Journal of Microbiology, Biotechnology and Food Sciences

Barboráková et al. 2012 : 1 (4) 466-477

REGULAR ARTICLE

EFFECT OF GLUCOSE CONCENTRATION AND GROWTH CONDITIONS ON THE FUNGAL BIOMASS, PH OF MEDIA AND PRODUCTION OF FUMAGILLIN BY A NON-PATHOGENIC STRAIN *PENICILLIUM SCABROSUM*

Zuzana Barboráková[1], Roman Labuda[2], Georg Häubl[2], Dana Tančinová[1]*

Address: [1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

[2]Romer Labs Division Holding GmbH, Technopark 1, 3430 Tulln, Austria

*****Corresponding author: zuzana.barborakova@gmail.com

ABSTRACT

The aim of this study was to obtain the information on the fumagillin production by a strain *Penicillium scabrosum* on a selected synthetic medium, and to study the effect of different glucose concentrations and the cultivation conditions on the production of fumagillin, fungal biomass, and changes of pH in media. These parameters were observed on 7th, 14th, 21st, 28th, 35th, 42nd and 49th day of cultivation. TLC (thin-layer chromatography) and HPLC analysis (high-performance liquid chromatography) were used for confirmation of fumagillin production. Based on the results from this study, MM medium with 1% glucose and 42 days cultivation at room temperature was found to be the best synthetic medium for production of fumagillin by the strain *P. scabrosum* used (82.54 µg.mL^{-1}). Growth of fungal biomass reached its maximum in MM medium with 3% glucose on 14th day of cultivation at 25 °C. An increased concentration of glucose in MM medium (up to 3%) had positive impact on biomass growth, but it negatively influenced production of fumagillin. Furthermore, increasing concentration of glucose in media resulted in decreasing of pH and consequently increasing pH of media caused decreasing of fumagillin production.

Keywords: fumagillin, fungal biomass, *Penicillium scabrosum*, pH, synthetic medium

INTRODUCTION

Fumagillin is a metabolite of *Aspergillus fumigatus* and has a potent amoebicidal property **(Fekete *et al.*, 1995)**. The production and properties were firstly descirbed in 1951 **(Eble and Hanson, 1951; McCowen *et al.*, 1951)**, and the structure was published by **Tarbell *et al.* (1961)**. It has been widely used both in human and veterinary medicine. The first reported effect was the activity against the microsporidian pathogen *Nosema apis* in honey bees (*Apis mellifera* L.) which were fed with fumagillin dissolved in sugar syrup **(Bailey, 1953)**. This approach has also been adapted for other insect species **(Whittington and Winston, 2003)**. Administered in the diet, it was also used to treat microsporidiosis in fish **(Takeda Chemical Industries, 1983)**. In human, fumagillin was used more than 40 years ago for the treatment of intestinal amebiasis **(McCowen *et al.*, 1951)**, and it is effective when used in the treatment of microsporidial keratoconjuctivitis **(Wilkins *et al.*, 1994)**. It has appeared to be the most effective medicine in supressing cryptosporidiosis and microsporidiosis caused by *Enterocytozoon bieneusi*, which can be fatal for HIV-infected persons **(Molina et al, 2000)**. One of the most promising new approaches to cancer chemotherapy is the use of angiogenesis inhibitors **(Furness *et al.*, 2005)**. Fumagillin and the structurally related ovalicin are two of the most potent anti-angiogenic compounds **(Mazitschek *et al.*, 2005**). In fact, it has been reported that fumagillin inhibits the vascularization of solid tumours, which is promising to treat certain types of cancer **(Picoul *et al.*, 2003)**. Data on genotoxic effects of fumagillin obtained from *in vitro* studies showed discrepancy, being either positive **(Kulić, 2006)** or negative **(Heil *et al.*, 1996)**.

Fumagillin is known to be unstable in light **(Eble and Garrett, 1954)** and in heat **(Garrett, 1954)**. Significant degradation took place even in samples stored in freezer, therefore fumagillin drug substance should be stored below minus 60 °C **(Agner *et al.*, 2003)**. Surprisingly, fumagillin is very stable in honey **(Furgala, 1962)** even at higher temperatures. For example it was still detectable after 35 days at 80 °C **(Assil and Sporns, 1991)**.

A. fumigatus is an ubiquitous saprophytic fungus which plays an important role in recycling environmental carbon and nitrogen but it may also be an opportunistic pathogen **(Latgé, 1999)**. *A. fumigatus* is able to produce secondary metabolites which can be harmful (e.g. gliotoxin, helvoic acid) or of medical importance (e.g. fumagillin as an antibiotic) **(Boudra and Morgavi, 2005)**. Fumagillin is produced by other fungi, e.g. *Penicillium scabrosum*, as well. *P. scabrosum* was first time described by **Frisvad *et al.* (1990)** and the species was isolated from food, feedstuff and soil samples. Among the ca. 100 different

secondary metabolites produced by *P. scabrosum*, cyclopenin, cyclopenol, and viridicarin are antibiotically active and fumagillin is antiprotozoan too **(Cole and Cox, 1981)**.

Antibiotic fumagillin was used on therapy and prevention against *N. apis* until 2002. Nowadays, in accordance with **Regulation (EC) No 1831/2003** of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, it is not possible using antibiotics based on fumagillin in Europe Union coutries anymore **(Nagy *et al.*, 2007)**.

The objective of this study was to obtain the information on the fumagillin production by a strain *P. scabrosum* on a selected synthetic medium, and to study the effect of different glucose concentrations and the cultivation conditions on the production of fumagillin, fungal biomass, and changes of pH in media.

MATERIAL AND METHODS

Fungi, media and growth conditions

A strain of *P. scabrosum*, FCB 353 (Romer Labs Division Holding GmbH, Austria) was used in the study. The strain was inoculated in a 100 µL of spore suspension (concentration of spores: 3,0 x 10^6 per mL) into 50 mL of the Glucose Mineral Salts medium (MM medium) **(Wyss *et al.*, 2001)** with 1%, 2% and 3% glucose, in duplication. Static culture conditions in Erlenmayer flasks were used. The inoculated media were incubated in darkness at 25 °C, 28 °C, and under ambient daylight at room temperature (RT, 22 ± 0.5 °C) for 7, 14, 21, 28, 35, 42 and 49 days.

HPLC analysis

A total of 0.5 mL of sample following centrifugation on Biofuge pico (Heraeus instruments, 13x1000 rpm) and after filtration throught filter (13 mm Syringe Filter 0.2 µm PTFE; VWR International, USA) was used for detection of fumagillin on the Dionex Ultimate 3000 system with 250 x 3 mm, 5 µm Luna C_{18} II column (Phenomenex, Germany). The HPLC gradient consisted of eluent A (water + 0.1% H_3PO_4) and eluent B (acetonitrile) with a flow rate of 500 µL/min: 0 min 20% B, 1.5 min 20% B, 11.5 min 90% B, 15.5 min 90% B, 15.6 min 20% B, 18.5 min 20% B. The injection volume was 20 µL. The detection

occured at 331 nm with a photo diode array detector. Fumagillin standard was purchased from Romer Labs Division Holding GmbH (Austria).

TLC analysis

The production of fumagillin was monitored by TLC (thin-layer chromatography) method, as well. One mL of each samples from liquid media was transfered into 2.0 mL Eppendorf vials and extracted with 500 µL ethylacetate. For subsequent mixing, a vortex IKA® KS 4000 ic control was used. TLC was carried out on a precoated silica gel 60 TLC plates (0.25 mm thick; Merck, Germany). The volume of extracts, applied onto plates was 50 µL per spot. A developing solvent system was toluene-ethylacetate-formic acid (TEF, 5:4:3 v/v). For visualisation of fumagillin, 1.0% 4-nitrobenzyl pyridine (NBP) (Merck, Germany) in chloroform was applied. The presence of fumagillin was detected as a blue spot that appeared after heating of TLC plate at 150 °C for 8 min, following spraying of plate with solution of tetraetylenepentamin (TEPA) (Merck, Germany) in chloroform.

Quantification of fungal biomass and pH meassuring

Mycelial dry weights were obtained by harvesting the mycelium on pre-activated (at 80 °C for 16 hours) and pre-weighed filter papers (qualitative filter papers, Grade 1288, 150 mm, 84g/m^3; Sartorius Stedim Biotech, France) after autoclavation of liquid media. The mycelium was filtrated, washed twice with distilled water, dried at 80 °C for 16 hours and weighed again. The difference between initial and final weight, was taken as dry weight of fungal biomass. For meassuring of the naturally pH of each medium before autoclaving, a pH meter (Vario set/Vario pH; WTW, Germany) was used.

RESULTS AND DISCUSSION

A. fumigatus is mentioned as a main producer of an antibiotic fumagillin. It is a widespread thermophilic and xerotolerant species, known to occur naturally on decomposing, self-heating rooting plant material and organic debris from which it releases a high number of spores into the atmosphere **(Gravesen *et al.*, 1994).** Unfortunately, this species is regarded as a pathogenic fungus causing allergic bronchopulmonary

aspergillosis (ABPA), aspergillomonas and invasive pulmonary aspergillosis **(Gravesen *et al.*, 1994; de Hoog and Guarro, 1995)**.

Table 1 Production of fumagillin by strain *P. scabrosum* on MM medium with different glucose concentrations

Medium	Temperature of cultivation	Production of fumagillin (μg.mL^{-1})						
		7th day	14th day	21st day	28th day	35th day	42nd day	49th day
MM + 1% glucose	**25 °C**	< LOD	0.07	1.40	7.84	1.64	23.85	0.13
	28 °C	< LOD	0.01	0.41	1.90	1.39	< LOD	< LOD
	RT	0.01	0.17	2.64	13.32	9.06	82.54	15.83
MM + 2% glucose	**25 °C**	< LOD	2.04	4.14	8.88	0.35	1.64	0.20
	28 °C	< LOD	< LOD	0.35	< LOD	< LOD	9.207	< LOD
	RT	< LOD	0.13	10.40	22.69	9.92	70.19	41.09
MM + 3% glucose	**25 °C**	< LOD	3.01	1.91	1.91	< LOD	< LOD	< LOD
	28 °C	< LOD	0.34	< LOD	< LOD	< LOD	< LOD	< LOD
	RT	< LOD	10.00	0.35	20.65	14.35	61.72	21.47

Legend: LOD – limit of detection, RT – room temperature (22 ± 0.5 °C), MM - Glucose Mineral Salts medium

Non-pathogenic *P. scabrosum* strain was used as an alternative producer of fumagillin in previous study from 2010 **(Barboráková *et al.*, 2010)**. MM medium was the best synthetic medium for fumagillin production by this strain. Different concentrations of glucose (1%, 2% and 3%) in synthetic MM medium were used in the current study and the influence of different glucose concentration in medium and cultivation conditions on fungal biomass, pH of media before autoclaving and production of fumagillin were studied. The production of fumagillin by *P. scabrosum* strain studied is shown in table 1.

The highest concentration of fumagillin was obtained on 14th day in medium with 3% glucose at RT (10.0 μg.mL^{-1}). The amount 10.4 μg.mL^{-1} was obtained in MM

medium with 2% glucose at RT on 21st day. Moreover, this medium and cultivation conditions were the best after 28 days of cultivation (22.69 μg.mL^{-1}). The highest concentration of fumagillin was obtained in MM medium with 3% glucose at RT (14.35 μg.mL^{-1}) on 35th day of cultivation. The best results in production of fumagillin were obtained on 42nd day of cultivation in MM medium with 1% glucose at RT (82.54 μg.mL^{-1}). In this study, the highest amount of the antibiotic in MM medium with 2% glucose at RT (41.09 μg.mL^{-1}) was obtained on 49th day of cultivation. **Boudra and Morgavi (2005)** published that fumagillin concentration produced by *A. fumigatus* strain decreased by 35% after one week of incubation and at the end of the 8-week incubation period only 10% was recovered. The average of yield of fumagillin reached 7.72 μg.mL^{-1} in MM media with 1%, 8.63 μg.mL^{-1} in media with 2% and 6.46 μg.mL^{-1} in media with 3% glucose, but the best results in production of fumagillin were obtained on 42nd day of cultivation in MM medium with 1% glucose at RT (82.54 μg.mL^{-1}). In this study, it was shown that lower concentration of glucose in MM medium is better for biosynthesis of fumagillin. The best conditions for production of this antibiotic were found to be RT (22 ± 0.5 °C) under ambient daylight (82.54 μg.mL^{-1} in medium with 1%, 70.19 μg.mL^{-1} in medium with 2% and 61.72 μg.mL^{-1} with 3% glucose). The literature deals only with production of fumagillin by *A. fumigatus* strains. **Boudra and Morgavi (2005)** reported about 25 μg.mL^{-1} from *A. fumigatus* grown in submerged fermentation. The age of non-pathogenic *P. scabrosum* strain appears to be a good alternative in production of this antibiotic.

In the *P. scabrosum* strain, maximum growth was observed in medium with 3% glucose on 14th day of cultivation at 25 °C (Tab 2). The fungal biomass decreased in nearly all media on 21st day of cultivation in comparison with 14th day. The fungal biomass decreased (78% of cases) on the 28th and 35th day of cultivation, but it again increased during last 2 weeks of cultivation (42nd and 49th day of cultivation). *P. scabrosum* strain had the highest fungal biomass on MM medium with 3% glucose. Increasing concentration of glucose in MM medium had positive impact on increasing of mycelial dry weight, but negative impact on fumagillin production. The best production of fumagillin was found only in the case of lower biomass formation (Fig 1).

Table 2 Variation of mycelial dry weight in MM media with different glucose concentrations produced by strain *Penicillium scabrosum*

Medium	Temperature of cultivation	Mycelial dry weight ($g.L^{-1}$)						
		7th day	14th day	21st day	28th day	35th day	42nd day	49th day
MM + 1% glucose	25 °C	1.7	3.6	2.6	2.2	1.4	1.8	1.5
	< LOD 28 °C	1.3	3.3	3.2	2.3	1.0	1.3	1.4
	RT	1.4	3.2	2.6	2.6	1.7	1.7	1.5
MM + 2% glucose	25 °C	1.7	5.1	3.2	3.5	2.8	2.9	2.5
	< LOD 28 °C	1.5	4.4	4.7	3.0	1.8	2.6	3.6
	RT	1.8	4.8	4.9	3.7	1.7	2.6	2.6
MM + 3% glucose	25 °C	2.0	7.0	5.0	5.3	3.4	5.5	4.0
	28 °C	2.2	4.8	4.6	4.8	4.5	5.7	3.9
	RT	2.1	6.7	5.4	4.3	2.5	4.0	3.3

Legend: RT – room temperature (22 ± 0.5 °C), MM - Glucose Mineral Salts medium

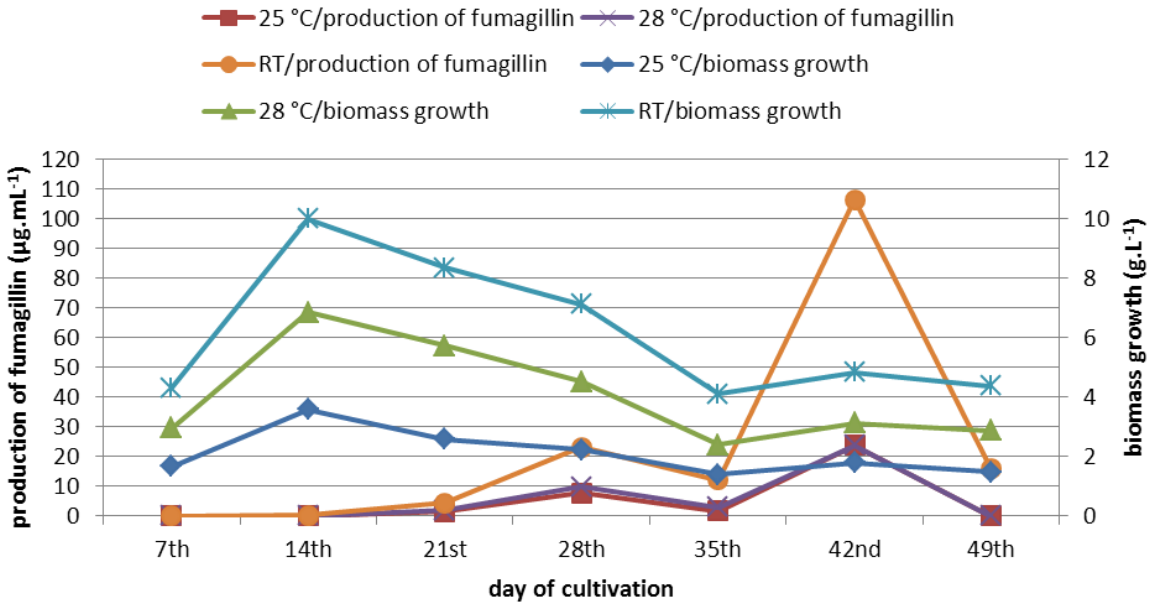

Figure 1 Dependence between production of fumagillin by *Penicillium scabrosum* and biomass growth on minimal medium with 1% glucose

The pH of media before autoclaving ranged from 4.1 (MM medium with 2% glucose, 28 °C, 14 days) to 7.6 (MM medium with2 % glucose, RT, 35 days). The media with 1% and 2% glucose showed higher pH than those with 3% glucose (Tab 3), what could be explained by the fact, that lower amounts of glucose in media resulted in decreasing of pH. The pH showed an increasing tendency between 7 to 35 days of cultivation in 81% of cases. Decreasing of pH (78%) was observed on 42nd and 49th day again increasing of pH in all MM media when compared it with that on 42nd day. The average pH in media with 1% glucose was 6.5, 6.1 in media with 2% glucose, and 6.11 in media with 3% glucose. These pH values seem to be related to the fumagillin production. Naturally declining values of pH had positive effect on the fumagillin production.

Table 3 Variation of the pH in MM media with different glucose concentrations produced by strain *Penicillium scabrosum*

Medium	Temperature of cultivation	pH						
		7th day	14th day	21th day	28th day	35th day	42th day	49th day
MM + 1 % glucose	**25 °C**	4.5	6.3	6.5	6.6	7.3	7.1	7.1
	< LOD **28 °C**	6.4	6.0	6.6	6.8	6.8	6.7	6.8
	RT	4.6	6.3	6.4	6.6	6.8	6.7	6.8
MM + 2 % glucose	**25 °C**	4.2	5.3	6.3	6.7	6.9	6.8	6.9
	< LOD **28 °C**	6.3	4.1	5.8	6.4	6.7	5.7	5.7
	RT	4.5	5.1	6.6	6.7	7.6	6.8	7.0
MM + 3 % glucose	**25 °C**	5.2	5.3	6.7	6.6	6.0	5.7	6.0
	28 °C	6.4	6.1	6.5	6.1	6.1	6.5	6.6
	RT	4.6	6.2	4.4	7.2	6.5	6.8	7.0

Legend: RT – room temperature (22 ± 0.5 C), MM - Glucose Mineral Salts medium

CONCLUSION

Based on the results obtained during this study, minimal medium containing 1% glucose and 42 days cultivation at RT was found to be the best option medium

for production of fumagillin by non-pathogenic fungus *P. scabrosum* (82.54 μg.mL^{-1}). These results can be applied directly to the biotechnology, e.g. production of reference standard for this antibiotic. Growth of fungal biomass was maximum in medium with 3% of glucose on 14th day of cultivation at 25 °C, however it negatively influenced the total yields of fumagillin. Increasing concentration of glucose in MM medium had positive impact on increasing of biomass growth, but negative impact on fumagillin production. The pH in media ranged from 4.1 to 7.6. Increasing concentration of glucose resulted in decreasing of pH, also in the production of fumagillin, as well as lower yields of fumagillin.

Acknowledgments: This study was performed in the laboratories of Romer Labs Division Holding GmbH in Tulln (Austria), where a scholarship of the author Zuzana Barboráková was realized via SAIA n.o., National scholarship programme of the Slovak republic.

REFERENCES

AGNER, G. – GYÓRBIRÓ, A. – VALKÓ, I. – VASVÁRI-DEBRECZY, L. et al. Light- and temperature-induced degradation of fumagillin. In *Acta Pharmaceutica Hungarica*, vol. 73, no. 1, 2003, p. 41-45.

ASSIL, H. I. – SPORNS, P. 1991. ELISA and HPLC methods for analysis of fumagillin and its decomposition products in honey. In *Journal of Agricultural and Food Chemistry*, vol. 39, 1991, p. 2206-2213.

BARBORÁKOVÁ, Z. – LABUDA, R. – HÄUBL, G. 2010. Production of an antibiotic fumagillin by *Penicillium scabrosum* on synthetic media. In *V. vedecká konferencia doktorandov s medzinárodnou účasťou, Nitra 26.11.2010*, 2010, p. 43-47.

BAILEY, L. 1953. Effect of fumagillin upon *Nosema apis* (Zander). In *Nature*, vol. 171, 1953, p. 212-213.

BOUDRA, H. – MORGAVI, D. P. 2005. Mycotoxin risk evaluation in feeds contaminated by *Aspergillus fumigatus*. In *Animal Feed Science and Technology*, vol. 120, 2005, p. 113-123.

COLE, R. J. – COX, R.H. 1981. Handbook of toxic fungal metabolites. In FRISVAD, J. C. 1990. A new species of *Penicillium*, *P. scabrosum*. In *Persoonia*, vol. 14, no. 2, 1990, p. 177-182.

de HOOG, G. S. – GUARRO, J. 1995. In *Atlas of Clinical Fungi*. Centraalbureau voor Schimmelcultures, Baarn and Delft, Universitat Rovira I Virgili, Reus, Spain, 1995, 720 p.

EBLE, T. E. – HANSON, F. R. 1951. Fumagillin, an antibiotic from *Aspergillus fumigatus* H-3. In *Antibiotics and Chemotherapy*, vol. 1, 1951, p. 54-58.

FEKETE, J. – ROMVÁRI, Z – SZEPESI, I. – MOROVJÁN, G. 1995. Liquid chromatographic determination of the antibiotic fumagillin in fish meat samples. In *Journal of Chromatography A*, vol. 712, 1995, p. 378-381.

FRISVAD, J. C. – SAMSON, R. A. – STOLK, A. C. 1990. A new species of Penicillium, *P. scabrosum*. In *Persoonia*, published by the Rijksherbarium, Leiden, vol. 14, no. 2, 1990, p. 177-182.

FURGALA, B. 1962. Residual fumagillin activity in sugar syrup stored by wintering honeybee colonies. In *Journal of Apicultural Research*, vol. 1, 1962, p. 35-37.

FURNESS, M. S. – ROBINSON, T. P. – EHLERS, T. et al. 2005. Antiangiogenic Agents: Studies on Fumagillin and Curcumin Analogs. In *Current Pharmaceutical Design*, vol. 11, 2005, p. 357-373.

GARRETT, E. R. 1954. Studies on the Stability of Fumagillin. III. Thermal Degradation in the Presence and Absence of Air. In *Journal of the American Pharmaceutical Association*, vol. 43, no. 9, 1954, p. 539-543.

GARRETT, E. R. – EBLE, T. E. 1954. Studies on the Stability of Fumagillin. I. Photolytic Degradation in Alcohol Solution. In *Journal of the American Pharmaceutical Association*, vol. 43, no. 7, 1954, p. 385-390.

GRAVESEN, S. – FRISVAD, J. C. – SAMSON, R. A. 1994. In *Microfungi,* Munksgaard, Copenhagen, 1995, 168 p.

HEIL, J. – REIFFERSCHEID, G. – WALDMANN, P. – LEYHAUSEN, G. – GEURTSEN, W. 1996. Genotoxicity of dental materials, In *Mutation Research*, vol. 368, p. 181-194.

KULIĆ, M. 2006. Research on genotoxic potential of dicyclohexylamine *in vivo* an *in vitro*. PhD. Thesis, Faculty of Biology, University of Banjulaka, 2006.

LATGÉ, J. P. 1999. *Aspergillus fumigatus* and aspergillosis. In *Clinical Microbiology Review*. vol 12, 1999, p. 31-350.

MAZITSCHEK, R. – HUWE, A. – GIANNIS, A. 2005. Synthesis and biological evaluation of novel fumagillin and ovalicin analogues. In *Organic and Biomolecular Chemistry*, vol. 3, 2005, p. 2150-2154.

MOLINA, J. M. – GOGUEL, L. – SARFATI, C. et al. 2000. Trial of oral fumagillin for the treatment of intestinal microsporidiosis in patients with HIV infection. In *AIDS*, vol. 14, 2000, p. 1341-1348.

McCOWEN, M. C. – CALLENDER, M. E. – LAWLIS, J. F. 1951. Fumagillin (H-3), a new antibiotic with amebicidal properties. In *Science*, vol. 113, 1951, p. 202-203.

NAGY, J. – TOPORČÁK, J. – POPELKA, P. 2007. Hodnotenie rizika výskytu rezíduí liečiv v mede. Internet: http://www.mpsr.sk/sk/index.php?navID=193&id=1034.

PICOUL, W. – BEDEL, O. – HAUDRECHY, A. – LANGLOIS, Y. 2003. Progres in fumagillin synthesis. In *Pure and Applied Chemistry*. vol. 75, 2003, p. 235-249.

REGULATION (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Internet: http://eur-law.eu/EN/Regulation-EC-1831-2003-European-Parliament-Council-22,437026,d.

TAKEDA CHEMICAL INDUSTRIES, 1983, Jpn. Patent 43 204, In *Chemical Abstracts*, vol. 98, 1983, P46950m.

TARBELL, E. S. – CARMAN, R. M. – CHAPMAN, D. D. et al. 1961. The chemistry of fumagillin. In *Journal of the American Chemical Society*, vol. 83, 1961, p. 3096-3113.

WILKINS, J. H. – JOSHI, N. – MARGOLIS, T. P. – CEVALLOS, V. – DAWSON, D. R. Microsporidial keratoconjunctivitis treated successfully with a short course of fumagillin. In *Eye*, vol. 8, 1994, p. 703-704.

WHITTINGTON, R. – WINSTON, M. L. 2003. Effects of *Nosema bobmi* and its treatment fumagillin on bumle bee (*Bombus occidentalis*) colonies. In *Journal of Invertebrate Pathology*, vol. 84, 2003, p. 54-58.

WYSS, G. S. – CHARUDATTAN, R. – DeVALERIO, J. T. 2001. Evaluation of agar and grain media for mass production of conidia of *Dactylaria higginsii*. In *Plant Disease*, vol. 85, 2001, p. 1165-1170.