Laser nephelometry applied in an automated microplate system to study filamentous fungus growth

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Reports

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By contrast with photometry (i.e., the measurement of light transmitted through a particle suspension), nephelometry is a direct method of measuring light scattered by particles in suspension. Since the scattered light intensity is directly proportional to the suspended particle concentration, nephelometry is a promising method for recording microbial growth and especially for studying filamentous fungi, which cannot be efficiently investigated through spectrophotometric assays. We describe herein for the first time a filamentous fungi–tailored procedure based on microscale liquid cultivation and automated nephelometric recording of growth, followed by extraction of relevant variables (lag time and growth rate) from the obtained growth curves. This microplate reader technique is applicable for the evaluation of antifungal activity and for large-scale phenotypic profiling.

Introduction

Since the yeast *Saccharomyces cerevisiae* was sequenced in 1996 (1), the number of available fungal genome sequences has increased by one order of magnitude. Over 100 complete fungal genomes have been publicly released or are currently being sequenced, thus representing the widest sampling of genomes from any eukaryotic kingdom (2). This fungal genomics boom has greatly expanded our view of the genetic and physiological diversity of these organisms. However, the function of many proteins encoded by the genome has not yet been experimentally determined. Targeted inactivation of the corresponding genes and subsequent phenotypic characterization of resulting mutants are frequently the first important step toward determining the cellular role of proteins. Deletion strain collections of all genes in the genome are now available for model organisms such as *S. cerevisiae* (EUROSCARF, Frankfurt, Germany) and the filamentous ascomycete *Neurospora crassa* (Fungal Genetics Stock Center, Kansas City, MO, USA). These initiatives have paved the way to the development of phenomics: large-scale quantitative phenotypic analysis of genotypes on a genome-wide scale (3). Procedures based on microscale liquid cultivation and optical recording of growth in automated microplate systems have been developed for high-throughput quantitative phenotypic profiling of yeasts (4,5). Concerning filamentous fungi, assays based on the analysis of colony expansion rates on solid media or on microscopic measurement of the length of hyphae can generate quantitative phenotypic data. However, such screenings are cumbersome and time-consuming, and therefore not suitable for large-scale phenotypic analysis. Moreover, the specific effects of a particular mutation and/or environment on different fungal growth features (such as the lag phase, rate of growth, and growth yield) cannot be deduced from data obtained on solid media. Despite the fact that they are theoretically only applicable for unicellular organisms (6), spectrophotometric assays have also been developed to monitor the growth of filamentous fungi in microbroth systems over time (7,8). However, the accuracy of spectrophotometric readings may be hampered by the presence of clumps of hyphae and the nonhomogeneous growth of filamentous fungi in liquid media. Moreover, a major drawback of spectrophotometric methods, irrespective of the kind of cells under analysis, is that extinction in cell suspensions is proportional to the cell density only at rather low values. Usually, it is considered that this lack of proportionality is significant at OD values >0.5 and that only corrected photometric readings should be used for values beyond the range of proportionality (7,9,10). In contrast to spectrophotometry, which measures the transmission of light, nephelometry (another light-based technique for measuring medium opacity) uses light scattering. This parameter is directly proportional to the cell density and is suitable for a broad range of cell densities. Since its first description for counting yeast suspensions (11), nephelometry has been routinely used to study the growth of *S. cerevisiae*, and a microbroth kinetic system was recently developed to monitor the growth of some *Candida* yeast species (12,13). As it has also been successfully used for quantifying particles in nonhomogeneous suspensions, we assumed that nephelometry might be suitable for plotting accurate growth curves for filamentous

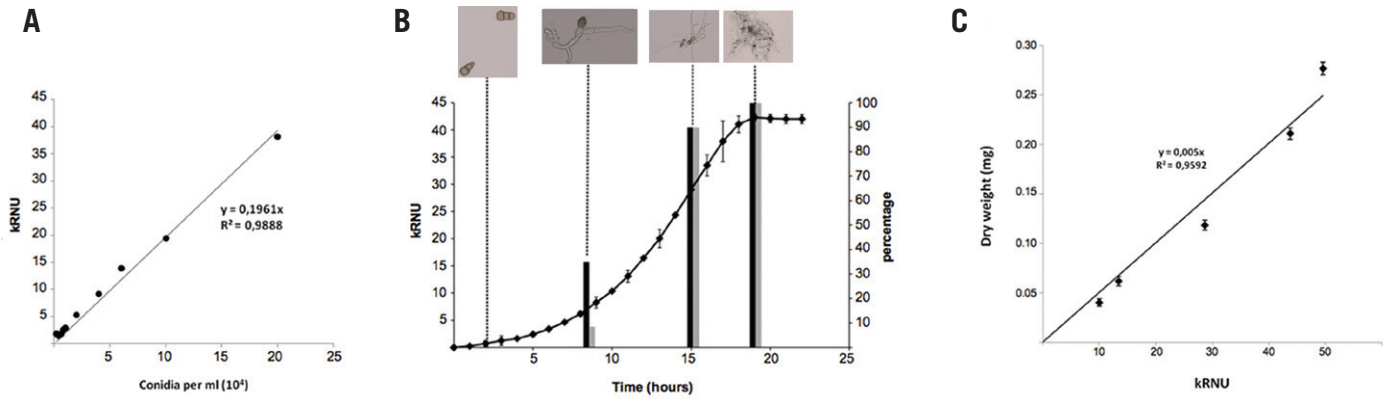

Figure 1. Use of laser nephelometry for fungal propagule determination and growth curve monitoring. (A) Correlation between the number of conidia of *A. brassicicola* and RNU values. RNU values were obtained from serial dilutions of conidia. Conidia were harvested in sterile water from PD agar plates and conidial densities were determined microscopically using a Thoma's counting chamber. Conidial suspensions were then diluted to obtain the desired concentrations (600–60,000 conidia/well). RNU measurements were done immediately without incubation for each calibrated suspension. (B) Nephelometric growth curve of *A. brassicicola* in PDB. The initial suspension was adjusted to 10^5 conidia/mL and the nephelometric values were automatically recorded for each well every hour. The microscopic morphology was examined at different time points and the percentage of conidia developing hyphae (black column) or branching (gray column) was estimated in duplicate at 2, 8, 15, and 19 h. (C) Regression curves of kRNU versus dry weight for *A. brassicicola*. The mycelial mass was collected at different time points of the growth curve and dry weight values were determined for the total biomass from 12 wells. Error bars, SD.

fungi. In this study, we took advantage of the commercial availability of a laser-based microplate nephelometer to develop a method based on microscale liquid cultivation for automated recording of fungal growth. This procedure was evaluated with phytopathogenic (*Alternaria brassicicola*) or human pathogenic (*Aspergillus fumigatus* and *A. terreus*) filamentous fungi and the yeast *Candida glabrata*.

Materials and methods

Fungal strains and cultivation media

The *A. brassicicola* wild-type strain *Abra43* used in this study was isolated from *Raphanus sativus* seeds. Two disruptants—*Abhog1Δ1* and *Abnik1Δ3*—were obtained from the wild-type strain *Abra43* as previously described (14). Strains of *A. terreus* and *C. glabrata* were recovered from clinical samples (15) and deposited at the Institute of Hygiene and Epidemiology Mycology section (IHEM; Brussels, Belgium) culture collection. The reference strain 18963 of *A. fumigatus,* used for sequencing the genome of this species, was obtained from IHEM. *A. brassicicola* and *Aspergillus* strains were cultivated at 24°C on potato dextrose (PD) agar medium (Cat. no. 213200; Becton Dickinson, Franklin Lakes, NJ, USA). *C. glabrata* strains were grown at 30°C on yeast peptone dextrose (YPD) (Cat. no. Y1500, Sigma-Aldrich, St. Louis, MO, USA) agar.

Growth curve measurements

For inoculum preparation, conidia of filamentous fungi were collected from 8-day-old solid cultures by adding PD broth followed by gentle scraping of the agar plates. They were then counted in a Thoma's chamber and the conidial suspensions were diluted to obtain the desired concentrations. Overnight precultures of *C. glabrata* isolates were harvested by centrifugation (2000× *g*), washed in sterile water, and inoculated to OD 0.10–0.15 in fresh YPD medium. The microplate wells were filled with calibrated suspensions (300 μL/well).

Growth was automatically recorded for ≥30 h at 25°C for filamentous fungi and at 30°C for yeast using a nephelometric reader (NEPHELOstar Galaxy, BMG Labtech, Offenburg, Germany), equipped with a 635-nm laser as radiation source. During incubation, the 96-well plates were subjected to shaking at 175 rpm for 5 min every 10 min. Measurements were done every hour with a gain value of 90 and a percentage of the maximum value of 20%. Each well was measured for 0.1 s with a laser beam focus of 2.5 mm.

Analysis of growth data

Data were exported from Nephelostar Galaxy software in ASCII format and further processed in Microsoft Excel 2008 (Version 12.2.4; Redmond, WA, USA) and R 2.6.1 (http://cran.r-project.org). All data presented herein was obtained from two independent biological repetitions. Each repetition included three technical replicates. Both lag time and maximal growth rate variables were calculated from the growth curves using a calculation method derived from that reported for yeast cultures (5). First, an initial relative nephelometric unit (RNU) value was calculated as the mean of the initial three measurements and then subtracted from each curve value. For each point of the curve, a slope was calculated using measurements made 2 h before and 2 h after that time point. The lag phase was defined as the time required to obtain a slope value of 1. The maximal growth rate was defined as the highest slope. Homoscedasticity and normality of residues were checked using Breusch-Pagan and Shapiro-Wilk tests, respectively.

Dry weight measurements

The initial conidial suspension was adjusted to 10^5 conidia/mL (i.e., 30,000 conidia/well) in PD broth. The mycelial mass was collected at different time points of the growth curve of *A. brassicicola*. Dry weight values were determined for the total biomass from 12 wells after freeze-drying until constant weight.

Results and discussion

Proportionality between nephelometric values and cell densities

The nephelometric values were tested for linearity through serial dilution of conidia of *A. brassicicola*. At all tested densities (ranging 600–60,000 conidia/well), there was a linear relationship between the number of conidia and the RNU (R^2 = 0.988) at least until saturation of the light detector [which was adjusted to 50,000 RNU (Figure 1A)]. A direct consequence of the linear relationship between the number of fungal propagules and RNU is that the raw values do not need to be converted to corrected values before growth curve analysis. Moreover, the

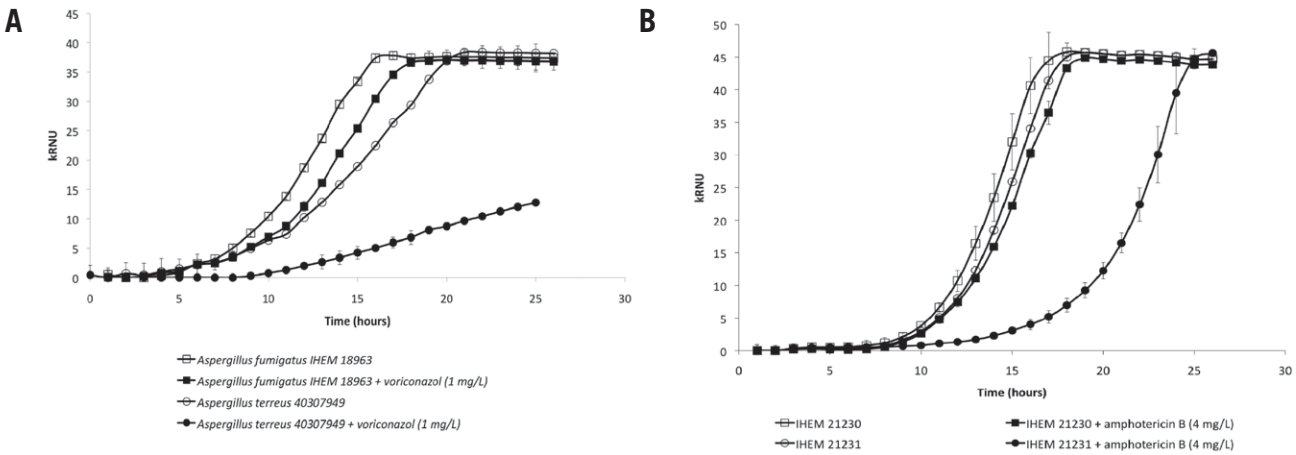

Figure 2. Nephelometric monitoring of growth for antifungal susceptibility testing. (A) Growth curves describing the effect of voriconazole (1 mg/L) on isolates of *Aspergillus fumigatus* (IHEM 18963) and *A. terreus* (IHEM 23439). (B) Growth curves describing the effect of amphotericin B (4 mg/L) on two isolates of *C. glabrata* (wild-type IHEM 21231 and clinical isolate IHEM 21230). Error bars, SD.

nephelometer can be used as an accurate tool for calibrating fungal inoculum or evaluating the capacity of mutants to sporulate.

General growth characteristics of filamentous fungi in a laser-based microplate nephelometer

Figure 1B shows a representative growth curve for *A. brassicicola* plotted from the nephelometric assays. A similar growth pattern was obtained from *N. crassa* or *Aspergillus* inocula (data not shown) and was reproducible in both the biological and technical replicates. The initial suspension was adjusted to 10^5 conidia/mL (i.e., 30,000 conidia/well) in PD broth and the nephelometric values were automatically recorded for each well every hour after shaking. In order to correlate the RNU changes with the morphology of the fungal colonies, the microplate well contents were harvested at given intervals and the hyphae and branching numbers were estimated by microscopic examination. Dry weight values were also determined for the mycelial mass at different time points of the growth curve.

Monitoring the filamentous fungi growth kinetics by nephelometry resulted

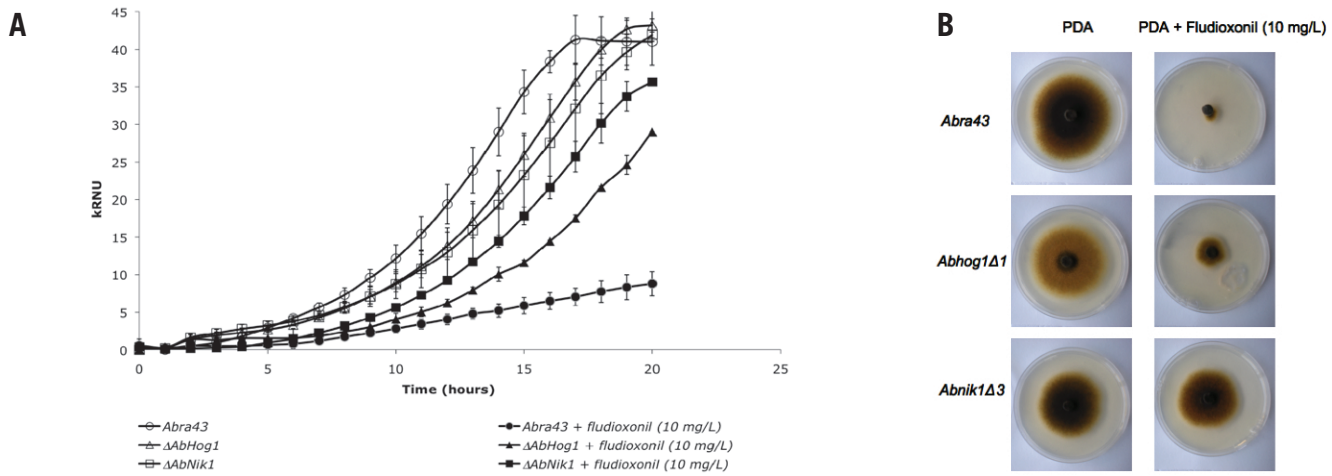

Figure 3. Determination of fludioxonil susceptibility in null mutants of *A. brassicicola* deficient in genes from the HOG-related pathway. (A) Nephelometric growth curves of wild-type and two deletion strains (*Abhog1Δ1* and *Abnik1Δ3*) following exposure to fludioxonil (10 mg/L) and under standard conditions. (B) Mycelium radial growth measurements on agar media. Agar disks were cut from the margin of a 7-day-old colony growing on PDA and were transferred onto the center of a PDA medium supplemented with fludioxonil at 10 mg/L. Growth was scored after 7 days of incubation at 24°C. Error bars, SD.

in typical microbial growth curves containing a lag phase, a maximal growth rate phase, and a plateau. In our experimental procedure, this last step cannot be considered as a classical stationary phase; rather, it corresponds to the saturation value of the detector (adjusted to 50,000 RNU). This saturation step did not have an impact on shape of the growth curves or on extrapolated growth variables (lag time and maximal growth rate). A biological plateau could be reached by lowering the nutrient source (data not shown). However, the use of a poor medium might result in erroneous data (as reported by Reference 8). Microscopic examinations revealed a correlation between the RNU variations and the extension and branching of hyphae from conidia. *A. brassicicola* conidia germination did not occur within 5 h of incubation, which was a period with low RNU variation (between 0 and 4000 RNU). After 18 h of incubation, all conidia had produced branched hyphae and a dense mycelium covered the bottom of the wells. Alongside the microscopy images, Figure 1C showed that dry weight values of mycelial biomass correlated with recorded nephelometry measures (R^2 = 0.959), providing evidence that laser nephelometry is an accurate indicator of the fungal biomass and can be used as reliable tool for the monitoring of fungal growth.

Growth curves in the presence of antifungal compounds

As extrapolated from the yeast absorbance growth curves (5), two standard growth variables for filamentous fungi could be defined: the maximum growth rate and the length of the lag phase (or lag time). (For calculation details, see the "Materials and methods" section.) These values provide precise parameters that can be used for analysis of growth inhibition curves and antifungal activity of natural or synthetic substances. Figure 2 shows two examples of antifungal susceptibility testing with important human pathogenic fungi. The sensitivity of a fungus to a drug could result in different types of growth aberration. A first effect is illustrated in Figure 2A. The sensitivity of a clinical *A. terreus* isolate (IHEM 23439) to voriconazole (a triazole molecule which is recommended for the treatment of invasive aspergillosis) resulted in a significant reduction of the maximum growth rate (from 3.66 kRNU/h ± 0.06 for the control to 1.91 kRNU/h ± 0.27 after exposure to voriconazole at 1 mg/L). By comparison, the maximum slope (4.39 kRNU/h ± 0.40) of the *A. fumigatus* reference strain (IHEM 18963) was only slightly affected when challenged with this drug at the tested concentration, which was in agreement with the minimum inhibitory concentrations (MICs) of voriconazole determined for these two *Aspergillus* strains using the E test method (0.064 mg/L for *A. terreus*, IHEM 23439 and 0.25 mg/L for *A. fumigatus*, IHEM 18963). Figure 2B shows another type of inhibitory effect caused by the application of amphotericin B (4 mg/L) on *C. glabrata* suspensions. When using the E test method on Casitone agar, the clinical isolate IHEM 21230 was shown to be less susceptible to polyenes than the wild-type isolate IHEM 21231 with a MIC of amphotericin B more than twice that reported for the wild-type isolate (15). The resistance of isolate 21230 was perfectly illustrated by nephelometric recording, since the growth curves obtained with or without amphotericin B were almost identical. By contrast, the sensitivity of the wild-type was clearly explained by a delayed entry into the log phase, while its maximum growth rate (6.48 kRNU/h ± 0.51) remained unaffected. Lag times were estimated at 6.50 h ± 0.71 and 14.50 h ± 0.71 for the control and treated conditions, respectively.

In an attempt to create a methodology for large-scale phenotypic profiling, we applied a strategy similar to that used for antifungal susceptibility testing to compare the phenotypes of deletion and wild-type strains on the basis of growth behavior during micro-cultivation. In this study, we tested two *A. brassicicola* null mutants (*ΔAbHog1* and *nik1Δ3*) and the corresponding wild-type (*Abra43*) for susceptibility to the phenylpyrrole fungicide fludioxonil (Figure 3A). It was previously reported that this compound exerts its toxicity in filamentous fungi through targeting of the high-osmolarity glycerol (HOG) pathway, a mitogen-activated protein kinase (MAPK) cascade involved in the response to high osmotic stress (14,16). The genes *AbNIK1* and *AbHOG1* encode an osmosensor group III histidine kinase and the MAP kinase of the HOG pathway, respectively. The wild-type strain was highly susceptible to the fungicide, according to the nephelometric growth records. At 10 mg/L of fludioxonil, the lag time was increased by 4× and the maximal slope was reduced by 6×, compared with control conditions. By contrast, *Abnik1Δ3* was found to be highly resistant (roughly similar lag time and maximum growth rate under both control and treated conditions), while *Abhog1Δ1* exhibited moderate resistance to this fungicide (the lag time was increased

by 1.5× and the maximum growth rate was decreased by 1.3× in the presence of 10 mg/L fludioxonil). The growth patterns obtained by nephelometric recording were confirmed by mycelium radial growth measurements on agar media (Figure 3B). We also observed distinct nephelometric profiles revealing various levels of susceptibility toward fludioxonil in different *N. crassa* mutants deficient in genes from the Hog/Os-related pathway (data not shown). These fungicide susceptibility patterns are in accordance with results obtained with deletion mutants of orthologous genes in the southern corn leaf blight fungus *Cochliobolus heterostrophus*, thereby confirming that some filamentous fungi have two pathways that additively control phenylpyrrole fungicide sensitivity (17). Both are regulated by a group III histidine kinase but only one controls HOG1-type MAPK phosphorylation.

In conclusion, we present evidence that a laser-based microplate nephelometer can be used as a reliable tool for monitoring filamentous fungus growth. In comparison with the current standard method based on radial growth measurements on solid media, the microplate reader of this system provides many advantages, such as fast and easy handling of large numbers of samples, a reduction in the amount of test substances, and a rapid and accurate measuring mode with computerized processing of the quantitative data. Moreover, the raw values do not need to be corrected for nonlinearity before quantitative analysis, and altered growth can be easily interpreted on the basis of a change in the length of the lag phase, the rate of growth, or both, which is not the case for colony-size measurements. We believe that this system could be successfully applied for a broad range of filamentous fungi, yeasts, and bacteria for large-scale screening based on quantitative changes in growth phenotypes under a wide variety of growth conditions. Thus, the method described herein paves the way for the development of a high-resolution quantitative phenomic approach for filamentous fungi, as is already available for yeast (18).

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Competing interests

The authors declare no competing interests.

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