). At all tested regimes more than 80% of initial concentration of ammonium ions and about 60% of nitrate ions were exhausted. The degree of utilization of phosphate ions slightly depended on conditions of cultivation. During the cultivation at the optimal regime (15 min flooding and 8 h stand-by, and 26 °C) of growth, the *L. aestivum* 80 shoot culture utilized about 70% of phosphate ions (Fig. 2). Data presented concerning the utilization of the main components of nutrient medium by *L. aestivum* 80 shoot culture showed that temporary immersion regime covered the main nutrient needs of the culture.

The highest volumetric yield of galanthamine (265 μg/RITA) was achieved in after 35 days of cultivation of *L. aestivum* 80 shoot culture at regime with 15 min flooding and 8 h stand-by periods and 26 °C (Figs. 3 and 4). The obtained results clearly underlined that both temperature and immersion frequency influenced the galanthamine production by *L. aestivum* 80 shoot culture and did not influence on the ratio between accumulated in the cells and secreted in the culture medium galanthamine. Unexpectedly, the optimal temperature for galanthamine production *L. aestivum* 80 shoot culture cultivated in a temporary immersion system RITA differ significantly from the average daily temperature (22 °C, http://iasas.government.bg) during maximal galanthamine biosynthesis by intact plants in nature. In parallel to the galanthamine, *L. aestivum* 80 shoot culture biosynthesized significant amounts of lycorine (Fig. 3). This pyrolophenanthridine alkaloid also attracts interest because of its strong antiviral effect [12, 13], antimitotic, and cytotoxic activities [14, 15]. The influence of immersion frequency on lycorine production by *L. aestivum* 80 shoot culture was significant (Fig. 3), as 15 min immersion and 10 h stand-by periods (at 26 °C) seems to be the most appropriate regime for its biosynthesis. At these conditions *L. aestivum* 80 shoot culture produced the maximal amounts (1,699 μg/RITA) lycorine. Increase or decrease of stand-by periods of the cultivation led to inhibition of the lycorine accumulation. The obtained results, concerning influence of immersion frequency on galanthamine and lycorine production, showed that alkaloid biosynthesis of *L. aestivum* 80 shoot culture could be manipulated through changes of the regime of cultivation. In contrast, the optimal temperature for lycorine production was the same (26 °C) as for galanthamine production (Fig. 4). Beside galanthamine and lycorine production by *L. aestivum* 80 shoot

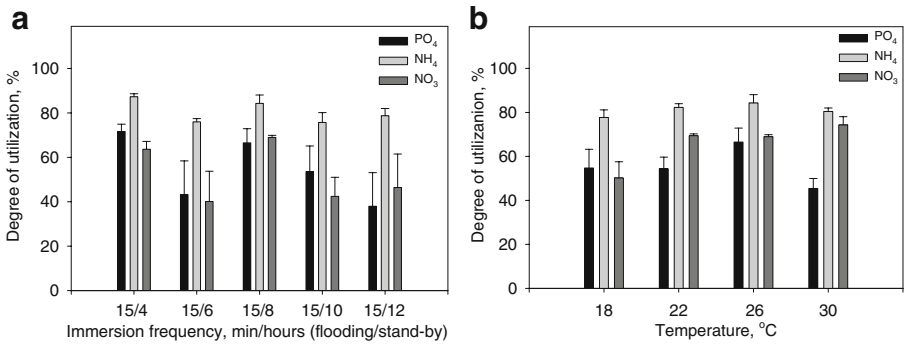

Fig. 2 Degree of utilization of phosphate, ammonium, and nitrate ions from *L. aestivum* 80 shoot culture during its cultivation at different immersion frequencies (**a**) and temperatures (**b**)

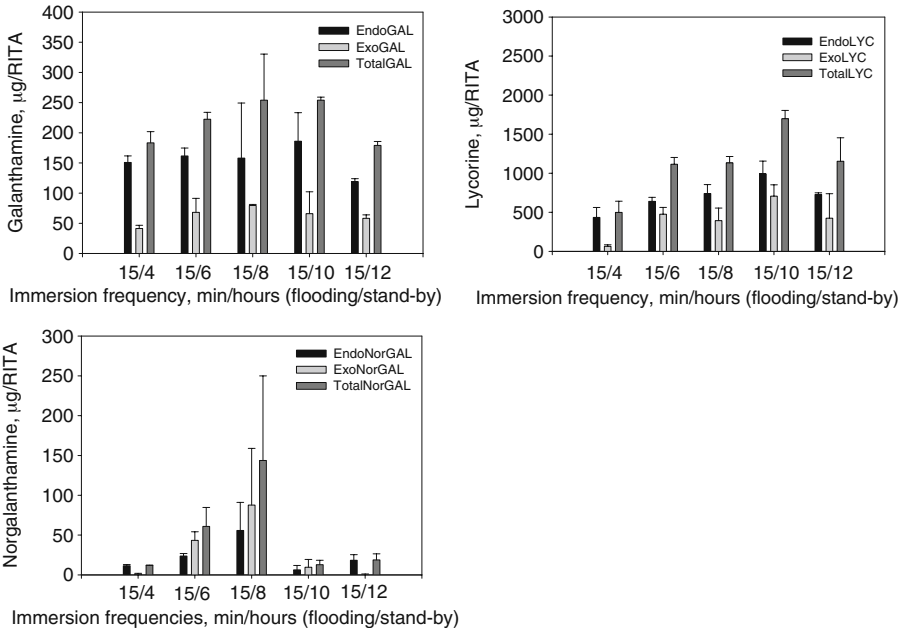

Fig. 3 Effect of the immersion frequency on galanthamine, lycorine, and norgalanthamine biosynthesis by *L. aestivum* 80 shoot culture. The experiments were performed at 26 °C

culture, the production of biosynthetic precursor of galanthamine–norgalanthamine was investigated as well. Expectably, the maximal amounts of norgalanthamine (225 μg/RITA) *L. aestivum* 80 shoot culture biosynthesized during its cultivation in RITA apparatus at 15 min immersion and 8 h stand-by periods (the same immersion frequency as in the case with galanthamine; Fig. 3). It should be underlined that at other investigated regimes of cultivation, *L. aestivum* 80 shoot culture synthesized insufficient amounts of norgalanthamine. It is worth of remark that the optimal temperature for norgalanthamine production (22 °C) was different than this for galanthamine production (26 °C; Fig. 4). This fact was probably due to that the enzyme activity, catalyzing the last step of galanthamine biosynthesis (methylation of norgalanthamine, [30]), possesses temperature optimum different than 22 °C. At this temperature *L. aestivum* 80 shoot culture produced 388 μg/RITA norgalanthamine. This is a very important result because recent studies of Kim and co-authors [31] showed that norgalanthamine possess strong promotion effect on hair growth, as well as it is not difficult to transform the norgalanthamine to galanthamine using simple chemical methylation [30].

One of the most powerful tools for the optimization of secondary metabolite production by plant in vitro systems is statistical optimization of both content of nutrient media [32] and environmental conditions in the cultivation systems [33].

The results described above clearly outlined that the investigated variables (immersion frequency and temperature) significantly influenced alkaloids biosynthesis by *L. aestivum* 80 shoot culture. As far as the galanthamine is the most valuable alkaloid among investigated ones, statistical analyses were performed concerning its production. The statistical regression model was obtained, taking in to account the

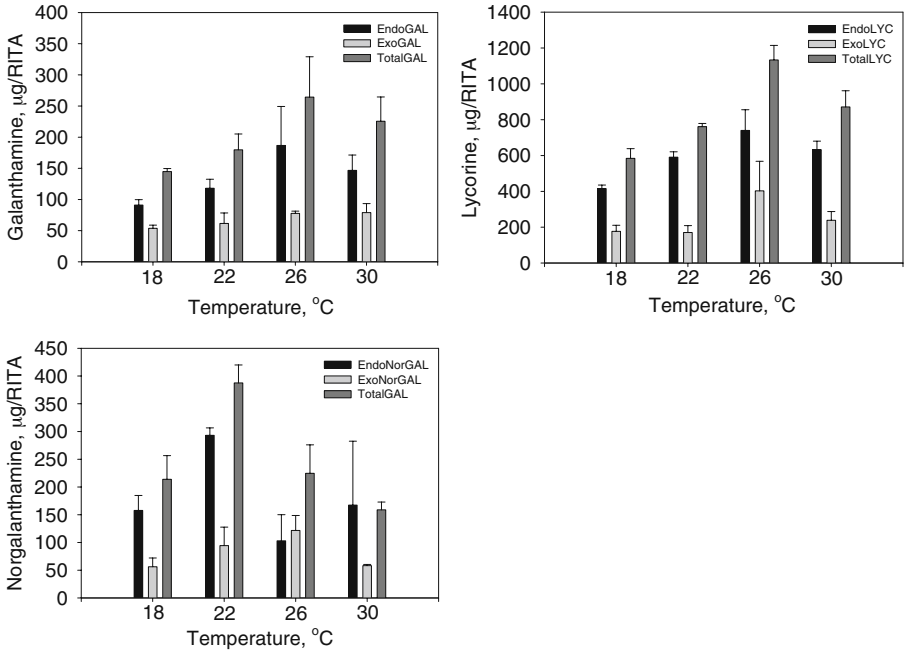

Fig. 4 Effect of the temperature on galanthamine, lycorine, and norgalanthamine biosynthesis by *L. aestivum* 80 shoot culture. The experiments were performed with 15 min immersion and 8 h stand-by periods

influence of the stand-by periods (X_1) and temperature (X_2) on the amount of total produced galanthamine (Y, Eq. 1).

$$Y = -932.40 + 41.06X_1 + 76.09X_2 - 2.65X_1^2 - 1.44X_2^2 \quad (1)$$

$$R^2 = 71.2$$

The received coefficient of determination (R^2) was good enough taking into account the degree of differentiation of the investigated plant in vitro systems.

The optimization procedures carried out using "Response optimizer" of MINITAB 14 software gave the following values of variable X_1 and X_2 for maximum biosynthesis of galanthamine (Y) by *L. aestivum* 80 shoot culture:

$$X_1^* = 7\,\text{h}\,43\,\text{min}; \quad X_2^* = 26.5$$

$$\hat{Y}_{\max}^* = 1,171.95\mu\text{g/L}$$

The deviation between the theoretically studied maximal amounts of galanthamine and experimentally obtained (at 15 ;min flooding and 8 h stand-by periods, Fig. 3) was only 149.72 μg/L. On this basis we propose 15 min flooding and 8 h stand-by periods and 26 °C as optimal for galanthamine production by *L. aestivum* 80 shoot culture in temporary immersion cultivation RITA systems.

In conclusion, the presented study shows that temporary immersion technology is appropriate for bioactive substances production by differentiated plant in vitro systems. The high yield of galanthamine obtained, as well as revealed possibilities for manipulation of the biosynthetic process through environmental conditions, are good base for scaling-up and development of a biosynthetic process for obtaining valuable Amaryllidaceae alkaloids.

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References

1. Gabrielsen, B., Monath, T. P., Huggins, J. W., Pettit, G. R., Groszek, G., Hollingshead, M., et al. (1992). Antiviral (RNA) activity of selected Amaryllidaceae isoquinoline constituents and synthesis of related substances. *Journal of Natural Products, 55*, 1569–1581.
2. Pettit, G. R., Pettit, G. R., III, Groszek, G., Backhaus, R. A., Doubek, D. L., Barr, J., et al. (1995). Antineoplastic agents, 301. An investigation of the Amaryllidaceae genus *Hymenocallis*. *Journal of Natural Products, 58*, 756–759.
3. Luttmann, E., Linnemann, E., & Fels, G. (2002). Galanthamine as bis-functional ligand for the acetylcholinesterase. *Journal of Molecular Modeling, 8*, 208–216.
4. Sweeney, J. E., Puttfarcken, P. S., & Coyle, J. T. (1989). Galanthamine, an acetylcholinesterase inhibitor: A time course of the effect on performance and neurochemical parameters in mice. *Pharmacology Biochemistry and Behaviour, 34*, 129–137.
5. Thomsen, T., & Kewitz, H. (1990). Selective inhibition of human acetylholinesterase by galanthamine *in vitro* and *in vivo*. *Life Sciences, 46*, 1553–1558.
6. Thomsen, T., Zendeh, H., Fischer, J. P., & Kewitz, H. (1991). *In vitro* effects of various cholinesterase inhibitors on acetyl and butyrylcholinesterase of healthy volunteers. *Biochemical Pharmacology, 41*, 139–141.
7. Harvey, A. L. (1995). The pharmacology of galanthamine and its analogues. *Pharmacy and Therapary, 68*(1), 113–128.
8. Radicheva, N., Vydevska, M., & Mileva, K. (1996). Nivalin P-induced changes in muscule fiber membrane processes. *Methods and Finding in Experimental and Clinical Pharmacolody, 18*, 301–308.
9. Novikova, Y. U., & Tulaganov, A. A. (2002). Identification and evaluation of purity of the parent substance and medicinal form of galanthamine hydrobromide. *Pharmaceutical Chemistry Journal, 36*(7), 396–397.
10. Heinrich, M., & Teoh, H. L. (2004). Galanthamine from Snowdrop—The development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of Ethnopharmacology, 92*, 147–162.
11. Ptak, A., Tahchy, A. E., Dupire, F., Boisbrun, M., Henry, M., Chapleur, Y., et al. (2009). LCMS and GCMS for the Screening of alkaloids in natural and *in vitro* extracts of *Leucojum aestivum*. *Journal of Natural Products, 72*, 142–147.
12. Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., et al. (2005). Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Research, 67*(1), 18–23.
13. Szlávik, L., Gyuris, A., Minarovits, J., Forgo, P., Molnar, J., & Hohmann, J. (2004). Alkaloids from *Leucojum vernum* and antiretroviral activity of Amaryllidaceae alkaloids. *Planta Medica, 70*(9), 871–873.
14. Liu, J., Hu, W. X., He, L. F., Ye, M., & Li, Y. (2004). Effacts of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. *FEBS Letters, 578*(3), 245–250.
15. Weniger, B., Italiano, L., Beck, J., Bastida, J., Bergónon, S., Codina, C., et al. (1995). Cytotoxic activity of Amaryllidaceae alkaloids. *Planta Medica, 61*, 77–79.
16. Berkov, S., Georgieva, L., Kondakova, V., Atanasov, A., Viladomat, F., Bastida, J., et al. (2009). Plant sources of galanthamine: Phytochemical and biotechnological aspects. *Biotechnology and Biotechnological Equipment, 23*(2), 1170–1176.

17. Pavlov, A., Berkov, S., Courot, E., Gocheva, T., Tuneva, D., Pandova, B., et al. (2007). Galanthamine production by *Leucojum aestivum in vitro* systems. *Process Biochemistry, 42*, 734–739.
18. Selles, M., Bergonon, S., Viladomat, F., Bastida, J., & Codina, C. (1997). Effect of sucrose on growth and galanthamine production in shoot-clumps cultures of *Narcissus confusus* in liquid shake medium. *Plant Cells, Tissue and Organ Cultures, 49*, 129–138.
19. Diop, M., Ptak, A., Chrétien, F., Henry, M., Chapleur, Y., & Laurain-Mattar, D. (2006). Galanthamine content of bulbs and *in vitro* cultures of *Leucojum aestivum* L. *Natural Product Communications, 1*(6), 475–479.
20. Georgiev, V., Berkov, S., Georgiev, M., Burrus, M., Codina, C., Bastida, J., et al. (2009). Optimized nutrient medium for galanthamine production in *Leucojum aestivum* L. *in vitro* shoot system. *Zeitschrift für Naturforschung, 64C*, 219–224.
21. Debnath, S. (2009). Characteristics of strawberry plants propagated by *in vitro* bioreactor culture and ex vitro propagation method. *Engineering in Life Sciences, 9*(3), 239–246.
22. Pavlov, A., & Bley, Th. (2006). Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in a temporary immersion cultivation system. *Process Biochemistry, 41*, 848–852.
23. Berkov, S., Pavlov, A., Ilieva, M., Burrus, M., Popov, S., & Stanilova, M. (2005). CGC-MS of alkaloids in *Leucojum aestivum* plants and their *in vitro* cultures. *Phytochemical Analyses, 16*, 98–103.
24. Berkov, S., Pavlov, A., Georgiev, V., Bastida, J., Burrus, M., Ilieva, M., et al. (2009). Alkaloid accumulation in *Leucojum aestivum in vitro* cultures. *Natural Product Communication, 4*(3), 359–364.
25. Pavlov, A., & Bley, Th. (2005). Betalams biosynthests by *Beta vulqans* L hairy root culture in different bioreactor systems. *Scientific works of University of Food Technologies, Plovdiv, Bulgaria, 52*(2), 299–304.
26. Perez-Alonso, N., Wilken, D., Gerth, A., Jaehn, A., Nitzsche, H.-M., Kerns, G., et al. (2009). Cardiotonic glycosides from biomass of *Digitalis purpurea* L cultured in temporary immersion systems. *Plant Cell, Tissue and Organ Cultures, 99*, 151–156.
27. Teisson, C., Alvard, D., Berthouly, M., Cote, F., Escalant, J., Etienne, H., et al. (1996). Simple apparatus perform plant tissue culture by temporary immersion. *Acta Horticulture, 440*, 521–525.
28. Perez, A., Napoles, L., Carvajal, C., Hernandez, M., & Lorenzo, J. C. (2004). Effect of sucrose, inorganic salt, inositol, and thiamine on protease excretion during pineapple culture in temporary immersion bioreactors. *In Vitro Cellular and Development Biology Plant, 40*, 311–316.
29. Javed, F., & Ikram, S. (2008). Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes. *Pakistan Journal of Botany, 40*(4), 1487–1495.
30. Eichhorn, J., Takada, T., Kita, Y., & Zenk, M. (1998). Biosynthais of the Amaryllidaceae alkaloid galanthamine. *Phytochemistry, 49*(4), 1037–1047.
31. Kim, S.-C., Kang, J.-I., Kim, M.-K., Hyun, J.-H., Boo, H.-J., Park, D.-B., et al. (2010). Promotion effect of norgalanthamine, a component of *Crinum asiaticum*, on hair growth. *European Journal of Dermatology, 20*(1), 42–48.
32. Pavlov, A., Georgiev, V., Marchev, A., & Berkov, S. (2009). Nutrient medium optimization for hyoscyamine production in diploid and tetraploid *Datura stramonium* L. hairy root cultures. *World Journal of Microbiology & Biotechnology, 25*, 2239–2245.
33. Pavlov, A., Georgiev, M., Panchev, I., & Ilieva, M. (2005). Optimization of rosmarinic acid production by *Lavandula vera* MM plant cell suspension in a laboratory bioreactor. *Biotechnology Progress, 21*, 394–396.