

## Protopine Production by *Fumaria* Cell Suspension Cultures: Effect of Light

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**Abstract** Protopine biosynthesis in *Fumaria rostellata* and *Fumaria officinalis* cell suspensions was investigated. For the first time, we reported for calli and cell suspensions obtained from *F. rostellata* and *F. officinalis*. Callus induction was initiated on a Murashige and Skoog medium, supplemented with sucrose and various concentrations of plant growth regulators:

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2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). The best morphological characteristics, growth behavior, and protopine biosynthesis were observed for two callus lines (5FRL14 and 12FOL1) cultivated under submerged conditions, at low concentration of 2,4-D (0.2 and 0.5 mg/L) and higher concentration of BAP (2.0 and 3.0 mg/L). The maximal yield of protopine was accumulated from cell suspension of *F. rostellata* (line 5FRL14) cultivated under illumination—49.6 mg/L. Time courses of utilization of sucrose, ammonium, nitrate, and phosphate ions in cultural liquid and acetylcholinesterase inhibitory activity of alkaloid extracts of studied suspensions are also presented.

**Keywords** Callus · Cell suspensions · *Fumaria officinalis* L. · *Fumaria rostellata* Knaf. · Isoquinoline alkaloids · Protopine

## Introduction

Plants produce a variety of chemical structures responsible for their biological activities. In *Fumaria* spp., one of these substance classes are isoquinoline alkaloids. Protopine (7-methyl-6,8,9,16-tetrahydrobis[1, 3]benzodioxolo[4,5-c:5',6'-g]azecin-15(7H)-one) is the primary member of this group. This alkaloid attracts the scientific attention with its wide range of pharmacological activities, such as reduction of intracellular calcium flux, inhibition the release of arachidonic acid, and synthesis of thromboxane A<sub>2</sub> [1–3]. Protopine also possesses anti-bacterial, anti-histaminic and anti-cholinergic activities [4, 5]. It was shown that protopine intake downregulated glutamine level in rat brain through activation of glutamate dehydrogenase [6] and could facilitate the protection against transient cerebral ischemic damage [7].

Plants of the genus *Fumaria* have been used widely in traditional medicine as antihypertensive, diuretics, hepatoprotectants, laxatives, and in the treatment of skin conditions (rashes or conjunctivitis) [8, 9]. In Bulgaria, the genus *Fumaria* is represented by ten wild growing species [10]. Numerous investigations have been focused on their alkaloid profiling [8, 9, 11–13], phenolic compounds [14], and biological activities [1, 15–17]. However, there is limited data available concerning study of *Fumaria officinalis* L. and *Fumaria rostellata* Knaf. grown in Bulgaria. A previously developed TLC densitometry assay showed that both species have high potential in protopine accumulation [18]. However, in addition to protopine, *Fumaria* plants produced accompanying mix of alkaloids, such as *F. rostellata* grown in Romania, which synthesized eight alkaloids (alloycryptopine, chelidonine, protopine, bicuculine, sanguinarine, cheliditine, stylophine, and hydrastine) [19]. It should be noted, that there is no available records about in vitro culture initiations from *F. officinalis* and *F. rostellata*. Development of such in vitro cultures would be a powerful tool for fundamental study of their metabolism and for the commercial production of protopine. It is well known that the light could have a tremendous impact on secondary metabolism of plant cells [20, 21]. Study on the effect of light on protopine accumulation in *Fumaria* in vitro cells may provide a basic tool for manipulating the yields and creating eco-friendly biotechnology for its production. In this study, we describe the effect of light on callus induction and protopine production by cell suspension cultures of *F. officinalis* and *F. rostellata*.

## Materials and Methods

### Plant Material

*F. officinalis* and *F. rostellata* plants were collected in May 2013 from their natural habitats near Blagoevgrad and Sozopol, Bulgaria. Identification of the plant species was made through the references deposited in Herbarium of the Institute of Biodiversity and Ecosystem Research in Sofia and Herbarium of Sofia University.

### Callus Induction

Leaves, flowers, and stems from collected plants were carefully washed and then sterilized by using 70 % ethanol for 20 sec and 7 % (w/v) sodium hypochlorite for 20 min. Sterile explants were triple-washed in sterile water, dried with sterile paper, and transferred on callus induction media. Various combinations of induction media were used, all based on Murashige and Skoog (MS) medium, supplemented with 30.0 g/L sucrose, 5.5 g/L plant agar (Duchefa, The Netherlands), and differed in concentrations (0.2, 0.5, 1.0, 2.0, and 3.0 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma, USA) and 6-benzylamynopurine (BAP, Duchefa). Callus induction was performed in a culture chamber at 26 °C. Half of the explants were cultivated under illumination (16-h light/8-h dark) with light intensity of 110  $\mu\text{mol}/(\text{m}^2 \text{ s})$  (SYLVANIA Gro-Lux fluorescent lamps, F18W/GRO-LUX), and the other half were grown in darkness. The obtained calli were separated from explants and transferred for self growth in petri dishes under conditions of cultivation corresponding to their initiation and with a subculturing period of 21 days.

### Suspension Cultures

Friable parts from selected callus lines were transferred in liquid MS media, composed with the same combinations of auxins and cytokinins, used for initiation of corresponding calli. To obtain stable suspensions, a consistent increase of flasks volume (from 50 to 200 mL) and decrease of used inoculum (from 50 %v/v to 20 %v/v) was applied. The obtained stable suspensions were supported for at least 6 months before conducting the experiments with a subculturing period of 10 days. The cultivation was carried out on rotary shaker (110 rpm) at 26 °C in darkness or under illumination (16-h light/8-h dark) depending of experimental conditions. Samples were taken daily.

### Analyses

#### *Cell Growth Evaluation*

The growth of cell suspensions was evaluated by using both the accumulated dry biomass (ADB) [22, 23] and changes in nutrient medium conductivity with a pH/conductivity meter (INOLAB, WTW, Germany).

#### *Quantification of Protopine*

Lyophilized biomasses (0.15 g) were triple-extracted with 5 mL ethanol in ultrasonic bath for 15 min. The combined extracts were concentrated under vacuum and dissolved in 3 % sulfuric

acid (2×2 mL). Before performing HPLC analyses, the alkaloids were partially purified from the crude extract by using pH differential method [18]. The obtained alkaloids fractions were dissolved in 1 mL 1 N HCl in ethanol and filtrated by 45- $\mu$ m syringe filters before analyses. Quantitative determination of protopine was performed as previously described by Vrancheva et al. [18]. Shortly, HPLC system: Waters 1525 Binary Pump HPLC systems, Waters 2484 dual  $\lambda$  absorbance detector (Waters, Milford, MA, USA), equipped with Supelco Discovery HS C18 column (5  $\mu$ m, 25 cm×4.6 mm), and Breeze 3.30 software were used. The mobile phases used were as follows: A, 10 % solution of triethylamine with pH 2.5 (80 %) and acetonitrile (20 %), and B, 10 % solution of triethylamine with pH 2.5 (40 %) and acetonitrile (60 %). UV detection at 290 nm and the volume of injection 20  $\mu$ L, at 26 °C and flow rate 1.0 mL/min.

### *GS-MS Analyses*

GC-MS analyses were carried out on gas chromatograph Agilent Technology Hewlett Packard 7890A, coupled with a mass detector Agilent Technology 5975 inert XL EI/CI MSD at 70 eV). Separation of alkaloids was carried out on HP-5MS column (30 m×0.25 mm×0.25  $\mu$ m, Agilent Technology) with the following temperature program: from 100 °C with 15 °C/min to 180 °C and from 180 °C with 5 °C/min to 300 °C then hold on 300 °C for 10 min. The injection volume was 1  $\mu$ L at temperature of 250 °C, and the flow rate of carrier gas (helium) was 1.0 mL/min. Additionally, temperatures of MS Sorse was 230 °C, MSD transfer line, 250 °C, and MS Quad, 150 °C. Scan parameters were as follows: low mass 42.0 *m/z* and high mass 550.0*m/z*.

The Kovats retention index (RI) was recorded with a standard n-hydrocarbon calibration mixture (C9-C36, Restek, Teknokroma, Spain). The obtained mass spectra were analyzed using 2.64 Automated Mass Spectral Deconvolution and Identification System (AMDIS, NIST Gaithersburg, MD, USA). Protopine in samples was identified by comparing its GC-MS spectra and RI with those of both protopine standard and the reference data in NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0, 208), National Institute of Standardization and Technology (Gaithersburg, MD, USA).

### *Sucrose, Glucose, and Fructose Assay*

Changes in amounts of sucrose, glucose, and fructose in culture media during cultivation of cell lines were monitored by Shimadzu HPLC system (LC-20 AD pump, coupled with Shimadzu RID-10A refractive index detector, and LC solution version 1.24 SP1 software; Shimadzu Corporation, Kyoto, Japan). Separation was carried out on a Shodex® Sugar SP0810 with Pb<sup>2+</sup> column (300 mm×8.0 mm i.d.), coupled with a guard column (50×9.2 mm i.d.), and placed in thermostat LCO 102 (ECOM, Czech Republic). The mobile phase was deionized water, and isocratic flow rate 1.0 mL/min was used as described previously [24].

### *Nitrate, Ammonium, and Phosphate Assay*

Nitrate, ammonium, and phosphate ions were determined by chemical test combinations (Spectroquant, MERCK, Germany, cat. nos. 1.14773.0001, 1.14752.0001, and 1.14842.0001).

## Inhibition of Acetylcholinesterase

The acetylcholinesterase inhibition activities of extracts were evaluated using spectrophotometric method [25], following the procedure described by Georgiev et al. [26]. The results were presented as  $IC_{50}$  (mg).

## Statistical Analyses

Presented data are average values and standard deviations calculated from two independent experiments repeated twice. Data was analyzed by *t* test with a level of significance of  $P \leq 0.05$  using SigmaPlot v. 11.0 Software (Systat Software, Inc., USA).

## Results and Discussion

### Callus Induction and Selection of Protopine-Producing Cell Lines

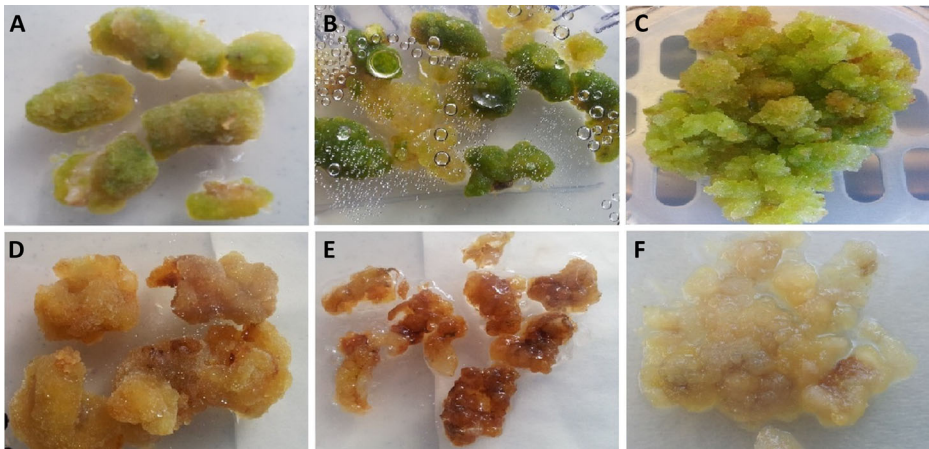
In this study, we reported a protocol for callus induction of wild growing *F. officinalis* and *F. rostellata*. Different plant organs, including leaves, stems, and flower buds were used as explants. All of the investigated media variants were able to induce callus in 2 weeks after planting of explants.

Light had significant influence on both explant survival and next callus formation abilities. However, the effect seems to be species specific. Data showed that the survival rate of *F. rostellata* was higher when the explants were cultivated under illumination (73 %) than in darkness (53 %). In contrast, the numbers of survived explants of *F. officinalis* were almost unaltered by the light (81 % when cultivated under illumination vs 88 % when grown in darkness). Moreover, all of the survived explants from the both species formed a callus after 2-weeks cultivation under illumination, whereas only 87 % of the explants from *F. rostellata* retained their callus induction abilities when cultivated in darkness. The obtained calli showed high variations in growth, morphology, and color (Fig. 1). In general, it was found that calli from *F. rostellata* were preferably green colored, whereas the calli from *F. officinalis* were brown (Fig. 1). Beside illumination conditions, the color seems to be species specific, since no variations between the calli from the same species, but grown on different media variants have been observed.

After separation from explants for independent growth most of the calli arrested their growth. Ten callus lines from *F. rostellata* and two callus lines from *F. officinalis* showed intensive growth and were selected for the further screening of protopine content (Table 1).

It is well known that the level of secondary metabolites could vary in wide range among the different callus lines, obtained from the same plant [27]. The presence of protopine in the obtained in vitro cultures was confirmed by using GC-MS (Fig. 2), and the quantification was done by HPLC (Table 1). Further, it should be noted that calli grown under illumination accumulated significant higher amounts of protopine (Table 1).

Five of the obtained *F. rostellata* lines biosynthesized protopine between 31 and 907  $\mu\text{g/g}$  DW, and the two lines of *F. officinalis* accumulated protopine between 25 and 43  $\mu\text{g/g}$  DW (Table 1). The observed huge deviations in protopine accumulation could be due to differences in growth regulators used in culture initiation, polyploidization and somaclonal variability, or cellular heterogeneity of callus cultures [27, 28].

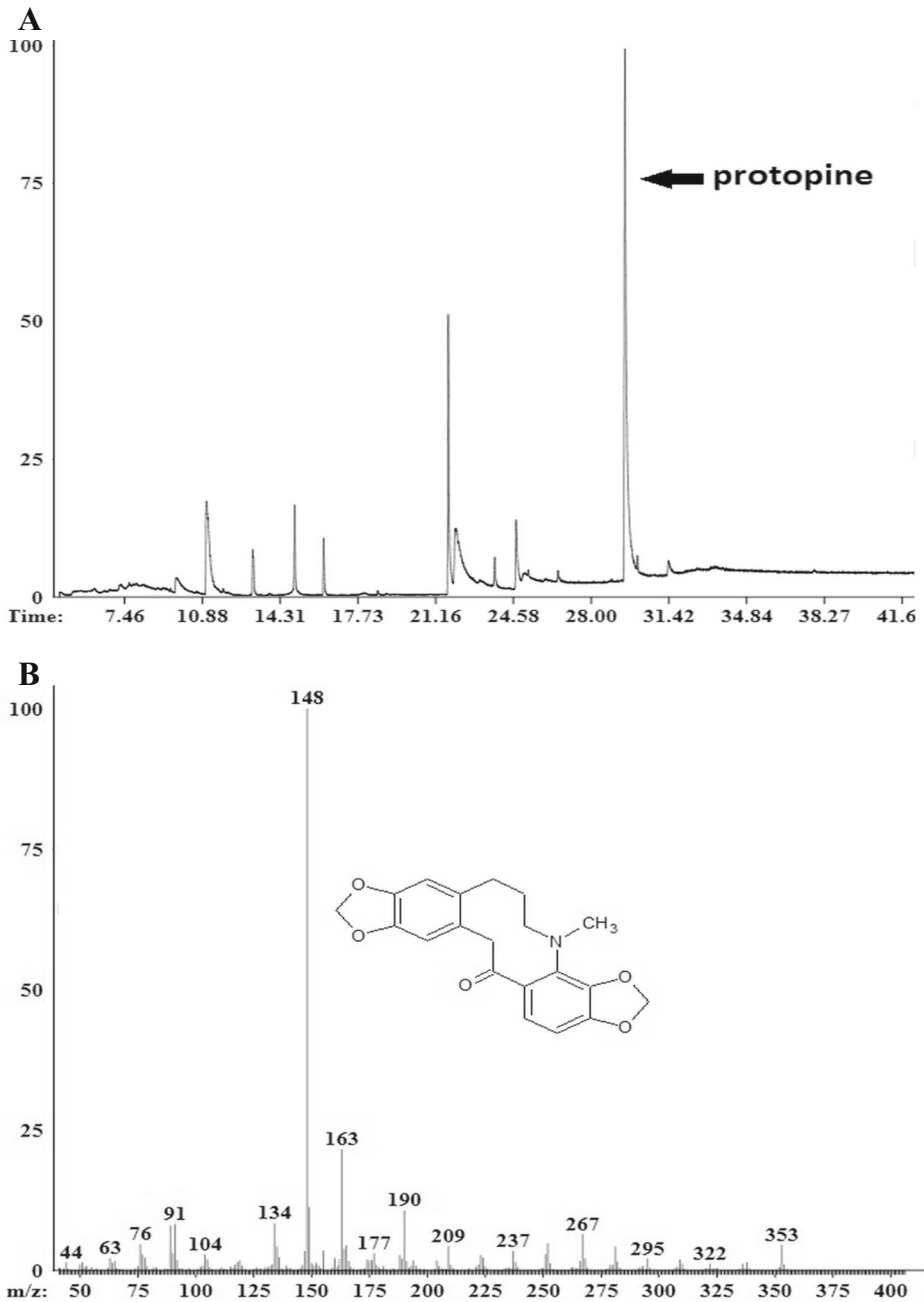


**Fig. 1** Morphology of callus cultures from *F. rostellata* and *F. officinalis*, cultivated under illumination (a–c) and in darkness (d–f). Hard and very compact callus (a, d); mixtures of compact and friable callus (b, e); friable callus (c, f)

The callus lines which produced protopine were selected for initiation of cell suspensions. Initially, for the purpose of screening, suspensions were cultivated in darkness. Adaptation of selected lines to submerged conditions of cultivation resulted in significant increase in their protopine production capacity (Table 2). The highest protopine contents were registered in line 5FRL14 (2603  $\mu\text{g/g}$  DW, 14.1 mg/L) of *F. rostellata* and line 12FOL1 (571  $\mu\text{g/g}$  DW, 4.6 mg/L) of *F. officinalis* (Table 2). At submerged conditions of cultivation of these lines showed increase in protopine content of 2.9-fold and 13.3-fold, compared to the growth on solid

**Table 1** Characteristics of the selected callus lines of *F. rostellata* and *F. officinalis* and protopine content after 21-day cultivation

Callus line	Explant	Conditions of cultivation	Plant growth regulators		Protopine in callus culture ( $\mu\text{g/g}$ DW)
			2,4-D	BAP	
<i>Fumaria rostellata</i> Knaf.					
5FRL14	Leaves	Light	0.2	2.0	907 $\pm$ 35
6FRL20	Leaves	Light	0.2	3.0	537 $\pm$ 51
11FRL106	Flowers	Dark	0.5	1.0	31 $\pm$ 5
16FRL114	Leaves	Dark	1.0	0.2	Trace
17FRL118	Leaves	Dark	1.0	0.5	Trace
17FRL119	Leaves	Dark	1.0	0.5	11 $\pm$ 2
18FRL56	Leaves	Light	1.0	1.0	165 $\pm$ 28
23FRL66	Leaves	Light	2.0	0.2	Trace
23FRL129	Leaves	Dark	2.0	0.2	Trace
23FRL130	Flowers	Dark	2.0	0.2	Trace
<i>Fumaria officinalis</i> L.					
12FOL1	Leaves	Light	0.5	2.0	43 $\pm$ 5
13FOL5	Leaves	Dark	0.5	3.0	25 $\pm$ 3



medium. The observed increase is probably due to improved mass transfers in suspension cultures. Both lines showed stable, uniform growth and were selected as perspective producers of protopine.

**Table 2** Protopine content in suspension cultures of *F. rostellata* and *F. officinalis* cultivated in darkness for 10 days

Line	Protopine	
	$\mu\text{g/g DW}$	mg/L
<i>Fumaria rostellata</i> Knaf.		
5FRL14	2603 $\pm$ 82	14.1 $\pm$ 1.7
6FRL20	681 $\pm$ 50	2.8 $\pm$ 0.4
11FRL106	1574 $\pm$ 67	4.6 $\pm$ 0.9
17FRL119	408 $\pm$ 28	1.5 $\pm$ 0.2
18FRL56	137 $\pm$ 17	0.6 $\pm$ 0.05
<i>Fumaria officinalis</i> L.		
12FOL1	571 $\pm$ 27	2.8 $\pm$ 0.2
13FOL5	198 $\pm$ 21	0.7 $\pm$ 0.09

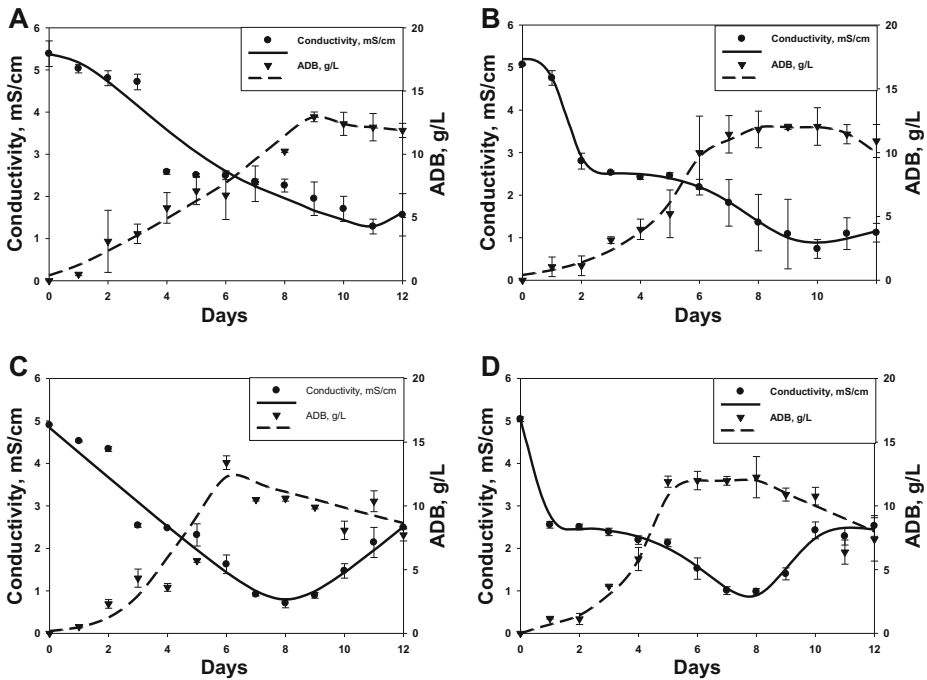
It should be noted that the accumulated level of protopine in biomass of *F. rostellata* line 5FRL14 biomass (2603  $\mu\text{g/g DW}$ ) was 7.7-fold and 16.7-fold higher than the reported in biomasses of intact plants grown in Bulgaria (335  $\mu\text{g/g DW}$ ) and Romania (156  $\mu\text{g/g DW}$ ) [18, 19]. A similar tendency was found in suspension line 12FOL1 of *F. officinalis*, where the protopine content (571  $\mu\text{g/g DW}$ ) was 2.0-fold higher than the protopine accumulated in intact plants (273  $\mu\text{g/g DW}$ ) [18]. The observed variations in protopine content confirmed the importance of quantitative phytochemical analyses during every step of the preliminary screening process for selection of high-yielding cell lines.

### Effect of Light on Growth and Protopine Biosynthesis by *F. rostellata* and *F. officinalis* Cell Suspensions

A maximal production of both biomass and desired metabolite is the main goal in the development of effective plant-cell-based biotechnologies. However, these two processes do not always occur in parallel [29]. A detailed study of time courses of changes in accumulated biomass and protopine production during cultivation of *Fumaria* cell lines could allow us to define the optimal harvest time for maximal system productivity. It is well known that light could have a significant effect on both biomass production and secondary metabolite accumulation by in vitro growing plant cells [20, 30]. In the case of isoquinoline alkaloid biosynthesis, the light could alter the production of their precursor tyrosine, in which biosynthesis is dependent on the photosynthesis [31, 32]. Moreover, light could act not only as energy source for photosynthesis but also as powerful abiotic elicitor of secondary metabolism in plant cells [21]. In this study, we investigated the influence of light on the growth and protopine biosynthesis by selected cell suspensions of *F. rostellata* and *F. officinalis*.

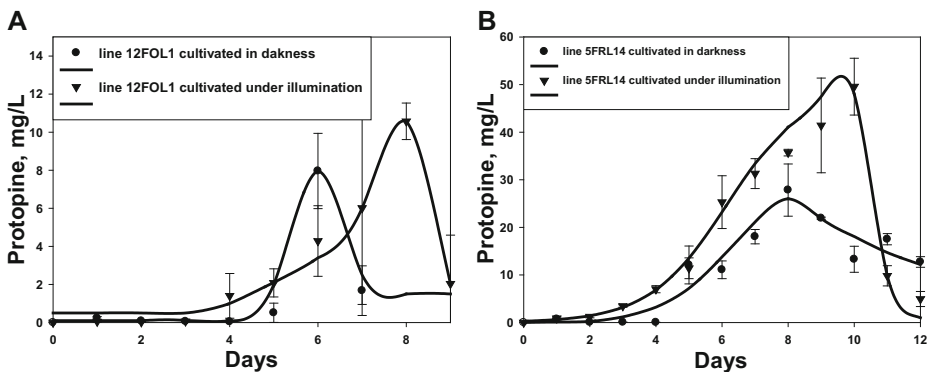
Both cell lines (*F. rostellata* 5FRL14 and *F. officinalis* 12FOL1) grew rapidly and the highest biomass accumulation was recorded on day 9 (13.0 g/L) and on day 6 (14.7 g/L) for the cell suspensions of *F. rostellata* and *F. officinalis* cultivated in darkness, respectively (Fig. 3a, c). Doubling times in this case were 3 and 2 days for *F. rostellata* and *F. officinalis*, respectively, while specific growth rates were equal, 0.3/days. It should be noted that at submerged cultivation under illumination, the growth cycle of both cell lines was characterized with longer stationary phase (Fig. 3b, d), and therefore, maximal amounts of accumulation biomasses (12.1 and 12.3 g/L for *F. rostellata* and *F. officinalis*, respectively)





**Fig. 3** Time courses of growth and changes in conductivity of media of *F. rostellata* (a, b) and *F. officinalis* (c, d) cultivated in darkness (a, c) and under illumination (b, d)

were reached latter on day 10 and day 8 (Fig. 3c, d). In this case, the doubling times were equal (3 days) and the specific growth rates were 0.2 and 0.3/days for *F. rostellata* and *F. officinalis*, respectively. Similar effect of illumination (prolonged growth cycle and decrease in biomass accumulation) was recently reported for cell suspension of *Artemisia absinthium* L. [30]. On the other hand, achieved levels of accumulated biomasses were comparable with values previously reported by Tanahashi and Zenk [11].

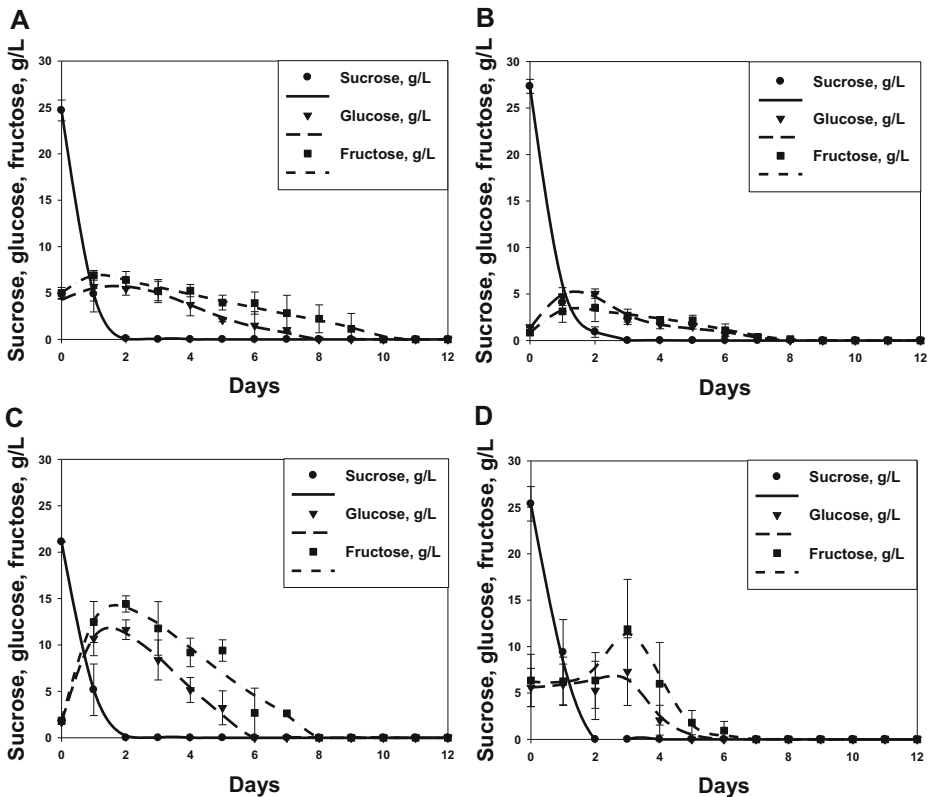


**Fig. 4** Time courses of biosynthesis by *F. officinalis* (a) and *F. rostellata* (b) cell suspensions, cultivated in darkness and under illumination

Conductivity of the medium decreased from 5 to 1 mS/cm (Fig. 3), and this decrease was inversely correlated with the increase of accumulated dry biomass ( $r^2 > 0.9$  in all cases, data are not shown); thus, conductivity of the medium is a potential indirect indicator for online monitoring of the growth cycle of the cultures, which is important for the future bioreactor cultivation. We have previously reported similar results also in different plant in vitro systems [33].

The maximal amount of biosynthesized protopine was achieved during the late exponential and stationary growth phases (Figs. 3 and 4). Light significantly influenced on protopine accumulation in both investigated cell suspensions (Fig. 4). *F. rostellata* cell suspension accumulated 27.8 and 49.6 mg/L (2.3-fold higher) protopine at the cultivated in darkness and under illumination, respectively. The same is the tendency in the case with *F. officinalis*. Maximal yield (10.6 mg/L) of accumulation protopine under illumination was 1.3-fold higher than this achieved during the cultivation in darkness (8 mg/L).

It should be noted that the maximal amount of protopine (49.6 mg/L) accumulated by *F. rostellata* under illumination is one of the highest amounts ever reported about alkaloid biosynthesized by plant cells cultivated in vitro. It is 9.6-fold higher than that accumulated in intact plants: 3193  $\mu\text{g/g}$  DW (in suspended cells) versus 335  $\mu\text{g/g}$  DW in intact plants [18]. At



**Fig. 5** Time courses of utilization of sucrose, glucose, and fructose from cell suspensions of *F. rostellata* (a, b) and *F. officinalis* (c, d) cultivated in darkness (a, c) and under illumination (b, d)

these conditions, achieved system productivity was 5 mg/(L day), which is a good base for further development of effective protopine production process based on *F. rostellata* cell suspension culture.

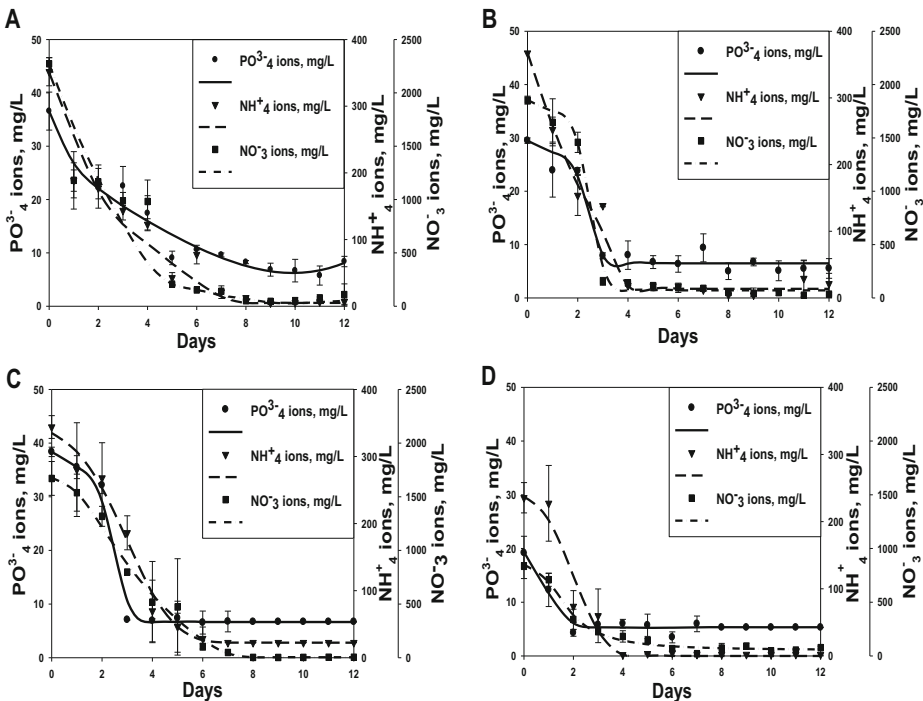
### Time Courses of Utilization of the Main Nutrient Elements

Sucrose is a common carbon source for growth and maintenance of cultured cells from various plant species. Plant cell and tissue culture metabolize sucrose to provide energy and carbon skeletons for every metabolic process [34, 35]. Hence, investigation of sucrose utilization is an important step of assessment of every production process.

An intensive hydrolysis of sucrose was observed (Fig. 5). After the second day of cultivation, there were no detectable amounts in all investigated cell suspensions. The consumption of glucose and fructose followed the growth of the cell suspensions (Figs. 3 and 5), and they were completely exhausted at the end of exponential growth phases when maximal amounts of biomasses and protopine were accumulated. Thus, the concentration of carbon source would be an important parameter in the further procedure of process optimization.

Other important macronutrients for the growth of cell suspension and secondary metabolite production are ammonium, nitrate and phosphate ions.

Nitrogen consumption followed the growth of the investigated cultures, and nitrate and ammonium ions were completely exhausted at the end of exponential phase of growth cycle.



**Fig. 6** Time courses of utilization of phosphate, nitrate and ammonium ions from cell suspensions of *F. rostellata* (a, b) and *F. officinalis* (c, d) cultivated in darkness (a, c) and under illumination (b, d)

**Table 3** AChE inhibitory activity of the tested alkaloid extracts expressed as IC<sub>50</sub> values

Sample	IC <sub>50</sub> (mg)
Protopine standard	3.9±0.9
Line 5FRL14 cultivated in darkness, 8 days	8.1±2.4
Line 5FRL14 cultivated under illumination, 10 days	12.6±3.6
Line 12FOL1 cultivated in darkness, 6 days	17.6±4.5
Line 12FOL1 cultivated under illumination, 8 days	18.2±8

Phosphate ions were utilized till the end of exponential phases of growth. After that, their concentrations remained constant till the end of cultivation (between 6 and 8 mg/L) (Fig. 6). The relationships between these ions and protopine accumulation is clearly visible; hence, their concentrations also would be important parameters in the optimization of the protopine biosynthesis by *Fumaria* cell suspensions.

### Acetylcholinesterase Inhibitory Activity

It is well known that huge numbers of plant alkaloids isolated from Fumariaceae are acetylcholinesterase inhibitors [36]. The development of in vitro techniques for screening and producing of alkaloids possess this activity is important by the means to continuous supply of these substances to pharmacy. The acetylcholinesterase inhibitory activity (AChE activity) of alkaloid extracts from investigated plant cell suspensions was determined at the day of maximal protopine accumulation (Table 3).

AChE activity of analyzed samples were between IC<sub>50</sub> 8.2 mg and IC<sub>50</sub> 18.2 mg, as the extract from *F. rostellata* cell suspension cultivated in darkness showed the highest AChE activity (Table 3). This was an unexpected result, because the cell suspensions cultivated in darkness produced lower amounts of protopine, and at the same time, protopine itself possessed higher AChE activity (IC<sub>50</sub> 3.9 mg, Table 3). These results clearly indicated that AChE inhibitory activity is a result of unknown interaction between alkaloids in the complex alkaloid extracts. For better understanding of this phenomenon, further metabolite analyses (based on GC-MS) should be performed.

### Conclusion

In conclusion, an effective protocol for obtaining *F. rostellata* and *F. officinalis* cell suspensions was developed. These are the first in vitro systems of *F. rostellata* and *F. officinalis* ever reported. *F. rostellata* 5FRL14 cell suspension was selected as the best producer of protopine. During its cultivation, under illumination, the achieved volumetric yield and system productivity were 49.6 mg/L and 5 mg/(L day), respectively. These results are a good base for further nutrient medium optimization, bioreactor, environmental condition optimization, and scale-up of the process as a part of development of effective biotechnology for protopine production.

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**Conflict of Interest** The authors declare that there is no conflict of interest.

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