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Aspergillus fumigatus branching complexity *in vitro*: 2D images and dynamic modeling

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ABSTRACT

Background: *Aspergillus fumigatus* causes serious infections in humans, and its virulence correlates with hyphal growth, branching and formation of the filamentous mycelium. The filamentous mycelium is a complex structure inconvenient for quantity analysis. In this study, we monitored the branching of *A. fumigatus* filamentous mycelium *in vitro* at different points in time in order to assess the complexity degree and develop a dynamic model for the branching complexity.

Method: We used fractal analysis of microscopic images (FAMI) to measure the fractal dimensions (*D*) of the branching complexity within 24 h of incubation.

Results: By photographing the filamentous mycelium dynamically and processing the images, the *D* variation curve of *A. fumigatus* complexity degree was obtained. We acquired the *D* variation curve which contained initial exponential period and stationary period of *A. fumigatus* branching. Further, the obtained data of *D* was modeled via the logistic model (LM) to develop a dynamic model of *A. fumigatus* branching for the prediction of the specific growth rate of branching value (0.23 h^{-1}).

Conclusions: Developed FAMI and LM models present a simple and non-destructive method of predicting the evolution of branching complexity of *A. fumigatus*. These models are useful as laboratory measurements for the prediction of hyphal and mycelium development, especially relevant to the pathogenesis study of aspergillosis, as well as pathogenesis of other diseases caused by moulds.

1. Introduction

Aspergillus fumigatus causes serious infections in humans, and its virulence correlates with the hyphal growth, branching and formation of the filamentous mycelium [1,2]. The filamentous mycelium is a complex structure inconvenient for quantity analysis. Therefore, a better understanding of the role that hyphal growth and mycelium have in pathogenesis and in disease progression is very important [3,4]. Typically, filamentous microorganisms consist of long, often branched hyphae that form extended structures of characteristic morphology called mycelia [5]. Although the hyphal extension and fungal branching constitute one of the major morphogenetic events in the development of a mycelium, these aspects have not been adequately analyzed in previous studies [6,7]. Although the number of *in vitro* studies performed on this topic is increasing, monitoring and quantification of *A. fumigatus* growth is still difficult to perform.

Mandelbrot [8] formulated fractal geometry with the aim to describe the complexity of living forms found in nature. Mycelia are amenable to fractal geometry and the fractal dimension (*D*) can be used to quantify the extent to which mycelia permeate space relative to the extent of system [9–12]. So far *D* was used to quantify the relation between mycelia morphology and growth [10,13,14]. The fractal nature of mycelia was studied [9,10,15] at two distinct levels using the measures of the surface/border *D*, however, quantification of mycelia complexity is lacking. The attraction of fractal analysis lies in its ability to quantify aspects of development of a growing fungus [10,14]. This can be achieved easily via fractal analysis of microscopic images (FAMI) that has been applied to various complex biological structures

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[10,13,16–20].

However, we lack detailed knowledge of how the complexity of mycelia evolves and varies during time, mainly because these complex structures are not entirely suitable for quantity analysis.

Therefore, the *A. fumigatus* branching progress and estimations can be useful for assessment of filamentous mycelium fungal growth strategy and quantity analysis. Therefore, the aims of this study were to: i) monitor the *A. fumigatus* branching at different points in time, *in vitro,* by using microscopic 2D images; ii) measure *D* of the *A. fumigatus* branching complexity on 2D images; iii) develop a dynamic model of *A. fumigatus* branching complexity.

2. Materials and methods

2.1. Microorganism and culture conditions

A. fumigatus (PL-12/10) was obtained from the mould collection of the National Reference Medical Mycology Laboratory (Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia) and subcultivated on Potato dextrose agar (PDA) (Oxoid, Basingstoke, United Kingdom) at 28 °C. After 72 h, a spore suspension was prepared from the surface of the formed mycelium using a sterile cotton swab and then re-suspended in 0.1% (v/v) Tween 20 using a vortex mixer. The final spore suspension (1–2.5 × 10^6 CFU/mL) was made in a Sabouraud dextrose broth (SDB) supplemented with 8% glucose (Sigma-Aldrich, St Louis, MO).

2.2. Monitoring of A. fumigatus growth in vitro

The modified procedure described by Manavathu et al. [21] was used for *in vitro* cultivation of *A. fumigatus.* Three 22 mm sterile plastic microscopic cover slips (CS) were placed in six sterile plastic Petri dishes. Aliquots (5 mL) of *A. fumigatus* spore suspension (1–2.5 × 10^6 CFU/mL) were placed in each Petri dish, completely covering the CS. During static incubation at 28 °C, *A. fumigatus* spores germinated and formed hyphae on the CS.

Six Petri dishes, each containing three CSs covered with *A. fumigatus* spore suspension were incubated at six different points in time. In six different points in time (after 19, 20, 21, 22, 23 and 24 h of incubation), each Petri dish was removed from the incubator and the CSs with the grown fungi were washed three times with sterile distilled water. The CSs were transferred to a clean sterile Petri dish and fungi stained with lactophenol cotton blue (Sigma-Aldrich, St Louis, MO) for 30 min at 37 °C. The CSs were washed three times with sterile distilled water, air-dried, placed on a microscopic slide and samples were fixed. For each point in time, images were obtained using microscope magnification of 400× (Leica), and photographed by a Nikon Camera. The number of images obtained for each point in time (number of images per Petri dish) ranged from three to six, due to uneven germination and formation of hyphae on CS. Therefore, the representative images on which the growth process is most evident were selected. A total of 36 grayscale digital images were acquired.

2.3. Image acquisition and processing

The public domain software package 'Image J' (www.rsb.info.nih.gov/ij) was used for image analysis and the image processing procedure is illustrated in Fig. 1. The first step of procedure was conversion of grayscale images (Fig. 1a) to binary (black-and-white) images using an adaptive threshold selection (Fig. 1b). At the end of image processing, binary images were converted into skeleton images (Fig. 1c, Process-Binary-Skeletonize) which reduced the initial image into single pixel lines.

2.4. Fractal analysis of A. fumigatus images

The *D* of a skeletonized image of *A. fumigatus* (Fig. 1c) was calculated using FAMI, i.e. the box-counting method in 'Image J' software [22,23]. Briefly, the box-counting method consisted of 'covering' the image outline with sets of squares (Fig. 2A). Each set is characterized by the value "*r*" of the square edge. The corresponding number of squares "*N*" that is necessary to cover the image outline, is presented as a function of *r* (Fig. 2b), while *D* is the absolute value of the slope of the log–log relationship between *N*(*r*) and *r* (Fig. 2b). In this method, the box sizes are scaled to the base of 2; that is, 2^1, 2^2 … 2^k; where *k* continues until *N* equals unity [22,23]. Depending on the image size, box-sizes were taken from 2 to 512 pixels.

2.5. Dynamic model

Dynamic of branching complexity growth of *A. fumigatus in vitro* was described empirically by the logistic model (LM):

$$\frac{dD}{dt} = \alpha D\left(1 - \frac{D}{D_m}\right) \tag{1}$$

After integration, Eq. (1) has the form

$$D = \frac{D_m}{1 + \left(\frac{D_m}{D_0} - 1\right)e^{-\alpha t}} \tag{2}$$

where *t* is *A. fumigatus* growth time and *α* is the specific growth rate of branching; D_m - the maximum value of the branching complexity; D_0 - the value of the branching complexity at zero time.

2.6. Statistical analysis

All measurements of images resulted in multiple of values for *D* for each point in time. Therefore, the multiplicate statistical analysis of the data was used to determine whether there is a statistically significant difference between *D* values for each point in time as well as the impact of the point in time has on *D* value. The image analysis data was evaluated via analysis of variance (ANOVA) using MATLABR2013a (demo version). Fisher statistic (*F*-values) was estimated at the level of significance of 0.05 ($p < 0.05$).

The LM validity was statistically evaluated using analysis of variance (ANOVA), coefficient of determination (R^2) and the mean relative percentage deviation (*MRPD*) [24]. The values of the LM parameters (*α*, D_0, and D_m) were determined using curve fitting (non-linear regression method) of Eq. (2).

3. Results

3.1. The fractal dimension images of A. fumigatus hyphal growth

Images of *A. fumigatus* growth mycelium *in vitro* monitored at different points in time are shown in Fig. 3. Growth mycelium was reflected in variations of morphology from freely dispersed hyphae to

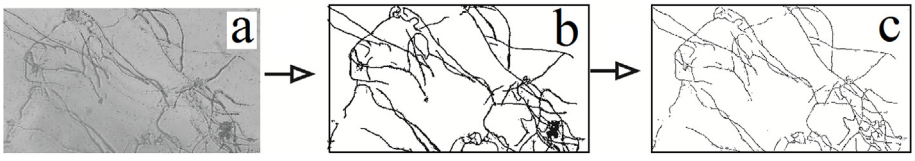

Fig. 1. Schematic representation of image processing procedure for *A. fumigatus* images: grayscale (a), binary (b), skeletonized (c) digital images.

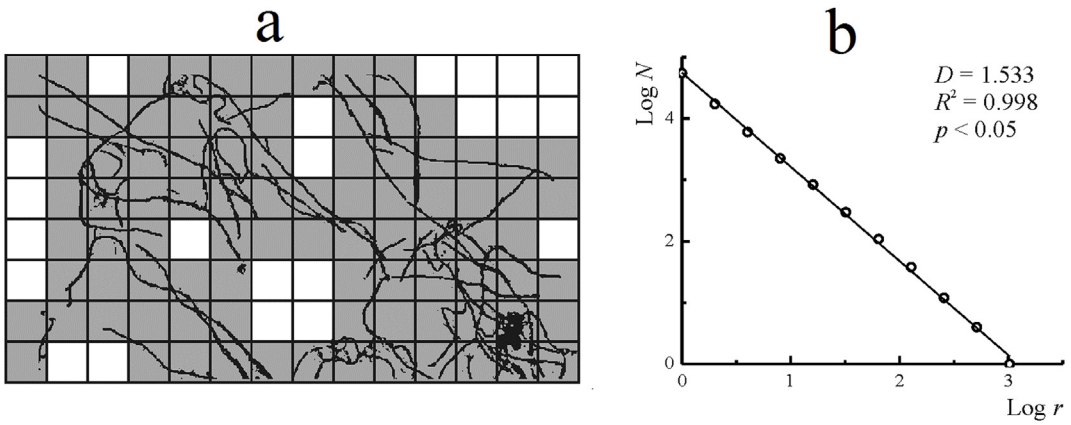

Fig. 2. Model for calculating the fractal dimension (D) of the skeletonized *A. fumigatus* image (D_B) (schematic presentation). A - squares that cover contour; B - log–log plot between the number of squares (N) and square size (r) fitted by a straight line; line slope represents D -fractal dimension, R^2 is the corresponding determination coefficient, p is significance level.

aggregated 'clumped' forms (Fig. 3).

Presented images (Fig. 3) were processed to obtain D. The results for multiplicate analysis showed no significant difference for three to six repeated measurements for each point in time (F-value was not statistically significant at the level of significance of 0.05). Additionally, it was concluded that time (t) had a significant impact on the D of *A. fumigatus* growth (F-value was statistically significant at the level of significance of 0.05).

3.2. Logistic model of fractal dimensions of A. fumigatus hyphal growth

The fractal dimension (D) data of *A. fumigatus* growth was successfully fitted by logistic model (LM), presented with Eq. (2) (Fig. 4a). The statistical significance of the model is shown in Table 1, as well as statistical parameters obtained by non-linear regression method of Eq. (2) and their values.

The statistical significance of the LM was estimated based on the difference between calculated and tabulated F value (F_{tab} = 2.21), and the p-value was less than 0.05. The LM was statistically significant at the confidence level of 95%. The R^2 values (Table 1) close to unity proved a good fit by Eq. (2). A good agreement between the predicted and actual values of the D was observed, as the *MRPD* values (± 2.5) were less than ± 10% (Fig. 4b). These results confirmed that the LM was valid for the prediction of the D, during the *A. fumigatus* growth. The estimated values of the LM parameters, the specific growth rate of the branching complexity (α) and the initial (D_0) and maximum (D_m) values of the branching complexity are shown in Table 1.

4. Discussion

In this study, it is clear that *A. fumigatus* growth is reflected in variations of the branching complexity in two-dimensional projections (Fig. 3) typical for *Aspergillus* [7,10,14]. Since D gives a measure of the degree of complexity of an object [17,18], we quantified variations of the branching complexity by measuring D.

By photographing the filamentous mycelium of *A. fumigatus* dynamically and processing the images, the variation curve of fractal dimension of complexity degree was obtained (Fig. 4). The variation curve contained the initial exponential period and a stationary period of branching complexity growth within 24 h of incubation (Fig. 4). Our research confirmed the hypothesis of Trinci [7] that after spore germination growth is initially completely integrated, but as the mycelium increases in size the degree of integration will become progressively less as parts of the mycelium become independent. These phases are merged into one and successfully mathematically described by LM (Eq. 3). Various structured models have been developed, including those using hyphal length and number of hyphal tips as morphological parameters [7]. However, the existing models were unable to explain the observed growth characteristics, therefore this method was restricted only to period of 'continuous' hyphal growth [7]. Our work was based on the assumption that *A. fumigatus* filamentous mycelium develops as a surface fractal [10] and is used to predict the value of the specific growth rate of branching (0.23 h^{-1}). Our estimated value coincided with the result of Trinci [7] who reported that the specific growth rate of hyphal length and number of hyphal tips were 0.23 and 0.25 h^{-1} for *A. nidulans*, respectively. FMAI offers promising perspectives for early detection of filamentous bulking because the morphology parameter of branching complexity respond rather fast to changing fungal growth process. The variations of D and fungi growth time were assessed previously [15,25], but a direct relationship was not presented. Yingyi and associates [14] used LM and Fractal analysis to predict fungal biomass growth, but their research was based on a macroscopic view of the mycelia-matrix. In this study, mycelia branching complexity during fungal growth was analyzed via microscopic examination and the direct relationship of D and incubation time was presented by LM. This paper aims to exploit LM parameters to predict the evolution of branching complexity, which could be used as a laboratory measurement for fungal growth prediction.

A. fumigatus has the ability to form filaments within the lungs, producing dense intertwined mycelial balls which are difficult to treat [26]. It may be more prudent to start treatment before the multicellular structure is established. Our study describes the method of exploration

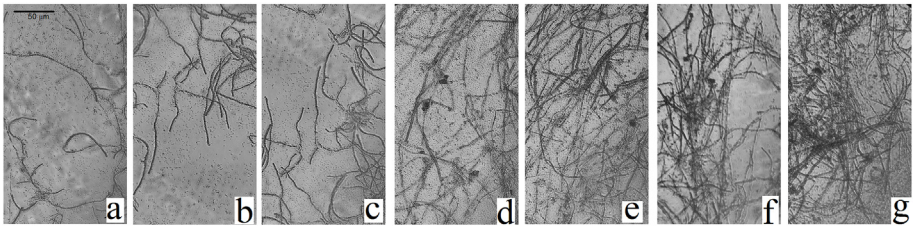

Fig. 3. *A. fumigatus* growth captured at different time points: 15 h (a), 19 h (b), 20 h (c), 21 h (d), 22 h (e), 23 h (f) and 24 h (g).

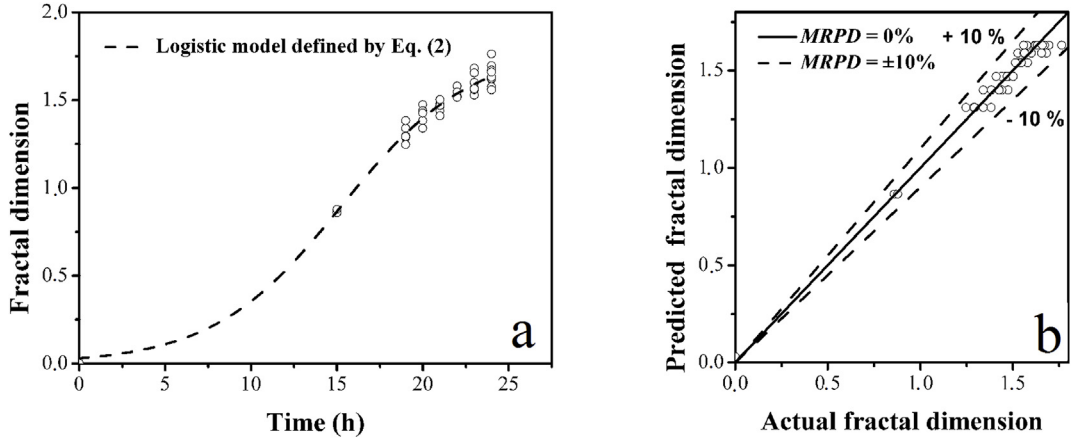

Fig. 4. Changes in fractal dimensions (D) of *A. fumigatus* images in time after inoculation (t). (a): Logistic model (LM) defined by Eq. (2); values of the model parameters: specific growth rate of the branching complexity (α) and initial (D_0) and maximum (D_m) values of the branching complexity are shown in Table 1. (b): Good agreement between actual and values predicted by LM shown with mean relative percentage deviation (*MRPD*) values less than ± 10%, indicating validity of the LM.

Table 1
Coefficients and parameters logistic model.

D_0[a]	α[a] (h^{-1})	D_m[a]	F	R^2	*MRPD* (%)
0.03 ± 0.02	0.27 ± 0.03	1.79 ± 0.05	13665[b]	0.976	± 2.5

D_0-the initial value of branching complexity; α - the specific growth rate of the branching; D_m - the maximum value of branching complexity; F - Fisher statistic; R^2 - the coefficient of determination; *MRPD* - the mean relative percentage deviation;

[a] Values ± standard error.

[b] Statistically significant at the confidence level of 95%.

of the growth characteristics of filamentous *A. fumigatus* through the development of an *in vitro* model that could be utilized to examine the antifungal susceptibility profiles before formatting multicellular structures. Aspergillosis in patients, in addition to allergic reactions and invasive forms, could be manifested as a fungus ball in existing cavities such as sinuses or lungs. The fungus ball is not an invasive form of infection, but rapid growth of moulds can cause mechanical damage by augmentation of the fungus ball, which in the case of localization in sinuses even leads to the destruction of bone tissue [2,27]. Radiological analysis (imaging analysis) do not provide sufficient data that can prove the possible fungal etiology of the determined lesions/changes in patients [28]. Monitoring of growth rate, using the methods presented here, would significantly contribute to the clarification of the etiology and can be the prediction factor in the case of a formed fungus ball.

The microbial growth can be measured in a number of different ways using mathematical analysis of images [7,14]. It is known that mould-infected humans exhibit symptoms and signs that vary in intensity depending on growth complexity, morphology of moulds and formed fungal biomass, and previous studies have suggested that reduced virulence in *A. fumigatus* is associated with a reduced growth rate [3]. Therefore, it is important to monitor, predict and control fungal growth especially in tracking of the treatment success, in order for further development of 2D images and dynamic modeling to be useful.

5. Conclusion

Our study focused on promoting the use of two mathematical models that can be valuable for pathogenesis study of aspergillosis, as well as pathogenesis of other diseases caused by moulds. The first model - FAMI was used to quantify the morphological aspects of development of *A. fumigatus* branching complexity. The second model - LM was applied to mathematical modeling of *A. fumigatus* branching and used to predict value of the specific growth rate of branching. Results observed by both models are promising, and, therefore, they can be used to measure the microbial growth as a function of the branching complexity in general, as well as to study pathogenesis of other diseases caused by moulds.

Conflicts of interest statement

None declared.

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References

[1] D.W. Denning, Invasive aspergillosis, Clin. Infect. Dis. 26 (1998) 781–803.
[2] S.R. Pekic, V.S. Arsic-Arsenijevic, M.K. Skender-Gazibara, T. Milojevic, I.P. Pendjer, M. Stojanovic, V.P. Popovic, What lurks in the sellar? Lancet 375 (2010) 432-432.
[3] D. Paisley, G.D. Robson, D.W. Denning, Correlation between in vitro growth rate and *in vivo* virulence in *Aspergillus fumigatus*, Med. Mycol. 43 (2005) 397–401.
[4] J.C. Rhodes, *Aspergillus fumigatus*: growth and virulence, Med. Mycol. 44 (2006) S77–S81.
[5] P.W. Cox, G.C. Paul, C.R. Thomas, Image analyses is of the morphology of filamentous microorganisms, Microbiology+ 144 (1998) 817–827.
[6] J.I. Prosser, A.P.J.A. Trinci, Model for hyphal growth and branching, J. Gen. Microbiol. 111 (1979) 153–164.
[7] A.P.J. Trinci, A study of the kinetics of hyphal extension and branch initiation of fungal mycelia, J. Gen. Microbiol. 81 (1974) 225–236.
[8] B.B. Mandelbrot, The Fractal Geometry of Nature, WH Freeman and Co, New York, 1983.
[9] L. Boddy, J.M. Wells, C. Culshaw, D.P. Donnelly, Fractal analysis in studies of mycelium in soil, Geoderma 88 (1999) 301–328.
[10] M. Papagianni, Quantification of the fractal nature of mycelial aggregation in *Aspergillus niger* submerged cultures, Microb. Cell Fact 5 (2006) 5–18.
[11] D.J. Barry, Quantifying the branching frequency of virtual filamentous microbes using fractal analysis, Biotechnol. Bioeng. 110 (2013) 437–447.
[12] Z. Ji, K.J. Card, F.B. Dazzo, CMEIAS JFrad: a digital computing tool to discriminate the fractal geometry of landscape architectures and spatial patterns of individual cells in microbial biofilms, Microb. Ecol. 69 (2015) 710–720.
[13] J. Blackledge, D. Barry, Morphological analysis from images of hyphal growth using a fractional dynamic model, in: H. Carr, I. Grimstead (Eds.), Theory and Practice of Computer Graphics, Warwick University, EG UK, 2011, pp. 17–24.
[14] D. Yingyi, W. Lan, C. Hongzhang, Digital image analysis and fractal based kinetic modelling for fungal biomass determination in solid-state fermentation, Biochem. Eng. J. 67 (2012) 60–67.

[15] M. Obert, P. Pfeifer, M. Sernetz, Microbial growth patterns described by fractal geometry, J. Bacteriol. 171 (1990) 1180–1185.
[16] M.R. Golinski, W.J. Boecklen, A.L. Dawe, Two-dimensional fractal growth properties of the filamentous fungus *Cryphonectria parasitica*: the effects of hypovirus infection, J. Basic Microbiol. 48 (2008) 426–429.
[17] N.T. Milošević, D. Ristanović, R. Gudović, K. Rajković, D. Marić, Application of fractal analysis to neuronal dendritic arborisation patterns of the monkey dentatenucleus, Neurosci. Lett. 425 (2007) 23–27.
[18] N.T. Milošević, D. Ristanović, H.F. Jelinek, K. Rajković, Quantitative analysis of dendritic morphology of the alpha and delta retinal ganglion cells in the rat: a cellclassification study, J. Theor. Biol. 259 (2009) 142–150.
[19] K. Rajković, D. Marić, N. Milosević, S. Jeremić, V. Arsic-Arsenijević, N. Rajković, Mathematical modeling of the neuron morphology using two dimensional images, J. Theor. Biol. 390 (2016) 80–85.
[20] X. Yang, H. Beyenal, G. Harkin, Z. Lewandowski, Quantifying biofilm structure using image analysis, J. Microbiol. Methods 9 (2003) 109–119.
[21] E.K. Manavathu, D.L. Vager, J.A. Vazquez, Development and antimicrobial susceptibility studies of in vitro monomicrobial and polymicrobial biofilm models with *Aspergillus fumigatus* and *Pseudomonas aeruginosa*, BMC Microbiol. 14 (2014) 53–68.
[22] N.T. Milošević, D. Ristanović, D. Marić, K. Rajković, Morphology and classification of large neurons in the adult human dentate nucleus: a quantitative study, Neurosci. Lett. 468 (2010) 59–63.
[23] D. Ristanović, N.T. Milošević, B.D. Stefanović, D. Marić, K. Rajković, Morphology and classification of large neurons in the adult human dentate nucleus: a qualitative and quantitative analysis of 2D images, Neurosci. Res. 67 (2010) 1–7.
[24] M. Pekmezović, K. Rajković, A. Barać, J.L. Senerović, V. Arsić-Arsenijević, Development of kinetic model for testing antifungal effect of *Thymus vulgaris* L. and *Cinnamomum cassia* L. essential oils on *Aspergillus flavus* spores and application for optimization of synergistic effect, Biochem. Eng. J. 99 (2015) 131–137.
[25] R. Lejeune, G.V. Baron, Simulation of growth of a filamentous fungus in 3 dimensions, Biotechnol. Bioeng. 53 (1997) 139–150.
[26] E. Mowat, J. Butcher, S. Lang, C. Williams, G. Ramage, Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*, J. Med. Microbiol. 56 (2007) 1205–1212.
[27] Z. Pesic, S. Otasevic, D. Mihailovic, S. Petrovic, V. Arsic-Arsenijevic, D. Stojanov, M. Petrovic, Alternaria-associated fungus ball of orbit nose and paranasal sinuses: case report of a rare clinical entity, **M**ycopathologia 180 (2015) 99–103.
[28] A.M. Barac, M.G. Pekmezovic, K.M. Rajkovic, Z. B Rakocevic, A. Janovic, Dj Bracanovic, R.S. Stosovic, V. Spiric Tomic, Mathematical modeling and image analysis: possible clinical application in practice as a predictor of fungal rhinosinusitis, Allergy 71 (2016) 426-426.