Short Communication

Two-dimensional fractal growth properties of the filamentous fungus *Cryphonectria parasitica*: the effects of hypovirus infection

Michael R. Golinski[1,2], William J. Boecklen[1] and Angus L. Dawe[1,3]

[1] Department of Biology, New Mexico State University, Las Cruces, NM
[2] Antiviral Research Center, University of California, San Diego, CA
[3] Molecular Biology Program, New Mexico State University, Las Cruces, NM

Whole-colony two-dimensional fractal growth patterns produced by hypovirus-infected *Cryphonectria parasitica* (EP155/CHV1-EP713) were measured and compared with those produced by the isogenic virus-free strain (EP155) on solid medium. We have quantified statistically significant differences in the rates of expansion and spatial dynamics of colony growth between the two strains and concluded that fractal dimension is affected by the presence of the hypovirus. Therefore, fractal dimension measurement is an effective quantitative tool for testing the effects of mycovirus infection on fungal growth parameters.

Keywords: Fungal colony growth / Hypovirulence / Mycovirus

Received: January 19, 2008; accepted March 27, 2008

DOI 10.1002/jobm.200800017

Introduction

The ability of fungal hyphae to penetrate the growth medium and the interconnected nature of a mycelial colony form that permits translocation of nutrients from one zone of growth to another is a crucial advantage in the exploitation of resources [1, 2]. The description of a fungal mycelium in terms of its two-dimensional fractal properties has been successfully employed using solid medium [3] and can be expanded to model the growth of fungi in liquid culture [4, 5]. Analyses of the geometric structure of the apical zone of laboratory isolates of different species of filamentous fungi suggested that change in colony radius over time is associated with the fractal growth of hyphae [6]. These studies further concluded that as the colony radius of the fungus increased, the fractal dimension of hyphae changes, so that the mycelium can optimize space utilization and nutrient assimilation [7]. Such studies have been expanded recently to include the modeling of behavior under a variety of nutritional and other environmental changes [8] with the aim of developing tools for better prediction of mycelial growth in both natural and industrial applications.

The organism used in this study, *Cryphonectria parasitica*, is the agent responsible for the near-elimination of the American chestnut, *Castanea dentata*, from its natural range across the eastern United States. This fungus can be infected by a mycovirus that induces characteristic alterations in phenotype that include reduced virulence, reduced asexual sporulation and changes in colony growth rate (reviewed by [9]). Although associated with all major classes of fungi [10], the mycoviruses of the family *Hypoviridae* that infect the chestnut blight fungus *C. parasitica* represent the only viral agents for this entire host kingdom for which infectious cDNA clones have been developed [11–13]. Changes in colony expansion rate and morphology have been observed in hypovirus-infected mycelium of *C. parasitica*, but they have only been quantified by simple analytical methods such as radial growth measurement and biomass production [14, 15]. Since spatial dynamics relate to fungal mycelium spread and are determined by the rate of colony expansion [16], the goal of this study was to apply a quantitative measurement to whole-colony morphological properties of *C. parasitica* and determine if such a measure is impacted by hypovirus infection.

Correspondence: Angus L. Dawe, Department of Biology, MSC-3AF, PO Box 30001, New Mexico State University, Las Cruces, NM, 88003
E-mail: dawe@nmsu.edu
Phone: (575) 646-4003
Fax: (575) 646-5665

Materials and methods

C. parasitica strain EP155 (ATCC 38755) and the isogenic strain EP155/CHV1-EP713 (ATCC 52571) infected with the prototypic hypovirus CHV1-EP713 [12] were each maintained on solid medium (3.9% wt/vol Difco potato dextrose agar [PDA, Becton Dickinson]) at a temperature between 22 and 25 °C with a 12 h light/dark cycle at 1300–1600 l×. Inoculations for measurements used a single plug (~3 mm each side) cut from the periphery of an actively growing culture and placed in the center of the growth medium in 150 mm × 15 mm Petri plates (Fisher Scientific). Colony expansion (mm d^{-1}) was quantified by measuring the diameter at twenty-four hourly increments between 96 and 264 h post-inoculation. Data were collected from 14 replicates of EP155/CHV1-EP713 and 12 replicates of EP155. The diameter of each colony at each time point was determined as the mean of the furthest extent of hyphal growth in two perpendicular cross-sections. For photography, each culture was placed on a uniform white background, and images (3.2 megapixel) were acquired using 32 W transmitted light, a Compact VHS camcorder (model GR-AXM910) and Systemax PC. Data were then digitized by ATI video player software© and saved as a 24-pixel bitmap. Two-dimensional fractal dimensions were calculated by BENOIT™ 1.3 software (Trusoft International, Inc., St. Petersburg, FL) using box dimension analysis [17]. The box dimension is defined as the exponent value *D*2 in the relationship $N(d) \approx 1/d^{D2}$ where $N(d)$ is the number of boxes of linear size d, necessary to cover a data set of points distributed in a two-dimensional plane. In this study, the data set in question is the captured two-dimensional image of the fungal colony. The *D*2 value can range from 1 to 2 and quantitatively describes the complexity of fungal colony shape distributed over the growth medium [18], where a larger number indicates a more variable perimeter due to a reduced hyphal density. However, one of the most important limitations of the *D*2 assay is that similar values do not imply similar colony shapes, although measurement of fractal dimension does provide a relative ordering of complexity of related objects [19]. To measure *D*2, the number of boxes of linear size d necessary to cover the set for a range of values of d were counted and the logarithm of $N(d)$ plotted on the ordinate versus the logarithm of d on the abscissa. If the object is fractal, this plot will follow a straight line with a negative slope that equals $-D2$. One-factor, repeated-measures analysis of variance, with infection state as the main effect and time as the repeated measure, was used to determine whether hypovirus infection affected rates of colony expansion and *D*2.

Results and discussion

Colonies of EP155/CHV1-EP713 (n = 14) were found to be surface fractals with two-dimensional fractal dimensions in the range 1.7410–1.8120, while fractal dimensions for colonies of EP155 (n = 12) were in the range 1.5986–1.7168, as described in Fig. 1. The notches in the boxplot do not overlap, therefore it can be concluded with 95% confidence that the medians of each range did differ. The range of *D*2 for EP155/CHV1-EP713 colonies, when compared to the lower values of *D*2 for uninfected EP155, indicated that hypovirus infected colonies are composed of less densely packed hyphae at the colony edge than uninfected mycelium. These values are consistent with less accurately quantified trends of *C. parasitica* growth (reviewed by [9]) and also the reduced biomass obtained from cultures of infected

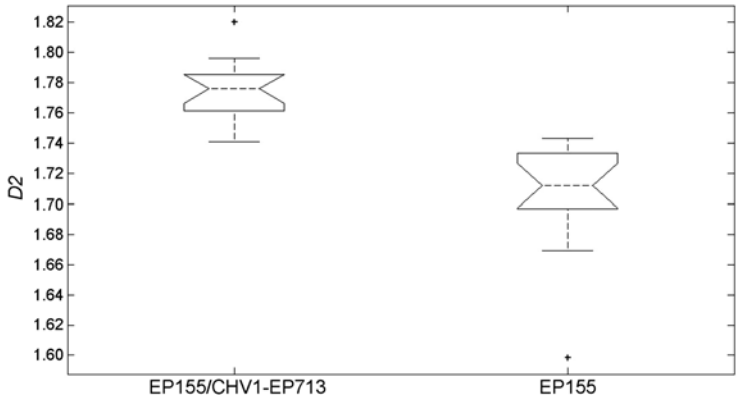

Figure 1. The ranges of *D*2 values measured from imaged colonies of the isogenic virus-infected and virus-free strains. The difference between the medians of the two groups is approximately 0.06. The length of the whiskers is approximately 0.06 times the interquartile range. Points beyond the whiskers are indicated by **+**.

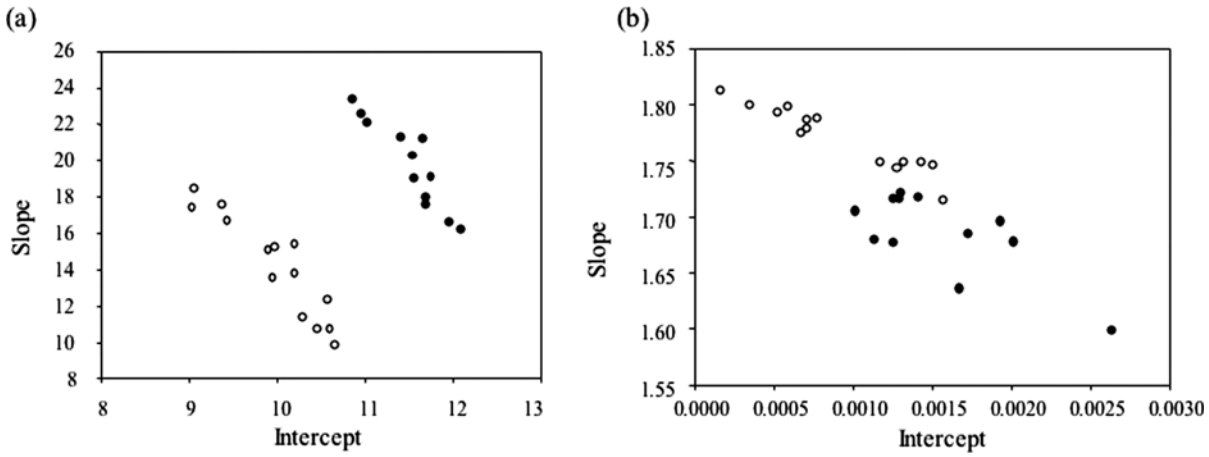

Figure 2. Slope vs. intercept values generated from repeated-measures analysis of variance. In both panels, filled circles represent values for EP155 and open circles for EP155/CHV1-EP713. (a) Colony expansion rates for EP155/CHV1-EP713 and EP155 ($p < 0.001$) do not differ significantly within samples of the same strain, but do differ between strains. (b) A correlation between rates of colony expansion and *D*2 value for EP155/CHV1-EP713 and EP155 ($p < 0.001$).

versus uninfected mycelium when harvested from cellophane-covered solid medium (Dawe laboratory, unpublished observations).

One-factor, repeated-measures analysis of variance showed that rates of colony expansion (mm d^{-1}) among replicates of the same strain (hypovirus infected or uninfected) were not significantly different. However, there was a significant difference ($p < 0.001$) between the rates of colony expansion between the infected and uninfected strains, as exhibited by the separate data groupings plotted in Fig. 2a. Statistical analysis also showed that the rate of colony expansion in each case was also a strong predictor of the value of *D*2 and that this relationship differed significantly ($p < 0.001$) between EP155/CHV1-EP713 and EP155 colonies (Fig. 2b).

This study has confirmed that colony expansion of EP155/CHV1-EP713 mycelium was significantly reduced when compared to uninfected EP155. We have established that both EP155/CHV1-EP713 and EP155 fungal colonies growing on a solid substrate developed colonies with surface fractal properties in the course of spreading to their surroundings. Our data identifies a statistical difference in fractal growth between mycovirus infected and uninfected colonies, which indicates quantitative differences in complexity of the colony margin that are induced by the hypovirus infection. Specifically, these differences between EP155 and EP155/CHV1-EP713 suggested a decreased hyphal density in the presence of hypovirus that is maintained throughout the growth of the colony. The correlation of reduced hyphal density to the previously observed reduced growth rate indicates that a reduction in colony expansion in the virus-infected strain may be due to a reduced ability to grow more densely and assimilate nutrients in an efficient manner. Such an alteration of growth pattern may represent a key feature that is reflected in reduced virulence of CHV1-EP713-infected mycelium on the host plant [12]. This growth pattern change is likely the result of physiological alterations induced by the large transcriptional differences between virus-free and virus-infected *C. parasitica* that have been observed using spotted cDNA microarrays [20]. These results further suggest that measurement of fractal dimension is an effective tool that expands the available quantitative methods for testing the impact of mycovirus infection on the growth of filamentous fungal colonies. This complements the availability of genetic and genomic tools for *C. parasitica* and infectious cDNA clones of three different members of the *Hypoviridae* family [11–13] facilitating further studies of specific phenotypic determinants through measurement of mycelial distribution.

Acknowledgements

The authors thank Victor Candelaria for technical assistance. This work was supported in part by grants from the National Science Foundation (DEB-0129630 to WJB and MCB-0718735 to ALD).

References

[1] Rayner, A.D.M., 1996. Interconnectedness and indiviualism in fungal mycelia. In: B.C. Sutton (ed.), A Century of Mycology, pp. 193–232. Univ. Cambridge Press, Cambridge.

[2] Rayner, A.D.M., Watkins, Z.R. and Beeching, J.R., 1999. Self-integration – an emerging concept from the fungal mycelium. In: N.A.R. Gow, G.D. Robson, G.M. Gadd (eds.), The Fungal Colony, p. 1–24. Cambridge University Press, Cambridge.

[3] Obert, M., Pfeifer, P. and Sernetz, M., 1990. Microbial growth patterns described by fractal geometry. J. Bacteriol., **172**, 1180–1185.

[4] Papagianni, M. and Papamichael, E.M., 2007. Modeling growth, substrate consumption and product formation of *Penicillium nalgiovense* grown on meat simulation medium in submerged batch culture. J. Ind. Microbiol. Biotechnol., **34**, 225–231.

[5] Papagianni, M., 2006. Quantification of the fractal nature of mycelial aggregation in *Aspergillus niger* submerged cultures. Microb. Cell Fact., **5**, 5.

[6] Rayner, A., Ramsdale, M. and Watkins, Z.R., 1995. Origins and significance of genetic and epigenetic instability in mycelial systems. Can. J. Bot., **73**, S1241–S1248.

[7] Bolton, R.G. and Boddy, L., 1993. Characterization of the spatial aspects of foraging mycelial cord systems using fractal geometry. Mycol. Res., **97**, 762–768.

[8] Boswell, G.P., Jacobs, H., Davidson, F.A., Gadd, G.M. and Ritz, K., 2003. Growth and function of fungal mycelia in heterogeneous environments. Bull. Math. Biol., **65**, 447–477.

[9] Dawe, A.L. and Nuss, D.L., 2001. Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. Annu. Rev. Genet., **35**, 1–29.

[10] Buck, K., 1986. Fungal Virology – An Overview. CRC Press, Boca Raton.

[11] Chen, B., Choi, G.H. and Nuss, D.L., 1994. Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science, **264**, 1762–1764.

[12] Choi, G.H. and Nuss, D.L., 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science, **257**, 800–803.

[13] Lin, H., Lan, X., Liao, H., Parsley, T.B., Nuss, D.L. and Chen, B., 2007. Genome sequence, full-length infectious cDNA clone, and mapping of viral double-stranded RNA accumulation determinant of hypovirus CHV1-EP721. J. Virol., **81**, 1813–1820.

[14] Chen, B., Geletka, L.M. and Nuss, D.L., 2000. Using chimeric hypoviruses to fine-tune the interaction between a pathogenic fungus and its plant host. J. Virol., **74**, 7562–7567.

[15] Hillman, B.I., Shapira, R. and Nuss, D.L., 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. Phytopathology, **80**, 950–956.

[16] Gilligan, C.A. and Bailey, D.J., 1997. Components of pathozone behaviour. New Phytol., **136**, 343–358.

[17] Kenkel, N.C. and Walker, D.J., 1996. Fractals in the biological sciences. Coenoses, **11**, 77–100.

[18] Ritchie, M.E. and Olff, H., 1999. Spatial scaling laws yield a synthetic theory of biodiversity. Nature, **400**, 557–560.

[19] Corbit, J.D. and Garbary, D.J., 1995. Fractal dimension as a quantitative measure of complexity in plant development. Proc. R. Soc. Lond., B, Biol. Sci., **262**, 1–6.

[20] Allen, T.D., Dawe, A.L. and Nuss, D.L., 2003. Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonetria parasitica* to infection by virulence-attenuating hypoviruses. Euk. Cell, **2**, 1253–1265.

