BRANCHING PATTERNS IN FUNGAL HYPHAE DURING THE COLONIZATION OF *QUERCU CERRIS* AND *QUERCUS PETRAEA* LITTER

MODELE ALE RAMIFICĂRII MICELIILOR ÎN TIMPUL COLONIZĂRII LITIEREI DE *QUERCUS CERRIS* ŞI *QUERCUS PETRAEA*

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Abstract: *Fungal mycelia are iterative and modular structures with different branching strategies according to the nature of the substratum and abundance of nutrients. Two types of experiments were developed to mimic nutrient poor and nutrient rich substrata: fragments were excised from litter leaves corresponding to substrate discontinuity and were sealed with cover slips to allow the developing mycelia to bridge the formed gap. In an alternative experiment, drops of agar malt medium were placed on leaves and incubated for 44 hours. CCD camera captured images of growing mycelia were visualized in polarized light and camera lucida drawings (scanned subsequently) were submitted to image analysis. Fractal analysis was performed on both types of images using HARFA program. The calculated fractal exponent is a good descriptor of mycelia branching and growth. In nutrient poor environment, the fractal exponent describes foraging type of mycelia branching due to explorative growth strategy (between D= 1.14 and 1.32) while in nutrient rich environment it describes the exploitative growth strategy (between D=1.62 and D=1.89)*

Rezumat: *Miceliile ciupercilor sunt structuri iterative şi modulare cu strategii diferite de ramificare în funcţie de natura substratului şi abundenţa nutrienţilor. Două tipuri de experimente s-au desfăşurat în care au fost imitate condiţiile unor substraturi sărace în nutrienţi, precum şi bogate în nutrienţi: frunze aflate în litieră au fost decupate, corespunzând unei discontinuităţi în substrat, fiind acoperite cu lamele microscopice pentru a permite dezvoltarea miceliilor care să acopere golul format. Într-un experiment alternativ, au fost plasate picături de mediu agar-malţ pe frunze care au fost incubate pentru 44 de ore. Imagini ale miceliilor în dezvoltare, preluate cu ajutorul unei camere de luat vederi din microscopie în lumină polarizată şi imagini scanate ale desenelor realizate la camera clară au fost supuse analizei. Analiza fractală a fost aplicată în cazul ambelor tipuri de imagini cu ajutorul programului HARFA. Exponentul fractal calculat este un descriptor bun al ramificării şi creşterii miceliilor. În medii cu un conţinut scăzut de nutrienţi, exponentul fractal descrie un tip exploratoriu al ramificării miceliene datorat unei strategii exploratorii(D=1.14 şi 1.32) în timp ce, în medii cu un conţinut ridicat de nutrienţi, acesta descrie o strategie de exploatare (D=1.62 şi D=1.89).*

Key words: *fungal mycelia strategies, fractals, foliar litter*
Cuvinte cheie: *strategii ale miceliilor de ciuperci, fractali, litieră foliară*

INTRODUCTION

Fungal hyphae are simple tubular structures which develop growth and branching strategies facilitating an effective exploration of surfaces and volumes representing substrata and subsequent exploitation of nutrients. The mycelia growth takes place in heterogeneous environments therefore, mycelia differentiate to form a complex interconnected network (GOODAY, 1995) having a modular and iterative nature (HARPER, 1977, ANDREWS, 1994). A modular mode of

growth in an organism is frequently coupled with the development of an enhanced structure and appears to be constructed in a regular form (BELL, 1986). Empirical studies and models were developed to describe the phenomena taking place during the colonization of natural substrata or in controlled, laboratory environment cultivation of fungi: percolation thresholds (BAYLEY et la., 2000), Lindenmayer systems (SODDELL et al., 1995), partial and non linear differential equations and stochastic models (KOTOV and RESHETNIKOV, 1990: EDELSTEIN, 1983: DAVIDSON, 2007), cellular automata (CASWELL and ETTER, 1993, HALLEY et al, 1994), fractal dimension (OBERT et al, 1990, RITZ and CRAWFORD, 1990: JONES et al, 1992: MIHAIL et al., 1994, BOLTON and BODDY, 1994: PAPAGIANI, 2006) and wavelet analysis (JONES, 1996).

The complexity of an object can be measured by the length of the shortest algorithm that it generates (KOLMOGOROV, 1968). Fractals are simple objects and consequently they represent useful developmental algorithms for modular, iterative structures such as plants or fungal mycelia. The fractal dimension D represents a measure of heterogeneity and when assigning a fractal dimension to complex objects it is assumed that a power law relationship exists between the parameters used to measure the structure (MANDELBROT, 1982: HASTINGS and SUGIHARA, 1994). In many growth processes of living organisms, regularly repeated structures are noticeable. On the conceptual level the distinctive feature of the fractal approach in plant and mycelia analysis is the emphasis on self similarity which offers a key to the understanding of complex looking compound structures suggesting a recursive developmental mechanism (PRUSINKIEWICZ and LINDENMAYER, 1990).

In terrestrial environments, the spatial distribution of nutrient resources is not uniform. The fungal mycelium represents an extremely efficient system for spatial exploration, resource capture and exploitation (RITZ and CRAWFORD, 1990). Plant litter in a detritic ecosystem such as forest is accumulating in considerable quantities. The decomposing communities dominated by fungi accomplish a fundamentally important task for the sustainable functioning of the ecosystem, the nutrient turn over. Consequently, mycelia strategies of foraging, capture and utilization of highly reluctant to decomposition compounds sequestrated in litter are of an outmost importance.

The aim of the present paper is to compare fractal behaviour of mycelia developing in nutrient poor and discontinuous environment with mycelia developing in punctual nutrient rich environment under two experimental settings conceived to mimic natural conditions occurring in the foliar litter.

MATERIALS AND METHODS.

First experiment: Litter samples of *Quercus cerris* from the first decomposition layer were collected in broad leaved mixed forest in the proximity of the city of Oradea, North-Western Romania. The leaves were repeatedly washed under tap water. Square fragments of 2×2 mm from the central area of each leaf (10 leaves were employed for the experiment) were excised and surface sterilized with hydrogen peroxide 3%. Smaller squares were subsequently removed from the excised fragments to obtain a U shaped fragment. These fragments were placed in sterile damp chambers and a cover slip was placed on each fragment. After 2 days, the cover slips were removed and placed on microscope slides in a drop of cotton blue - lactic acid dye. During a two month period sequentially, the cover slips were removed and analyzed for mycelia growth. Drawings at a camera lucida were performed during the examination of the developing mycelia under bright field microscopy. The drawings were scanned to be further processed for image based fractal analysis.

Second experiment: From the same location, litter leaves of *Quercus cerris* and *Quercus petraea* were repeatedly washed in tap water, surface sterilized with 3% hydrogen peroxide and placed in damp chambers for various time intervals (3 hours, 12 hours and 24 hours). After the incubation period allowing the initiation of fungal development from pre-existing decomposing litter community, molten malt agar drops were carefully placed on the surface of the incubated leaves. After several hours of incubation, the Petri dishes lids were removed. The desiccation of the agar drops took 2-3 hours. The peelings were placed on microscope slides in a drop of cotton - lactic acid dye and submitted to image analysis.

Polarized light microscopy is designed to observe and capture images of specimens which are visible primarily due to their anisotropic nature. It is a contrast enhancing technique that improves the quality of an image as compared to other microscopy techniques.

Image capture as performed using a CCD camera connected to a modified Zeiss microscope to incorporate polarizers. 4 positions of the same microscopic field in polarized light were digitally analyzed. Noise elements such as soil particles or trichomes were digitally cleaned. The captured images were processed in order to obtain highly contrastive images in a grey scale. For comparisons, bright-field microscopy was also employed. The calculated D values obtained from captured images using the two different types of microscopy were compared with the help of two sided, paired t test.

Captured images were analysed with HARFA Soft designed for fractal analyses (Buchniček et al., 2000). A digitized white/black image was used to calculate fractal dimension D of developing mycelia under both experiments by means of box counting method.

Box counting method is used to measure the fractal dimension of a curve by superimposing a regular grid of pixels of length δ on the object to be studied and by counting the number of occupied pixels (c) (HASTINS and SUGIHARA, 1994: KENKEL and WALKER, 1996). This procedure is repeated using different values of δ. The power-low relationship in this case is

$$C = K \cdot \delta^{-D}$$

where: D - fractal exponent of dimension, C - number of occupied pixels, δ - Length of the unit box, K - constant.

Box counting method is considered the most appropriate approach for measuring fractal dimension in filamentous type of growth (SODDELL and SEVIOUR, 1995).

Because there are frequently re-orientation of the grid that produce different values of c, grid placement replications are needed in order to obtain a distribution of D values. In the present study the employed δ were of 2, 4 and 6 pixels.

The method is sensitive to the range of box length δ used, thus the uppermost and lowermost limits must be set according to smallest digitally reproduced detail of the object and the whole scanned object. A supplementary graphical analysis was performed on the functional relationship between $\log D_f = f(\log 1/2)$ for every digitized image for $\delta = 2, \delta = 4, \delta = 6$ pixels. A small slope, almost parallel to the abscissa that contains the three points represents a normal situation. In case of a fractal object the slope approaches 0 and the correlation coefficient,

1. A high coefficient of determination R^2, near 0.995 indicates fractal behaviour of the analyzed object.

However, one limitation of this method resides in the fact that it is impossible to separate inactive, moribund hyphae from active growing, fractal dimension reflecting in this particular case the overall distribution of mycelia.

RESULTS AND DISCUSSION

First experiment: The incubation of the excised leaf fragments in damp chambers stimulated the development of fungal colonies and under the conditions of the first set of experiments, permitted also the sporulation and consequently the identification of the fungal species:

Alternaria alternata (Fr.) Keissler, *Arthrobothrys robusta* Duddington, *Aureobasidium pullulans* (de Bary) G. Arnaud, *Cladosporium cladosporioides* (Fr.) de Vries, *Epiccocum purpurascens* Ehrenb. Schlecht., *Fusarium oxysporum* Schleht, *Harzia acremonioides* (Harz.)Gost., *Helicosporium roseum* (Bon.) Saccardo, *Microyhyrium illicinum* De Not., *Monacrosporium sp.*, *Monochaetia dimorphospora* Yokahama, *Myrothecium roridum* Tode ex Fr. *Polyscitalum foecundissimum* Riess. These species are common pioneer members of the fungal litter communities on *Quercus spp*. leaves (Fodor, 1996).

Table 1

Mean fractal exponents D ± SD of growing mycelia on *Quercus cerris* leaf litter fragments, calculated by box/counting method, HARFA program.

Average fractal exponents (D)	SD
1.3206	0.0110
1.2130	0.0811
1.1476	0.0585
1.2106	0.0498
1.2482	0.0625
1.1575	0.0965
1.1809	0.0514
1.1466	0.0984
1.2011	0.0504
1.1636	0.1129

Mycelia growths developing on the cover slips reproducing naturally occurring nutrient gaps, were assigned to four morphological groups:

1. hyaline or pigmented crossing hyphae with rapid extension of the main axis and rare branching at right angles, also frequent scalariform anastomoses and hyphal bundles consisting of 2-3 coiling hyphae (Fig. 1, A),
2. mycelia of a restricted growth pattern, confined to the edges of the cover slip and frequently associated with hyphae of the first growth pattern type,
3. mycelia of restricted growth coiling around the leading hyphae of the first type and rapid sporulation,

4. multipolar mycelia growth pattern, restricted, initiating a rapid sporulation.

Ten drawings representing the four type of mycelia growth were subjected to fractal analysis using the box-counting method. The resulted fractal exponents are presented below (table 1).

The results of the second experiment, the estimation of fractal dimension of developing mycelia of malt-agar drops, as observed in polarized light and bright field microscopy are presented below (table 2).

Table 2.

Mean fractal exponents D ± SD of developing mycelia in agar drops placed on *Quercus petraea* litter leaves, in polarized light and bright-field microscopy.

Specimen/fields within drops	**D (polarized light)**	**SD**	**D (bright field)**	**SD**
Specimen 1/1	1.7795	0.0066		
Specimen 1/2	1.8123	0.0100		
Specimen 1/3	1.7862	0.0079		
Specimen 1/4	1.8210	0.0051		
Specimen 1/5	1.7758	0.0166		
Specimen 2/1	1.8788	0.0023		
Specimen 2/2	1.8327	0.0065		
Specimen 2/3	1.7039	0.0069		
Specimen 2/4	1.8303	0.0108		
Specimen 3/1	1.6683	0.0493	1.7630	0.0255
Specimen 3/2	1.7076	0.0342	1.8338	0.0199
Specimen 3/3	1.6615	0.0959	1.7490	0.0237
Specimen 3/4	1.6754	0.0147	1.8137	0.0212
Specimen 3/5	1.6857	0.0215	1.7980	0.0190

The paired, two sided t test comparing D values obtained under bright field and polarized microscopy showed an extremely significant difference at $P \leq 0.001$. The frequency distribution of the observed fractal exponents was approximately normal, the kurtosis (the curve is platykurtic) being a consequence of the low number of analyzed cases, only 68.

The coefficient of determination R^2 was high (around 0.995) in all cases confirming the fractal nature of the developing mycelia and the model represented by a power law.

As foraging theory stresses, foraging strategies support the maximization of the net production (COLINVAUX, 1986). Foraging in fungi permits the reallocation of mycelia biomass in a patchy environment such as foliar litter and is considered to be a highly adapted and selected network that can illuminate fundamental principles applicable to other supply networks (WATKINSON et al., 2005). Particularly effective foragers, able to cover the forest floor are basidiomycetous mycelia of cord and rhizomorph forming fungi (BODDY, 1993). Our experiments were conducted on micromycetes, mainly pioneer species displaying r survival strategy, short range dispersers, initiating sporulation soon after local exhaustion of the nutrients. Low D values under the conditions of the first experiment correspond to the exploratory strategy of the developing mycelia while the high values of D under the conditions of the second experiment that mimics nutrient rich substrate correspond to exploitative strategy of the mycelia growth.

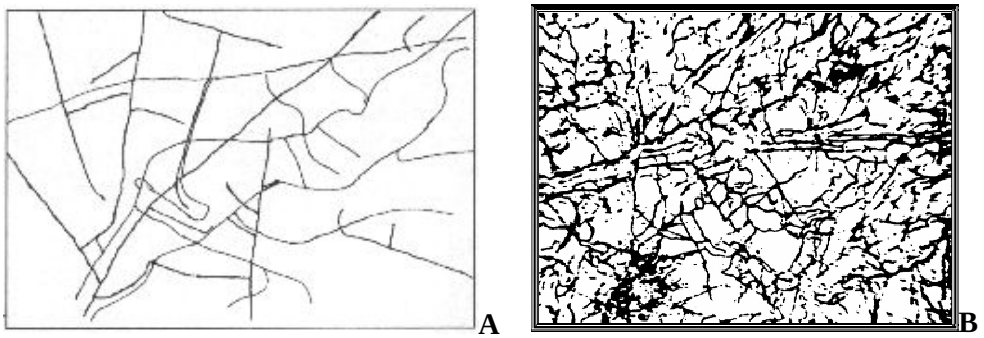

Figure 1. A. Mycelia developing on a cover slip (first experiment, see text) and **B.** Mycelia visualized in polarized light microscopy (second experiment, se text), *Quercus cerris* and *Quercus petraea* foliar litter.

Under the first experiment setting, mycelia were forced to cross the gap in the nutrient pool represented by the cover slip, consequently the growth was apically dominated, with leading hyphae and scarce branching unlike mycelia growing on culture media characterized by multipolar, uniform growth. The hyphal tip is an invasive, migratory structure (MOORE et al., 2005). Low values of the calculated fractal exponents confirm the exploratory strategy of mycelia growing at the edge of the cover slips. Branching is a strategy for the colonization of a maximal area using a minimum total mycelia length (GULL, 1975). Under the conditions of the second experiment the increase in the value of fractal exponents corresponds to intense branching due to the abundance of nutrients supplemented by malt-agar drops as well as a multidirectional growth of a rather sparse network of hyphae. Lower fractal exponents correspond to specimens allowed to develop in malt-agar drops for short time while higher values correspond to a longer exposure of developing mycelia to the nutrient pool. The situation is different compared with a standard multipolar growth provided by cultivation on agar media from initial central inoculum. The values of fractal exponents are even higher, close to 1.94 as in cultivation experiments conducted by other authors show (OBERT et al., 1990). Apparently, under natural conditions, the ideal nutrient supply provided by culture media together with uniform distribution of nutrients and uniform temperature plus humidity do not exist and mycelia of micromycetes are developing in manner closer to the conditions provided by our second experiment, that is in punctual rich environments. Fractal growth therefore maximizes nutrient capture by fungal colonies optimizing balance between exploratory and exploitative modes of growth (RITZ and CRAWFORD, 1990). To cross a gap, hyphae grow by apical domination, scarce branching together with the tendency to form bundles of 2 and 3 mono-specific or pluri-specific hyphae are consistent with the exploratory growth strategy. Hyphal systems are laterally consolidated by hyphal anastomoses in a scalariform manner permitting the translocation of metabolites and giving a positional information to the whole mycelium.

It is worth to mention that under different experimental settings, such as cultivation of fungal mycelia in submerged cultures (CLASSEN et al., 1996), the same pattern of fractal dimension is described: the increase from the early stages (low values of the fractal exponent, 1.334) to mature stages of colony growth (high values of the fractal exponent, 1.854).

The pluri-specific bundles of hyphae visualized on cover slips suggest a facilitation inter-specific relationship leading to the idea that most rapidly extending hypha plays a foretic role for slower partners, a hypothesis to be more attentively verified..

The differences in D values between bright-field microscopy and polarized light microscopy indicate that for the inflation of bright field values are responsible the lower quality of contrast and the background noise produced by other structures than hyphae. Polarized light is a better environment for the visualization of mycelia. The confirmation comes also from the significant difference between results in polarized and bright field microscopy as t test proved.

The value of camera lucida drawings, a classical graphical method is still high because of the clarity, being devised for reproducing sparse mycelia. The scanned drawings are easy to analyze using various programs. The combination of several methods gave relatively reliable results in our experiments.

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