RESEARCH ARTICLE

Andrey Marchev [1]
Vasil Georgiev [1,3]
Ivan Ivanov [1,2]
Atanas Pavlov [1,2]

Authors' addresses:
[1] Laboratory of Applied Biotechnologies, Institute of Microbiology "Stephan Angeloff", Bulgarian Academy of Sciences, 4000 Plovdiv, Bulgaria.
[2] Department of Organic Chemistry and Microbiology, University of Food Technologies, 4000 Plovdiv, Bulgaria.
[3] Center for Viticulture and Small Fruit Research, Florida A & M University, 6505 Mahan Drive, Tallahassee FL 32317, USA.

Correspondence:
Ivan G. Ivanov
Laboratory of Applied Biotechnologies, Institute of Microbiology "Stephan Angeloff", Bulgarian Academy of Sciences, 139 Ruski Blvd., 4000 Plovdiv, Bulgaria.
Tel.: +359 32 642430
e-mail: ivanov_ivan.1979@yahoo.com

Article info:
Received: 25 October 2012
Accepted: 10 December 2012

Cultivation of diploid and tetraploid hairy roots of *Datura stramonium* L. in stirred tank bioreactor for tropane alkaloids production

ABSTRACT

Biomass accumulation and tropane alkaloids production by diploid and tetraploid hairy root cultures of *Datura stramonium* L. cultivated in stirred tank bioreactor at different aeration rates were investigated. The maximal growth for both hairy root cultures (ADB = 8.3 g/L and 6.8 g/L for diploid and tetraploid line, respectively) was achieved at aeration rate of 15.0 L/(L.h). The corresponding growth indexes were remarkably high (GIDW = 9.0 and 7.8 for diploid and tetraploid line, respectively) compared to the values, usually reported for other hairy root cultures. The optimal aeration rate for biomass accumulation was also optimal for alkaloids biosynthesis. According to our survey, the achieved maximal amounts of accumulated hyoscyamine (35.0 mg/L and 27.0 mg/L for diploid and tetraploid line) were the highest reported in the scientific literature for *D. stramonium* L. hairy roots. During the cultivation in stirred tank bioreactor, the hairy roots biosynthesized pharmaceutically important alkaloid scopolamine in minor concentrations. This is an important observation since scopolamine was not detected during submerged cultivation of these hairy root lines in other bioreactor types. However, the ploidy level was found to be the most important factor concerning scopolamine production by *D. stramonium* L. hairy roots cultures. The present work demonstrated the effect of ploidity levels on biomass accumulation and tropane alkaloids production by *D. stramonium* L. hairy roots cultivated in stirred tank bioreactor. This investigation show that the stirred tank bioreactor could be successfully applied for both maximal biomass accumulations, as well as for manipulation of tropane alkaloids production by diploid and tetraploid *D. stramonium* L. hairy root cultures.

Key words: hyoscyamine, scopolamine, ploidy level, aeration rate

Introduction

Tropane alkaloids are produced by many Solanaceae species - *Mandragora*, *Brugmansia*, *Duboisia*, *Hyoscyamus*, *Datura*, *Atropa* and *Scopolia* (Palazón et al., 2008; Grynkiewicz et al., 2008). Some of them as hyoscyamine (its racemate mix called atropine), scopolamine and cocaine have valuable pharmacological activities and thus are widely used in medicine (Oksman-Caldentey & Arroo, 2000; Facchini, 2001). Hyoscyamine and scopolamine are competitive acetylcholine binding antagonists that possess antispasmodic and antitoxic properties (De Luca & St Pierre, 2000; Zhang et al., 2005). They are used as parasympatholytic agents, as antidote in cases of organophosphates poisoning, as drugs in controlling the symptoms of Parkinson's disease, in treatment of acute bronchitis, cardic and gastrointestinal diseases (De Luca & St Pierre, 2000; Oksman-Caldentey & Arroo, 2000). Scopolamine is active at lower therapeutic doses and has better activity on the central nervous system with less side-effect compared to atropine (Oksman-Caldentey & Arroo, 2000).

Hairy root cultures appear to be a promising alternative for industrial scale production of tropane alkaloids (Yang et al., 2011). Moreover, manipulation of their ploidy could be used as powerful tool for improving the yields of desired secondary metabolites (Berkov et al., 2002; Lavania, 2005).

RESEARCH ARTICLE

Assuring of higher and sustainable yields during bioreactor cultivation is also of great importance for economical effectiveness of tropane alkaloid production by biotechnological way. Since each hairy root line has special requirements, concerning oxygen mass transfer and shear stress tolerance, the right choice of bioreactor design is of critical importance (Wilson, 1997). Several different bioreactor systems have been used to study tropane alkaloids production by hairy roots of different *Solanaceae* species, but only few of them involves application of cultures with different ploidy levels (Ramakrishnan & Curtis, 2004; Williams & Doran, 2004; Eibl & Eibl, 2008; Georgiev et al., 2008; Pavlov et al., 2009a).

However, until now there is no single statement which bioreactor design is the most suitable for large scale cultivation of hairy root cultures, producers of tropane alkaloids. In this study, for the first time we investigate the role of ploidy levels on tropane alkaloids production by diploid and tetraploid hairy roots of *Datura stramonium* L., cultivated in stirred tank bioreactor. Data concerning the effects of aeration rates on biomass accumulation and maximal hyoscyamine and scopolamine production by both cultures is presented and discussed.

Materials and Methods

Plant material

Diploid and tetraploid hairy root cultures of *Datura stramonium* L., obtained previously (Pavlov et al., 2009a) were used. The cultures were supported on solid and liquid media as described elsewhere (Pavlov et al., 2009a).

Bioreactor cultivation

D. stramonium L. hairy roots were cultivated in 3 L (2 L working volume) stirred tank bioreactor (BioFlow 110, New Brunswick Scientific, Watford, Herts, UK), equipped with pitched blade impeller and standard sparger. The used media were optimized MS for diploid and tetraploid *D. stramonium* L. hairy roots as reported previously (Pavlov et al., 2009b). The experiments were performed at different aeration rates: 7.5; 15.0 and 22.5 L/(L.h) by using constant agitation speed (100 rpm) and temperature (26°C) for 21 days.

Alkaloids extraction

Intracellular and extracellular alkaloids were extracted from lyophilized hairy roots and culture liquids and analyzed according to procedure described elsewhere (Pavlov et al., 2009b).

HPLC analysis

The HPLC analyses of the alkaloids were performed according to Papadoyannis et al, (1993) with slight modification. The alkaloids were analyzed by HPLC system consisting of Waters 1525 Binary pump (Waters, Milford, MA, USA), Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA), controlled by Breeze 3.30 software. Supelco Discovery HS C_{18} column (5 µm, 25 cm × 4.6 mm) operated at 26°C was used for separation. The compounds were detected by monitoring at 210 nm and volume of the injected sample of 20 µL. Mobile phase was isocratic mixture of acetonitrile:methanol:0.05 M ammonium acetate (20.9 : 27.9 : 51.2) at flow rate of 1.0 mL/min.

Dry biomass determination

Hairy roots growth was evaluated through monitoring accumulated dry biomasses (ADB) and the corresponding growth indexes (GI). Dry biomass was determined according to the procedure described elsewhere (Dixon, 1985).

Determination of sugars and inorganic salts

Concentrations of sucrose, glucose and fructose in the culture liquids were determined by enzyme test kit (R-Pharm, Germany, Cat. No. 10716260035). Nitrate, ammonium and phosphate ions were determined by chemical test kits (Merck, Germany, Cat. Nos. 1.09713.0001, 1.00683.0001, 1.00798.0001, respectively).

Measurement of pH and conductivity

pH was monitored online, whereas the conductivity was measured offline by sampling and using external pH/conductivity meter (INOLAB, WTW, Germany).

Statistical analysis

The presented values are means with standard deviation from three independent experiments for each aeration rate (n=3).

Results

Diploid and tetraploid *D. stramonium* L. hairy roots showed rapid growth in stirred tank bioreactor and accumulates maximal amounts of biomass (ADB=8.3 g/L and ADB=6.8 g/L, respectively) when cultivated at aeration rate of 15.0 L/(L.h) (Figure 1). At these conditions the cultures accumulates maximal amounts of 35.0 mg/L and 27.0 mg/L hyoscyamine (for diploid and tetraploid line, respectivelly) (Figure 2).

RESEARCH ARTICLE

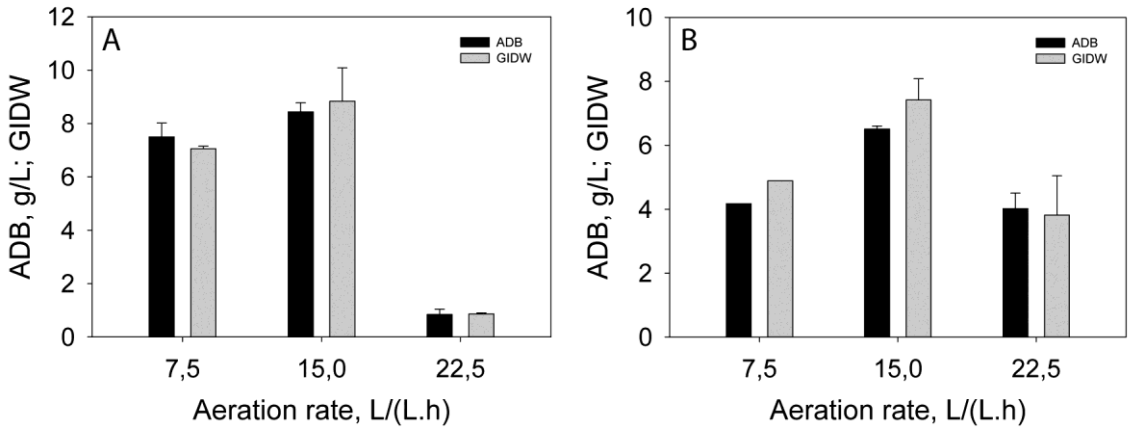

Figure 1. *Accumulated dry biomass and growth indexes achieved at the end of cultivation of diploid (A) and tetraploid (B) hairy root cultures from D. stramonium in stirred tank bioreactor.*

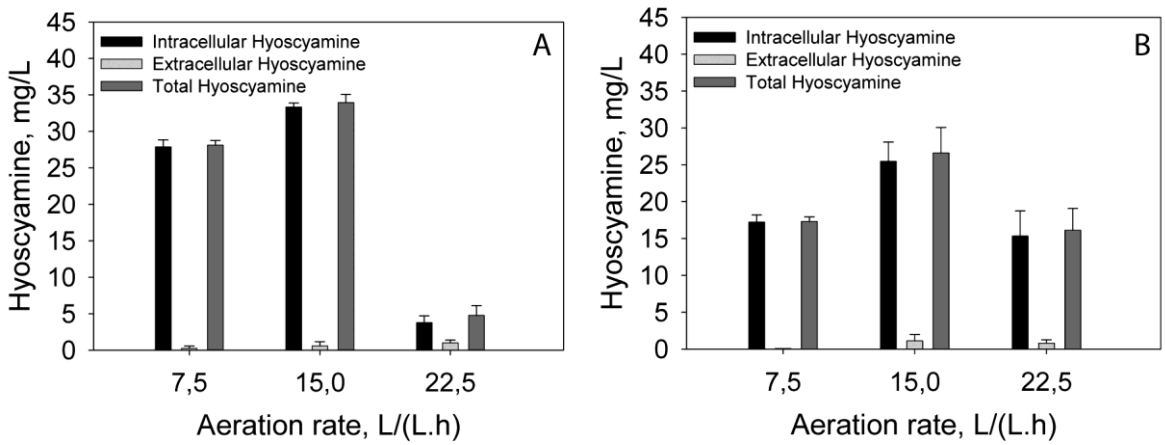

Figure 2. *Maximum amounts of biosynthesized hyoscyamine from diploid (A) and tetraploid (B) hairy roots root cultures from D. stramonium in stirred tank bioreactor.*

The time courses of utilization of the carbon source by diploid and tetraploid *D. stramonium* L. hairy root lines are presented on Figures 4 and 5. When aeration rate of 15.0 L/(L.h) was used, the sucrose in culture media was completely inverted after 18 days from the beginning of cultivation by the both cultures. The data for the degree of utilization of the main macronutrient ions (phosphate, nitrate and ammonium) is presented on Figure 6. Tetraploid culture showed better consumption of phosphate ions (90% of initial concentration), compared to diploid culture (70% of the initial concentration), whereas the diploid culture utilize more nitrate ions compared to tetraploid one (85%, compared to 60% for diploid and tetraploid, respectivelly) when aeration rate of 15.0 L/(L.h) was applied (Figure 6).

Discussion

In our previous studies we showed that diploid and tetraploid *D. stramonium* hairy root lines have stable growth and biosynthetic characteristics when cultivated in submerged conditions in shaking flasks and temporary immersion RITA systems (Georgiev et al., 2008; Pavlov et al., 2009a).

RESEARCH ARTICLE

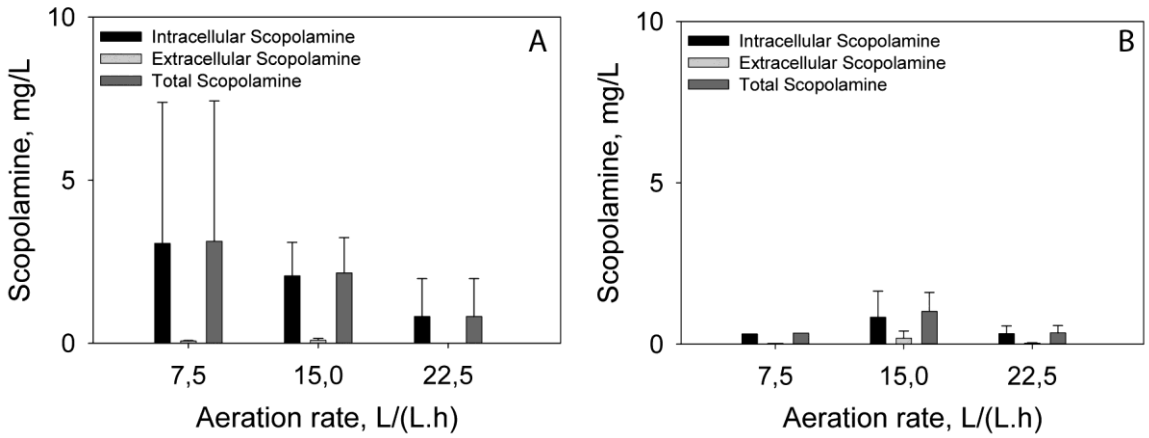

Figure 3. *Maximum amounts of biosynthesized scopolamine from diploid (A) and tetraploid (B) hairy roots root cultures from D. stramonium in stirred tank bioreactor.*

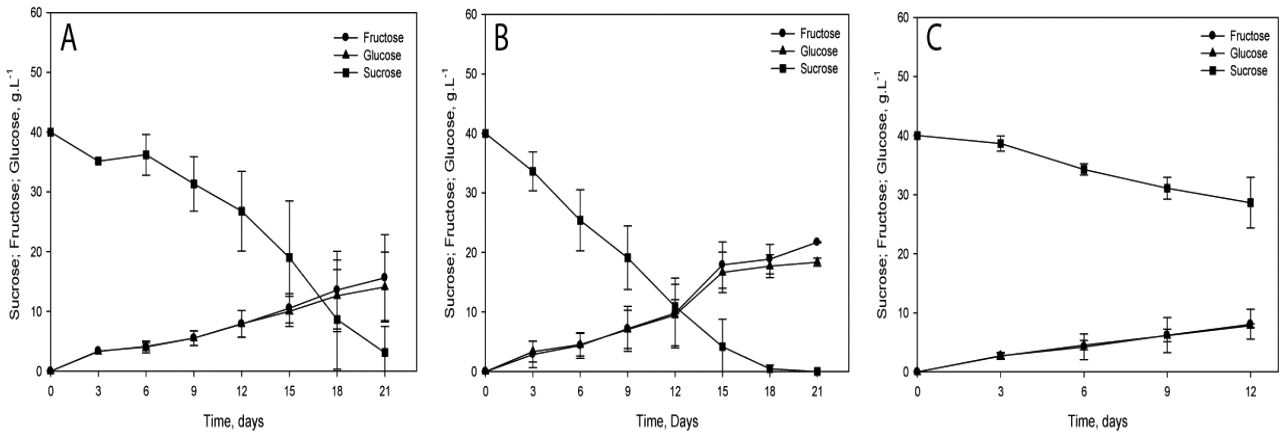

Figure 4. *Dynamics of utilization of sucrose, glucose and fructose of diploid hairy root culture from D. stramonium in stirred tank bioreactor at different aeration rates [A - 7.5; B – 15.0; C - 22.5 L/(L.h)].*

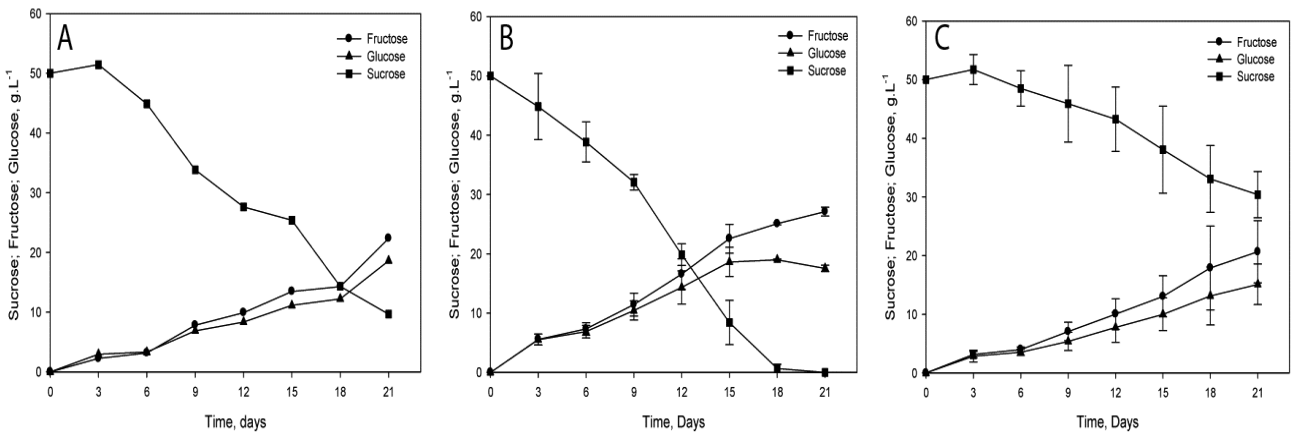

Figure 5. *Dynamics of utilization of sucrose, glucose and fructose of tetraploid hairy root culture from D. stramonium in stirred tank bioreactor at different aeration rates [A - 7.5; B – 15.0; C - 22.5 L/(L.h)].*

RESEARCH ARTICLE

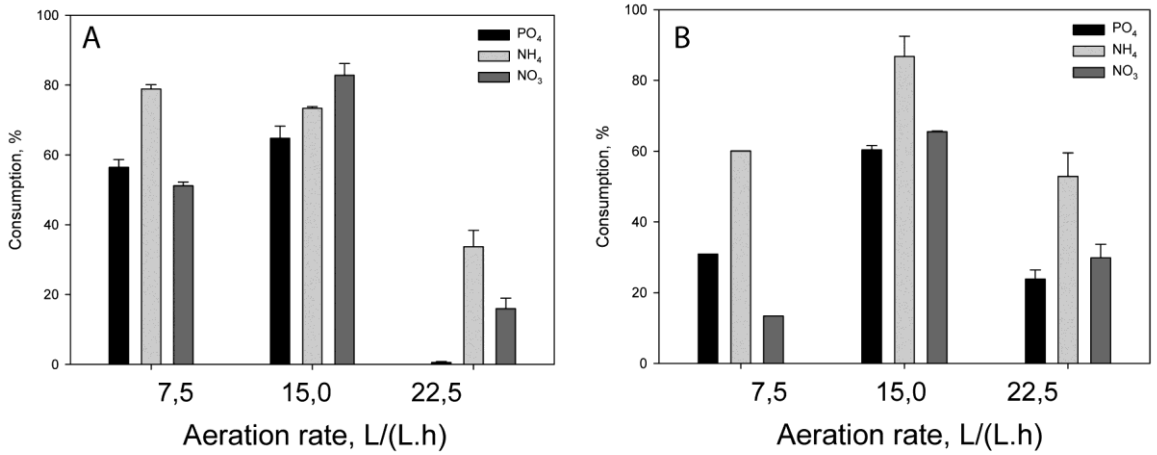

Figure 6. *Degree of utilization of ammonium, nitrate and phosphate ions of diploid (A) and tetrapolid (B) root culture from D. stramonium in stirred tank bioreactor at different aeration rates.*

It was demonstrated that both lines have different requirements to composition of nutrient medium for expressing maximal levels of tropane alkaloids (Pavlov et al., 2009b). In this study we investigated the biomass accumulation and alkaloid production by diploid and tetraploid *D. stramonium* hairy roots cultivated in conventional stirred tank bioreactor. Both cultures showed stable growth and morphological characteristics that did not change during the cultivation. In contrast with some early reports (Wilson, 1997) no disorganization of root tissue were observed which could be considered as a proof that the investigated lines were relatively tolerant to mechanical shear stress induced by the impeller. When cultivated at aeration rate of 15.0 L/(L.h) both diploid and tetraploid lines accumulated maximal amounts of dry biomass (Figure 1). Moreover, the calculated growth indexes were remarkably high (GIDW=9 and 7.8 for diploid and tetraploid line, respectively) compared to the values, usually reported about hairy root cultures (Figure 1). This is convincing indicator revealing the effectiveness of the process.

Aeration rate was found to have significant effect on hyoscyamine and scopolamine biosynthesis as well (Figure 2). It should be noted that the maximal hyoscyamine concentrations were achieved at the same aeration rate, which was found to be optimal for biomass production [15.0 L/(L.h)] (Figure 1 and Figure 2). This is an important observation since most of the hyoscyamine was found to be intracellular and only small amounts (approximately 3% from the total amount) were secreted into culture liquid (Figure 2). Moreover, when cultivated in stirred tank bioreactor diploid and tetraploid *D. stramonium* hairy roots produced also the pharmacologically important alkaloid scopolamine in minor concentrations (Figure 3). It is important to notice that scopolamine was not detected in both diploid and tetraploid hairy root lines when they were cultivated in temporary immersion systems (Georgiev et al., 2008).

For more detailed characterization of cultivation processes in stirred tank bioreactor the time courses of carbon source utilization and the percent of consumption of the main inorganic salts by diploid and tetraploid *D. stramonium* hairy root cultures were investigated (Figures 4, 5 and 6). The dynamics of carbohydrates depletions follow the dynamics of hairy root growths and tropane alkaloid biosynthesis for both diploid and tetraploid lines. The same tendency was observed also with the degree of consumption of the main inorganic ions, which were in correlations with the hairy roots growth and secondary metabolites production at every of the investigated aeration rates. In our previous study we showed the existence of strong relationships between the decrease of culture medium conductivity (triggered by the consumption of main nutrient ions) and the biomass accumulation by diploid and tetraploid *D. stramonium* hairy roots cultivated in shaking flasks (Pavlov et al., 2009a). However, when cultivated in stirred tank bioreactor, such correlations were not found for the both cultures, which mean that changes in conductivity cannot be applied as indirect method for growth monitoring in this case (data not shown).

Conclusion

Our results demonstrated that the conventional stirred tank bioreactor, equipped with pitched blade impeller is a

suitable cultivation system for commercialization of hyoscyamine production by diploid and tetraploid *D. stramonium* hairy root cultures. However, even the ploidy level of hairy root lines was found to have a significant influence over their tropane alkaloid profiles, to express their maximal biosynthetic potentials a precise optimization of cultivation conditions as well as on operational algorithms should be performed.

Acknowledgement

This research was supported by the Bulgarian Science Foundation, Bulgarian Ministry of Education and Science (Project ТК-Б-1605/06).

References

Berkov S, Pavlov A, Kovatcheva P, Stanimirova P, Philipov S. 2002. Alkaloid spectrum in diploid and tetraploid hairy root cultures of *Datura stramonium*. Z. Naturforsch., 58 (1-2): 42-46.

De Luca V, St Pierre B. 2000. The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci., 5(4): 168-173.

Dixon RA. 1985. Isolation and maintenance of callus and cell suspension cultures. - In: Dixon RA. (eds), Plant cell culture - a practical approach, Oxford: IRL, p. 1–20.

Eibl R, Eibl D. 2008. Design of bioreactors suitable for plant cell and tissue cultures. Phytochem. Rev. 7(3): 593-598.

Facchini PJ. 2001. Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annu. Rev. Plant. Physiol. Plant. Mol. Biol., 52: 29-66.

Georgiev V, Stukert A, Bley T, Pavlov A. 2008. Hyosciamine biosynthesis by diploid and tetraploid *Datura stramonium* L. hairy root cultures in a temporary immersion cultivation system. Advances in Bulgarian Science, 2(3): 42-47.

Grynkiewicz G, Gadzikowska M. 2008. Tropane alkaloids as medicinally useful natural products and their synthetic derivatives as new drugs. Pharmacol. Rep., 60(4): 439-463.

Lavania UC. 2005. Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. Plant Genet. Resour., 3(02): 170-177.

Oksman-Caldentey K-M, Arroo R. 2000. Regulation of tropane alkaloid metabolism in plants and plant cell cultures. - In: Verpoorte R & Alfermann AW. (eds), Metabolic engineering of plant secondary metabolism, Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 253-282.

Palazón J, Navarro-Ocaña A, Hernandez-Vazquez L, Mirjalili MH. 2008. Application of metabolic engineering to the production of scopolamine. Molecules, 13(8): 1722-1742.

Papadoyannis NI, Samanidou VF, Theodoridis GA, Vasilikiotis GS, Van Kempen GJM, Beelen GM. 1993. A simple and quick solid phase extraction and reversed phase HPLC analysis of some tropane alkaloids in feedstuff and biological samples. J. Liq. Chromatogr. Related Technol., 16(5): 975-998.

Pavlov AI, Berkov SH, Weber J, Bley T. 2009a. Hyoscyamine biosynthesis in *Datura stramonium* hairy root *in vitro* systems with different ploidy levels. Appl. Biochem. Biotechnol., 157: 210-225.

Pavlov AI, Georgiev VG, Marchev AS, Berkov SH. 2009b. Nutrient medium optimization for hyoscyamine productionin diploid and tetraploid *Datura stramonium* L. hairy root cultures. World J. Microbiol. Biotechnol., 25: 2239-2245.

Ramakrishnan D, Curtis WR. 2004. Trickle-bed root culture bioreactor design and scale-up: Growth, fluid-dynamics, and oxygen mass transfer. Biotechnol. Bioeng., 88 (2): 248-260.

Wilson PDG. 1997. The pilot-scale cultivation of transformed roots. - In: Doran PM. (ed), Hairy roots: culture and applications, Harwood Academic Publishers, Amsterdam, p. 179-190.

Williams GRC, Doran PM. 2000. Hairy root culture in a liquid-dispersed bioreactor: Characterization of spatial heterogeneity. Biotechnol. Prog., 16(3): 391-401.

Yang C, Chen M, Zeng L, Zhang L, Liu X, Lan X, Tang K, Liao Z. 2011. Improvement of tropane alkaloids production in hairy root cultures of *Atropa belladonna* by overexpressing pmt and h6h genes. Plant OMICS, 4(1): 29-33.

Zhang L, Kai G-Y, Lu B-B, Zhang H-M, Tang K-X, Jiang J-H, Chen W-S. 2005. Metabolic engineering of tropane alkaloid biosynthesis in plants. J. Integr. Plant. Biol., 47(2): 136-143.