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Research Article

Production of fumaric acid from *Fumaria* spp. plant *in vitro* systems

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Abstract

We report for the first time fumaric acid biosynthesis by *in vitro* systems of *Fumaria* spp. The highest amount of fumaric acid (137 mg/L) was accumulated by *Fumaria officinalis* L. plant cell culture, cultivated at submerged conditions in darkness. The prospects for efficient process development were also discussed.

Keywords: fumaric acid, *Fumaria officinalis* L., *Fumaria rostellata* Knaf., intact plants, callus, cell suspension, shoots

Abbreviations:

MS - Murashige and Skoog medium
2, 4-D - 2, 4-dichlorophenoxyacetic acid
BAP - 6-benzylamynopurine

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Introduction

Fumaric acid is a naturally occurring organic acid known as (E)-2-butenedioic acid or trans-1,2-ethylenedicarboxylic acid. It is an intermediate in the tricarboxylic acid cycle for organic acid biosynthesis. Main producers of fumaric acid are filamentous fungi from genus *Rhizopus*. Chemical synthesis of fumaric acid from maleic anhydride also has been reported (Roa Engel et al. 2008). Fumaric acid has numerous applications in the food industry (bread, fruit drinks, pie fillings, poultry, wine, jams, and jelly), pharmacy (manufacturing of psoriasis medicines) and chemistry (precursor for polymerization and esterification reactions). The fumaric acid has used as the acidity regulator in food and beverage, it has an E-number (E297) and it is classified as "practically nontoxic" (LD_{50} 6 $g.kg^{-1}$ for rat and 10 $g.kg^{-1}$ for chicken, respectively). (SCAN, 2003). Fumaric acid and its esters possess anti-inflammatory, hepatoprotective, analgesic (Shakya et al. 2014), antitumor and anti-intoxication activities (Kuroda et al. 1981), as well as strong antibacterial activity against *Staphylococcus aureus* and *Streptococcus*, *Escherichia coli* and *Salmonella* (He et al. 2011). The esters of fumaric acid (especially, dimethyl fumarate) have been used in the biomedical treatment of psoriasis, multiple sclerosis and granuloma annulare (Schweckendiek 1959; Kreuter et al. 2002; Moharregh-Khiabaniet et al. 2009; Das et al. 2016).The cultivation of plant cells and tissue cultures is well known prospective technology for production of valuable biologically active substances (Steingroewer et al. 2013). Despite of the fact that fumaric acid is of plant origin, up to now there are no data available for the plant *in vitro* systems producing it. In this manuscript we report for the first time accumulation of fumaric acid from *in vitro* systems of *Fumaria* spp. Further, we discuss the prospects for development of the efficient production process based on *Fumaria* cell suspension cultures.

Materials and Methods

Plant material and in vitro cultures. Collection and identification of *Fumaria officinalis* L. and *Fumaria rostellata* Knaf. intact plants were previously reported (Vrancheva et al. 2016). Voucher specimen numbers of the investigated plants are SOM 1030 (*Fumaria officinalis* L.) and SOM 1031 (*Fumaria rostellata* Knaf.). Samples were dried in shade at ambient temperature for 14 days and powdered by the homogenizer. The powdered materials were used for the extraction of fumaric acid. A protocol for obtaining calli and suspension cultures of *Fumaria rostellata* Knaf. and *Fumaria officinalis* L. was described previously by Georgieva et al. (2015). The obtained stable suspension cultures were cultivated in flasks (volume 200 mL) on Murashige and Skoog (MS) medium, supplemented with 30.0 $g.L^{-1}$ sucrose and 0.2 $mg.L^{-1}$ and 0.5 $mg.L^{-1}$ of 2,4-dichlorophenoxyacetic acid (2,4-D Sigma, USA), respectively, and 2 $mg.L^{-1}$ 6-benzylamynopurine (BAP, Duchefa) with subculturing period of 10 days. The cultivation was carried out on the rotary shaker (110 rpm) at 26°C in darkness or under illumination (16-h light/8-h dark) (SYLVANIA Gro-Lux fluorescent lamps, F18W/GRO-LUX) depending on experimental conditions. For callus cultures, similar media supplemented with 5.5% "Plant agar" (Duchefa) were used. Shoot cultures from both species were obtained and subcultivated in MS media supplemented with 30 $g.L^{-1}$ sucrose, 5.5 $g.L^{-1}$ "Plant agar" (Duchefa) and 0.2 mg/L 2,4-D and 3 $mg.L^{-1}$ of BAP for F. rostellata, and 0.5 $mg.L^{-1}$ 2,4-D and 2 $mg.L^{-1}$ BAP for F. officinalis. The formed shoot cultures were separated from the explants and transferred for self-growth in containers at the same conditions and cultivated under illumination (16 h light/8 h dark) with the sub-culturing period of 21 days.

Quantification of fumaric acid. Lyophilized biomass (0.1 g) was double-extracted with 1 mL 3% meta-phosphoric acid in ice cool ultrasonic bath for1 min. After centrifugation at 7000 rpm the supernatants were combined in volumetric flask 5 mL and were adjusted to 5 mL with 3% m-phosphoric acid. The extracts were filtrated through 45-μm syringe filters prior HPLC analyses.

Quantitative determination of fumaric acid was performed using Waters HPLC system: 1525 Binary Pump, Waters 2484 dual λ absorbance detector and Breeze 3.30 software (Waters, Milford, MA, USA), equipped with Supelco Discovery HS C18 column (5 μm, 25 cm×4.6 mm). The mobile phase used was 6 mM phosphoric acid with pH 2.1 and the gradient regime of flow rate as follow: from 0 min to 22 min (0.5 mL.min^{-1}) and from 23 min to 30 min (1.0 mL.min^{-1}). UV detection was set at 210 nm and the volume of injection 20 μL. The temperature of the column was 30°C. Presented data are average from two independent experiments repeated at least twice.

Results and Discussion

Biosynthesis of fumaric acid by Fumaria intact plants was previously reported in F. parviflora grown in Iran (Jowkar et al. 2011). Based on this, we investigated the accumulation of fumaric acid by *F. officinalis* and *F. rostellata* grown in Bulgaria. The determined contents were similar (0.26% w/w and 0.88% w/w for *F. officinalis* and *F. rostellata*, respectively) (Table 1). These results provoked our interest to investigate biosynthesis of fumaric acid by previously obtained and selected cell suspensions of *F. officinalis* and *F. rostellata* (Georgieva et al. 2015) at different culture conditions. Further calli and shoots of both species were also investigated for fumaric acid accumulation. Results are presented in Table 1.

Table 1. Production of fumaric acid by *Fumaria officinalis* and *Fumaria rostellata* and their plant *in vitro* systems.

Species	Concentration of fumaric acid, % *w/w*				
	Intact plant	*In vitro* cultures			
		Callus	Suspension culture		Shoot culture
			Dark	Light	
Fumaria officinalis	0.26 ± 0.02	0.35 ± 0.02	0.93 ± 0.03	0.12 ± 0.01	0.44 ± 0.01
Fumaria rostellata	0.88 ± 0.03	0.71 ± 0.03	0.14 ± 0.01	0.14 ± 0.01	0.33 ± 0.02

Callus and shoot cultures obtained from F. officinalis accumulated higher amounts of fumaric acid than intact plant 0.35% (1.4 fold higher) and 0.44% (1.7 fold higher), respectively, while in vitrocultures obtained from *F. rostellata* accumulated lower concentrations than intact plant - 0.71% and 0.33%, respectively (Table 1). It should be underlined that the highest content of fumaric acid were determined in suspension culture of *F. officinalis* cultivated in darkness (0.93%). At submerged conditions of cultivation this culture accumulated 2.6-fold higher amounts of fumaric acid than callus grown on solid medium. The obtained results were with high importance because the most appropriate plant *in vitro* system for the eventual next scale-up of the process is the cell suspension grown in darkness. Up to now, there are no data available in the scientific literature for fumaric acid production by *in vitro* plant systems. Thus, we investigated the time course of volumetric yields of fumaric acid synthesized by *F. officinalis* cell culture. Results are presented in Figure 1.

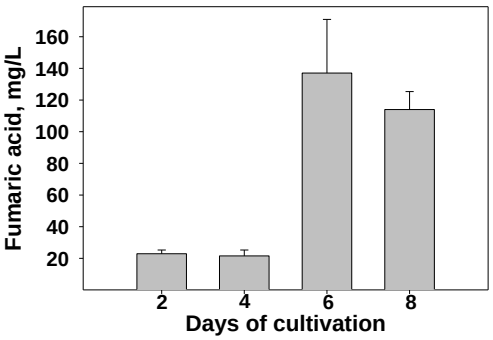

Figure 1. Time course of fumaric acid biosynthesis by Fumaria officinalis suspension culture cultivated in darkness.

F. officinalis cell suspension biosynthesized maximal amount of fumaric acid (137 $mg.L^{-1}$) on the 6th day of its cultivation at submerged conditions in darkness. At the cultivation under illumination, *F. officinalis* cell suspension accumulated 35.4 $mg.L^{-1}$ – 3.8 fold less. For comparison, *F. rostellata* cell suspension culture biosynthesized 18.1 $mg.L^{-1}$ and 23 $mg.L^{-1}$ fumaric acid at submerged cultivation under illumination and in darkness, respectively (data are not shown). The achieved yield of 137 $mg.L^{-1}$ fumaric acid during the cultivation of *F. officinalis* is significantly less than this reported for fumaric acid biosynthesis from fungi *Rhizopus ssp* (Roa Engel et al. 2008). However, achieved in our case results were during cultivation of *F. officinalis* cell suspension in a basic MS nutrient medium on a shaker. Many years experience in our group showed that after optimization of medium composition, elicitation and optimization of environmental conditions of cultivation these results could be significantly improved. Next scale-up of cultivation in different type bioreactors, as well as applied additional approaches for cultivation regime (such as two-phase cultivation) led to the additional improvement of yields of target metabolites by the plant in vitro systems. As successful examples could be noted biosynthesis of rosmarinic acid by *Lavandula vera* MM cell suspension up to 50 fold increase (Pavlov et al. 2005) and biosynthesis of galanthamine by shoots of *Leucojum aestivum* (1.36 fold increase) (Ivanov et al. 2013). Hence, for more detailed assessment of the capacity of *F. officinalis* cell suspension for development commercial process for fumaric acid production more detailed extensive experimental work should be performed. Despite of success in process optimization, development of cell suspension synthesizing fumaric acid is a perspective from point of view next fundamental (biochemical and genetic) investigations on fumaric acid biosynthesis in plant cells.

Conclusions

In this study, we report for the first time the ability of in vitro systems of *Fumaria* to synthesize and accumulate fumaric acid. It was established that plant cell suspension of *F. officinalis* cultivated under submerged conditions in darkness produce the highest amount of fumaric acid (137 $mg.L^{-1}$), which makes it the potential object for further experiments aiming optimization of volumetric yields using tools of bioprocessing engineering.

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