Use of morphology index histograms to quantify populations of the fungal pathogen *Paracoccidioides brasiliensis*

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To quantify the dimorphic process in wild and mutant strains of *Paracoccidioides brasiliensis*, we defined a morphology index (Mi) in terms of the maximum cell length (*l*), maximum cell diameter (*d*), and septal diameter (*s*), according to the equation $Mi = 2{\cdot}13 + 1{\cdot}13 \log_{10}(ls/d^2)$, whose intercept and slope were such that Mi was around 1 for yeast (spherical) cells or 4 for hyphal (elongated) cells. This discriminatory power was used to quantify morphological population mixtures through Mi histograms. During the temperature-induced dimorphic transition (either way), mean Mi ($\overline{Mi}$) varied linearly with time, suggesting a continuity in the process. Also, in wild strains and mutants thereof we found an inverse relationship between $\overline{Mi}$ and content of both cell wall chitin and 1,3-α-glucan.

Keywords: *Paracoccidioides brasiliensis*, morphology index, dimorphism, morphogenesis

INTRODUCTION

The dimorphic process characteristic of many infective fungi is relevant to pathogenesis, as only one of the forms is usually associated with the disease. The term dimorphism implies two distinct morphological phases, whose conversion from one to the other is triggered by environmental stimuli. However, many intermediate cell shapes occur frequently. An example is *Candida albicans* in which morphological transitions amount to gradations of form that lead to yeast cells, pseudohyphae, and true hyphae (Merson-Davies & Odds, 1989). A second example is *Paracoccidioides brasiliensis*, another pleomorphic fungus pathogenic for humans, that depends on temperature for phase expression. Morphological extremes are characterized by the yeast-like (Y) phase in tissue and *in vitro* at 37 °C, and by the mycelial (M) morphology below 25 °C; transitional forms with elongated structures, yeast-like chains or undefined structures, occur *in vitro* at intermediate temperatures (Carbonell & Rodríguez, 1965). Odd shapes such as septated short filaments, round cells with germ tubes, chalice-shaped forms, thick and short mycelia, and elongated yeast cells are present in samples of different origin (Lacaz, 1994; unpublished results) and in mutants defective in dimorphism (San-Blas *et al.*, 1981, 1983; Hallak *et al.*, 1982; San-Blas & San-Blas, 1992).

Merson-Davies & Odds (1989) quantified morphological forms of *C. albicans* to facilitate definition of cellular and molecular markers specific for cell development, and determine the distribution of morphological types in different environmental conditions. By relating the maximum length, and maximum and septal diameters of cells in a mathematical relationship, a morphology index (Mi) was established, ranging from 1 in spherical yeast cells to 4 in true hyphae, with elongated yeast cells and pseudohyphae giving intermediate values. In this way, the subjective descriptions of cell morphologies were substituted by objective parameters aimed to facilitate comparison of inter-laboratory data and a better distinction between phenotypes. With this in mind, we undertook the elaboration of a similar Mi specific for *P. brasiliensis* to compare morphological forms of wild and mutant strains and also to quantify the dimorphic transition.

METHODS

Fungal samples and growth. Light micrographs of *P. brasiliensis* (wild strains Pb9 and Pb73, and mutant strains thereof) were chosen from the collection formed throughout the years in our laboratory (Hallak *et al.*, 1982; San-Blas & San-Blas 1992; San-Blas *et al.*, 1981, 1983). They were grown as follows: *P. brasiliensis* wild strains IVIC Pb9 (ATCC 36324)

Abbreviations: M, mycelial morphology; Mi, morphology index; $\overline{Mi}$, mean morphology index; Mr, morphology ratio; Y, yeast-like morphology.

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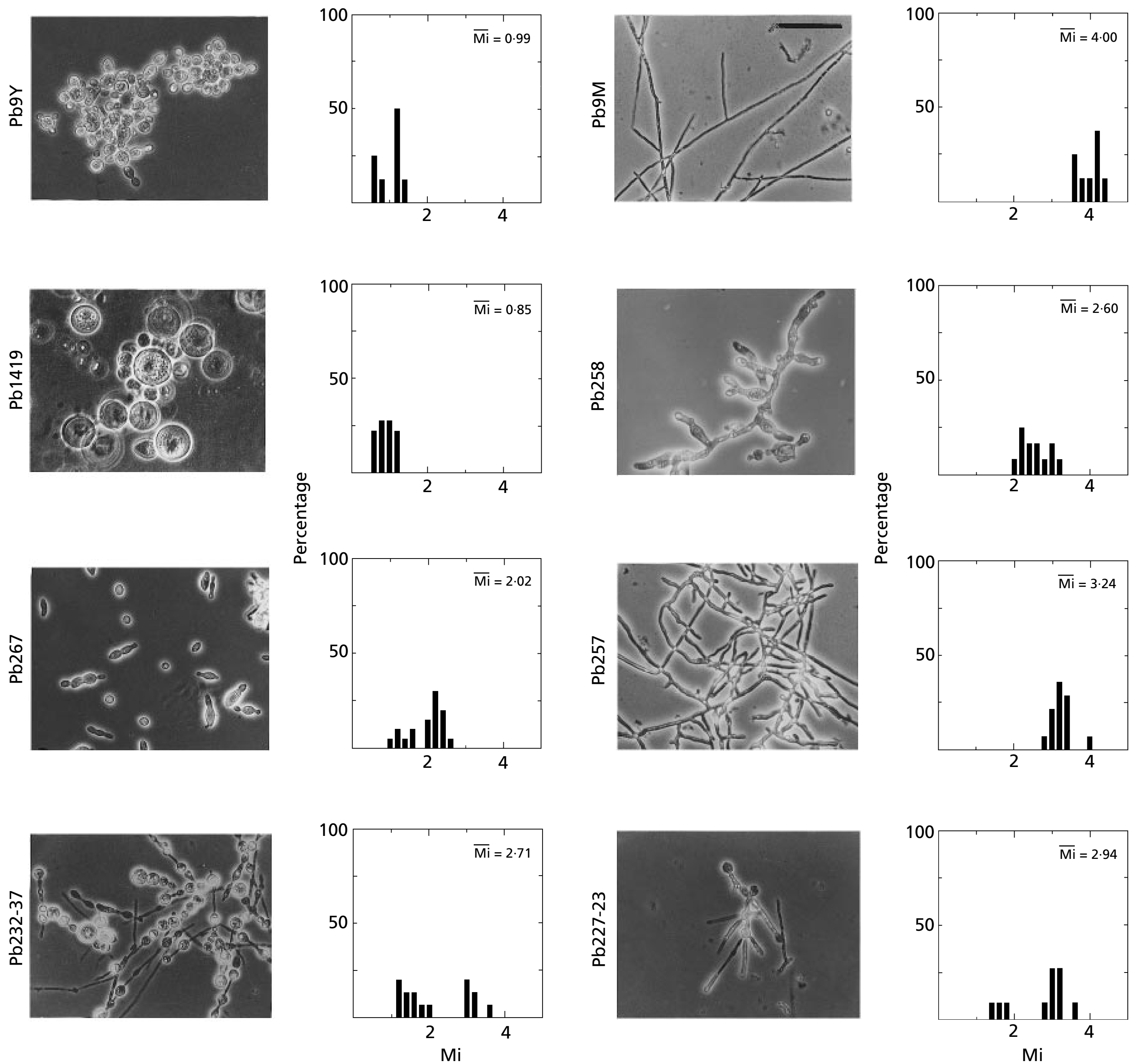

Fig. 1. Mi values and histograms of *P. brasiliensis*: wild strain Pb9, Y and M phases; mutant strains ex-Pb9: Pb257, Pb258, and Pb267; mutant strains ex-Pb73: Pb1419, Pb232-37, and Pb227-23 (Hallak *et al.*, 1982; San-Blas & San-Blas, 1992). Frequencies of Mi values are expressed as percentages. $\overline{Mi}$ is the $\overline{Mi}$ value of the sample. Bar, 20 µm. Light micrographs reproduced with permission.

and Pb73 (ATCC 32071) have been maintained in our laboratory on PYG medium (peptone, 5 g; yeast extract, 5 g; and glucose, 15 g; per l distilled water, final pH 7.0) agar slants for several years. They were grown in PYG liquid medium (200 ml in 500 ml Erlenmeyer flasks), after inoculation with 10 ml of a seed culture. They were incubated for three days at 37 °C (Y phase) or 23 °C (M phase), with continuous shaking on a gyratory shaker operated at 120 r.p.m. (Sorais-Landáez & San-Blas, 1993). Mutant strains were isolated by treatment of wild strains with NTG as described by San-Blas & San-Blas (1992). Micrographs were taken in a Carl Zeiss microscope. To follow Y → M transition, a drop of a Y-cell suspension was placed over mycosel agar (BBL) on a Neubauer chamber, a coverslip applied, incubated in a moist chamber at 37 °C for 18 h and transferred to 23 °C, and photographs taken at intervals (San-Blas *et al.*, 1980). To follow M → Y transition, an M culture was placed at 37 °C to start the process of transition to the Y form, and samples taken at intervals for microscopic observation and photographing. Since the transition process took several hours to start, the time required for sample manipulation under the microscope was insufficient to modify morphology.

Measurement of cells. This was done according to the method of Merson-Davies & Odds (1989), with modifications. On each micrograph, the length (l), maximum diameter (d) and the diameter at septal junctions (s) were determined with the use of a measuring magnifier. No fewer than thirty measurements in multiple micrographs were taken of each strain or morphological phase.

Morphology ratio (Mr). For each set of measured parameters (l, d, s) in every cell, the $\mathrm{Mr} = ls/d^2$ was calculated. Mr approached zero for spherical cells with near-zero septal diameter and increased exponentially, becoming large for elongated cells. The ratio ls/d^2 generated a unit-free index, and provided independence of measurements from the enlargements of micrographs.

Mi. To calculate the Mi of *P. brasiliensis* cells, the slope and intercept in the equation proposed for *C. albicans* by Merson-Davies & Odds (1989) were modified so that the new Mi was either 1 or 4 for *P. brasiliensis* yeast or hyphal cells, respectively. For this purpose, a PC computer program was developed to store sets of (l, d, s) data measured for each fungal sample and also to calculate Mr, Mi and mean Mi ($\overline{\mathrm{Mi}}$). To modify the slope and intercept parameters of the original Merson-Davies & Odds (1989) equation, two samples (pure yeast and pure mycelium of wild strain Pb9) were used as standards. The intercept and slope of the modified equation for *P. brasiliensis* were deduced. This resulted in the equation $\mathrm{Mi} = 2{\cdot}13 + 1{\cdot}13\ \log_{10}(ls/d^2)$, which allowed calculations of Mi and $\overline{\mathrm{Mi}}$, and also plotting of Mi histograms, using a 0·2 Mi window spread. The computer program permits the change of intercept and slope as needed for adapting Merson-Davies & Odds' equation to other specific fungi.

RESULTS AND DISCUSSION

Merson-Davies & Odds's approach (1989) of using a logarithm of Mr to calculate Mi in *C. albicans* opened the way to allow objective shape discrimination with other fungi, such as *P. brasiliensis*. While in the former, slope and intercept values were 1·78 and 2, respectively, in the latter, values of 1·13 and 2·13 were required for a similar Mi span between 1 (yeast phase) and 4 (mycelial form). Therefore, using the formula $\mathrm{Mi} = 2{\cdot}13 + 1{\cdot}13\ \log_{10}(ls/d^2)$, Mi values were calculated for wild and mutant strains of *P. brasiliensis* (Fig. 1). In this way, yeast-like cells had Mi around 1·0 (wild strain Pb9Y and mutant strain Pb1419, ex Pb73), and Mi approaching 4·0 as they departed from the round morphology, to produce either elongated yeast cells (mutant strain Pb267, ex-strain Pb9) or a variety of hyphal shapes (wild strain Pb9M, and mutant strains Pb257 and 258, ex-strain Pb9), so that the thinner the mycelium, the closer Mi was to 4·0. Some mutants produced mixed populations, such as Pb232-37 and Pb227-23 (both strains ex-Pb73). In these instances, the use of $\overline{\mathrm{Mi}}$ (2·71 and 2·94, respectively) was misleading, not only because of the apparent closeness to $\overline{\mathrm{Mi}}$ in different mutants (e.g. Pb258), but also because $\overline{\mathrm{Mi}}$ did not reflect the true nature of morphologically mixed mutants. To overcome this limitation, histograms were prepared for each strain. In this way, differences were clearly established between true yeast (Pb9Y and Pb1419) and mycelial (Pb9M) morphologies, atypical yeast-like (Pb267) or mycelial (Pb257 and Pb258) shapes (all of them having a single population group around a given Mi), and morphologically mixed mutants, which clearly separated into groups of different Mi (Pb232-37 and Pb227-23). This is in agreement with Merson-Davies & Odds (1989) who previously observed a considerable variation in the nature of environmentally induced morphologies in *C. albicans* that could be quantitatively expressed through the equation $\mathrm{Mi} = 2 + 1{\cdot}78\ \log_{10}(ls/d^2)$.

Table 1. Relationship between $\overline{\mathrm{Mi}}$ and cell wall polysaccharides in wild and derived mutant strains of *P. brasiliensis*

Strain	$\overline{\mathrm{Mi}}$	Concentration [μg (mg cell wall)$^{-1}$] of:		
		Chitin	1,3-α-Glucan	1,3-β-Glucan
Pb9Y[a]	0·99	434	450	76
Pb267[b]	2·02	235	233	91
Pb258[b]	2·60	191	26	69
Pb257[b]	3·24	189	77	69
Pb9 M[a]	4·00	133	0	355
Pb73Y[c]	1·68	297	262	68
Pb232-37[c]	2·71	135	179	91
Pb73M[c]	4·00	57	39	331

References: a, Kanetsuna *et al.* (1969); b, San-Blas & San-Blas (1992); c, Hallak *et al.* (1982).

Merson-Davies & Odds (1989) also found a direct correlation between Mi and cell wall chitin content in *C. albicans*, the latter considered a biochemical feature linked to morphology (Chattaway *et al.*, 1968; Merson-Davies & Odds, 1989). By contrast, in *P. brasiliensis* Mi values showed an inverse relationship with both cell wall chitin and 1,3-α-glucan (with the exception of strain Pb258 in the latter) when considering each wild-type and its mutants (Table 1). Unlike *C. albicans*, the *P. brasiliensis* Y phase has three times more chitin in its wall than the M phase (Kanetsuna *et al.*, 1969), leading to the observed inverse relationship with $\overline{\mathrm{Mi}}$. But the most prominent differential feature of *P. brasiliensis* morphogenesis is the substitution of 1,3-α-glucan by 1,3-β-glucan when the fungus changes from the Y to the M phase (Kanetsuna *et al.*, 1969; San-Blas & San-Blas, 1985). While 1,3-α-glucan showed an inverse relationship with $\overline{\mathrm{Mi}}$, 1,3-β-glucan did not, and concentrations increased sharply in the presence of a well-defined mycelial structure ($\overline{\mathrm{Mi}} > 3{\cdot}5$). This may suggest a requirement for 1,3-β-glucan in the final stage of hypha formation and a continuous, though variable, presence of the other two polysaccharides throughout the dimorphic process of *P. brasiliensis*, with major participation in the yeast-like phase.

Quantification of the dimorphic transition in both directions could be done with Mi calculations (Fig. 2). In the $\mathrm{Y} \rightarrow \mathrm{M}$ transition, histograms prepared from samples taken during the first 4 h revealed the formation of an intermediate population (Mi between 2 and 3)

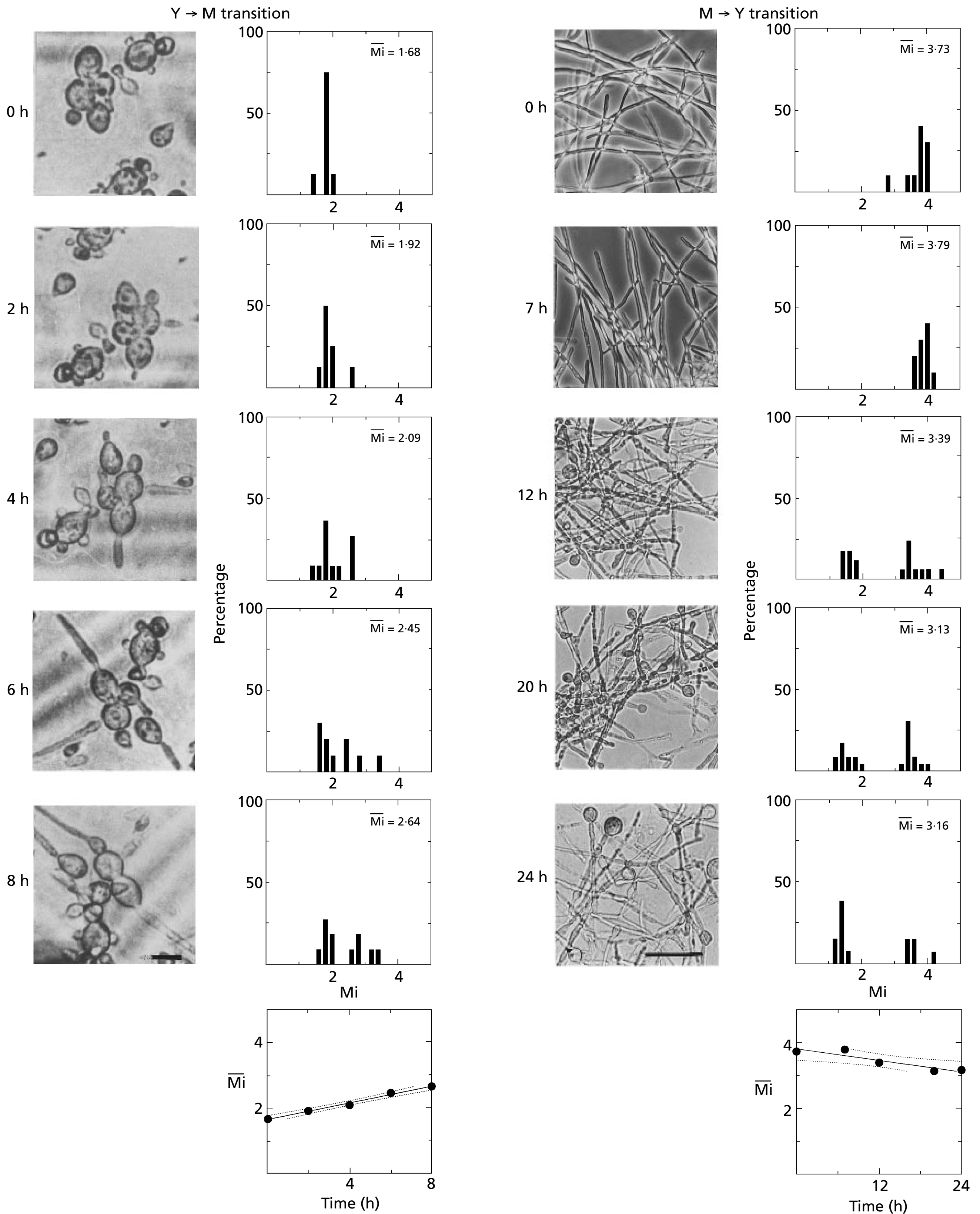

Fig. 2. Mi histograms and $\overline{\text{Mi}}$ values of *P. brasiliensis* strain Pb73 during dimorphic transition. Left lane: Y → M transition (37–23 °C); bar, 5 μm. Right lane: M → Y transition (23–37 °C); bar, 20 μm. Last panel in each side represents the linear variation of $\overline{\text{Mi}}$ with time. Light micrographs of Y → M transition correspond to San-Blas *et al.* (1980). Reproduced with permission.

from the original yeast cells (Mi between 1 and 2 for *P. brasiliensis* strain Pb73), followed by the appearance of a third population of true hyphal structures (Mi closer to 4) from 6 h onward. This process coincided with the model of Y → M transition proposed before (San-Blas & San-Blas, 1985) in which hyphal development starts 2 h after the change of temperature from 37 to 22 °C, with the formation of mycelial buds or primary hyphae which are thicker and shorter than typical hyphae (Mi between 2 and 3). Their cell walls are like the yeast wall, that is, they have 1,3-α- but not 1,3-β-glucan, because they originate from deformation of the yeast cell rather than true initiation of apical growth, as suggested by the halt in synthesis of macromolecules during the first 8 h of transition (San-Blas & San-Blas, 1985). Consequently, the fungus uses the yeast machinery in the initial steps of the dimorphic process towards a hyphal morphology. In a second step, a rearrangement of the cell system occurs, and true hyphae are produced (San-Blas & San-Blas, 1985).

Histograms were also used to quantify the dimorphic transition either way. $\overline{\mathrm{Mi}}$ correlated linearly with time in *P. brasiliensis* (Fig. 2), suggesting a continuity in the process. Actual extension of fungal growth reflects the incorporation of precursor materials, through the agency of apical vesicles, into pre-existing rigid walls which are subjected to continuous scission of bonds to allow incorporation of new material (Bartnicki-García, 1973) or through the assemblage of polymers synthesized at a plastic apex, rigidity developing while the wall segment moves in subapical directions during elongation (Wessels, 1986).

Bartnicki-García *et al.* (1989) postulated a vesicle supply centre (VSC) whose position and movement becomes the critical determinant of morphogenesis. When VSC remains stationary, vesicles reach the cell surface uniformly in all directions, and a spherical shape is generated. A sustained linear displacement of VSC generates the typical cylindrical shape of fungal hyphae with their characteristic rounded tips. These features were incorporated into a mathematical function [$y = x \cot (xV/N)$] in which N represents the amount of wall-destined vesicles released from VSC per unit time, and V, the rate of linear displacement of VSC. Therefore, fungal dimorphism is a graded response in which a morphological gradient dependent on V/N may correlate with the Mi values devised by Merson-Davies & Odds (1989) for *C. albicans* and applied by us to *P. brasiliensis*, to quantify the relative abundance of yeast, hyphal and intermediate forms in a mixed-morphology culture. Such a distribution is easily visualized with the use of histograms (Figs 1 and 2).

Mathematical approaches to fungal and plant cell enlargement have also been proposed (Ortega, 1990; Ortega *et al.*, 1995) by means of governing equations that describe the relevant physical processes in terms of biophysical and biomechanical parameters which, in turn, are controlled by relevant biological processes.

In conclusion, Mi and histograms thereof provide a simple objective estimation of morphology and dimorphism in *P. brasiliensis*. Extension of this method to other fungal species requires the adjustment of equations, as shown in *C. albicans* and *P. brasiliensis*. We hope that future application of this method may provide a means for determining morphology-specific regulatory processes at the molecular level.

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REFERENCES

Bartnicki-García, S. (1973). Fundamental aspects of hyphal morphogenesis. *Symp Soc Gen Microbiol* **23**, 245–267.

Bartnicki-García, S., Hergert, F. & Gierz, G. (1989). A novel computer model for generating cell shape: application to fungal morphogenesis. In *Biochemistry of Cell Walls and Membranes of Fungi*, pp. 43–60. Edited by P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey & L. G. Copping. Berlin: Springer.

Carbonell, L. M. & Rodríguez, J. (1965). Transformation of mycelial and yeast forms of *Paracoccidioides brasiliensis* in culture and in experimental inoculations. *J Bacteriol* **90**, 504–510.

Chattaway, F. W., Holmes, M. R. & Barlow, A. J. E. (1968). Cell wall composition of the mycelial and blastospore forms of *Candida albicans*. *J Gen Microbiol* **51**, 367–376.

Hallak, J., San-Blas, F. & San-Blas, G. (1982). Isolation and wall analysis of dimorphic mutants of *Paracoccidioides brasiliensis*. *Sabouraudia* **20**, 51–62.

Kanetsuna, F., Carbonell, L. M., Moreno, R. E. & Rodríguez, J. (1969). Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J Bacteriol* **97**, 1036–1041.

Lacaz, C. S. (1994). *Paracoccidioides brasiliensis*: morphology; evolutionary cycle; maintenance during saprophytic life; biology, virulence, taxonomy. In *Paracoccidioidomycosis*, pp. 13–25. Edited by M. Franco, C. S. Lacaz, A. Restrepo-Moreno & G. Del Negro. Boca Raton, FL: CRC Press.

Merson-Davies, L. A. & Odds, F. C. (1989). A morphology index for the characterization of cell shape in *Candida albicans*. *J Gen Microbiol* **135**, 3143–3152.

Ortega, J. K. E. (1990). Governing equations for plant cell growth. *Physiol Plant* **79**, 116–121.

Ortega, J. K., Smith, M. E. & Espinosa, M. A. (1995). Cell wall extension behavior of *Phycomyces* sporangiophores during the pressure response. *Biophys J* **68**, 702–707.

San-Blas, F. & San-Blas, G. (1985). *Paracoccidioides brasiliensis*. In *Fungal Dimorphism*, pp. 93–120. Edited by P. Szaniszlo. New York: Plenum.

San-Blas, F. & San-Blas, G. (1992). Mutants of *Paracoccidioides brasiliensis* strain IVIC Pb9 affected in dimorphism. *J Med Vet Mycol* **30**, 51–60.

San-Blas, F., San-Blas, G. & Inlow, I. (1980). Dimorphism in *Paracoccidioides brasiliensis*. In *Medical Mycology*, pp. 23–28 (*Zentralb Bakteriol* suppl. 8). Edited by J. H. Preusser. Stuttgart: Gustav Fischer Verlag.

San-Blas, F., San-Blas, G. & Yarzábal, L. (1981). A mycelial mutant of *Paracoccidioides brasiliensis* defective in dimorphism: chemical and immunological characterization. *Exp Mycol* **5**, 23–34.

San-Blas, F., San-Blas, G., Hallak, J. & Merino, E. (1983).

Ultrastructure and cell wall chemistry of a thermosensitive mutant of *Paracoccidioides brasiliensis*. *Curr Microbiol* **8**, 85–88.

Sorais-Landáez, F. & San-Blas, G. (1993). Localization of β-1,3-glucan synthetase in membranes of *Paracoccidioides brasiliensis*. *J Med Vet Mycol* **31**, 421–426.

Wessels, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. *Int Rev Cytol* **104**, 37–79.

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