Determination of ploidy of a dimorphic zygomycete *Benjaminiella poitrasii* and the occurrence of meiotic division during zygospore germination

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Ghormade, V., Shastry, P., Chiplunkar, J. and Deshpande, M.V. (2005). Determination of ploidy of a dimorphic zygomycete *Benjaminiella poitrasii* and the occurrence of meiotic division during zygospore germination. Journal of Agricultural Technology 1 (1) : 97-112.

Benjaminiella poitrasii is a zygomycetous, dimorphic fungus which exists in yeast or hyphal forms during the vegetative phase and produces asexual sporangiospores and zygopores during the reproductive stage. The zygospores germinate either by germ-sporangiophore formation or hyphal formation. However, the budding type germination of zygospores of *B. poitrasii* was observed in response to high glucose, 37°C and pH 4.0; conditions favouring the yeast-form. The yeast cells from the budding zygospore were analysed to ascertain time and occurrence of meiotic division and to understand the change in the ploidy levels in its life-cycle. The ploidy and nuclear behaviour of this fungus were studied at different stages in the life cycle using the vegetative yeast cells, the asexual sporangiospores, and the yeast cells from the budding zygospore. The uninucleate sporangiospores and the multinucleate yeast cells showed similar DNA contents/nucleus as estimated by spectrophotometric DNA content estimation, survival in the presence of ultraviolet radiation and flow cytometry. The sporangiospores and yeast cells were in the haploid state. DAPI staining of the zygospore showed that few nuclei fused in the zygospore and underwent the meiotic division. The haploid nature of the yeast cells from the budding zygospore indicated that meiosis had occurred before the initiation of germination. The understanding of spore germination in *B. poitrasii* and its use as a model to screen anti-fungal agents is also discussed.

Key words: *B. poitrasii*, DAPI staining, ploidy, DNA contents, flow cytometry

Introduction

Benjaminiella poitrasii (R.K. Benjamin) von Arx, is a zygomycetous, homothallic, dimorphic fungus that produces asexual sporangiospores and sexual zygopores and a vegetative phase with reversible yeast to hypha morphological transition in response to glucose and temperature (Deshpande *et*

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al., 1997; Chitnis and Deshpande, 2002). Extensive biochemical studies using the parent strain and its morphological (monomorphic yeast-form) mutant have been carried out with the correlation of cell wall metabolising enzymes such as chitinase, chitin synthase and glutamate dehydrogenase to the morphological outcome (Khale *et al*., 1990; Ghormade *et al*., 2000; Chitnis *et al.,* 2002; Amin *et al*., 2004). However at the genetic level not much is known about this organism. The determination of the ploidy holds an important position in the genetic characterization of eukaryotic organisms (Carle and Olson 1985; Shepherd *et al*., 1985). The life cycle of *B. poitrasii* is described in Fig. 1.

In zygomycetous fungi, zygospore germination is reported to be either by hypha formation or by germ- sporangiophore formation (Gauger, 1965). Though the dormancy period of zygospores before germination is reported to be around 2 months, in *Phycomyces blakesleeanus* it was observed to be about 8 months (Gauger, 1977; Mehta and Cerdá-Olmedo, 2001). In *B. poitrasii* the zygospores germinated in 45 days either by hypha formation or by germ-sporangiophore formation in conditions favouring the hyphal form i.e. yeast extract-peptone medium (YP) at 28°C. The zygospore of *B. poitrasii* germinated into budding yeast-like cells under yeast- favouring conditions (37°C, YP containing 1% glucose) (Ghormade and Deshpande, 2000). In zygomycetous fungi, the zygospore is formed by the union of gametangia that contribute several nuclei to the zygospore. On the basis of number of nuclei fusing and the meiotic division in the zygospores, Cutter (1942) demonstrated 4 patterns of nuclear behaviour: a) the nuclei from the two gametangia mixed, but there was no fusion, e.g. *Sporidinia grandis,* b) fusion of few nuclei as well as the reduction division took place at the time of zygospore germination e.g. *P. blakesleeanus*, c) the nuclei fused in the 6 day zygospore and the reduction division was delayed until 45 days, i.e. until the start of germination e.g. *Absidia glauca,* d) nuclear fusion and the reduction division took place consecutively in the 6 day old zygospore e.g. *Mucor hiemalis*.

While studying germination of the immature zygospore in *Rhizopus stolonifer* Gauger (1977) reported that the process of germination induced meiotic divisions. In the heterothallic, *P. blakesleeanus,* the auxotrophic marker method used to detect the occurrence of the reduction division could not determine the time of the meiosis during zygospore germination (Eslava *et al*., 1975; Eslava and Alvarez, 1996). In *Mucor racemosus*, Lasker and Borgia (1980) studied the meiotic event using protoplast fusion technique; they reported the formation of prototrophic heterokaryons but did not detect karyogamy.

The aim of this investigation was to address the ploidy change and detect the occurrence as well as time of meiotic division during the life cycle of *B.*

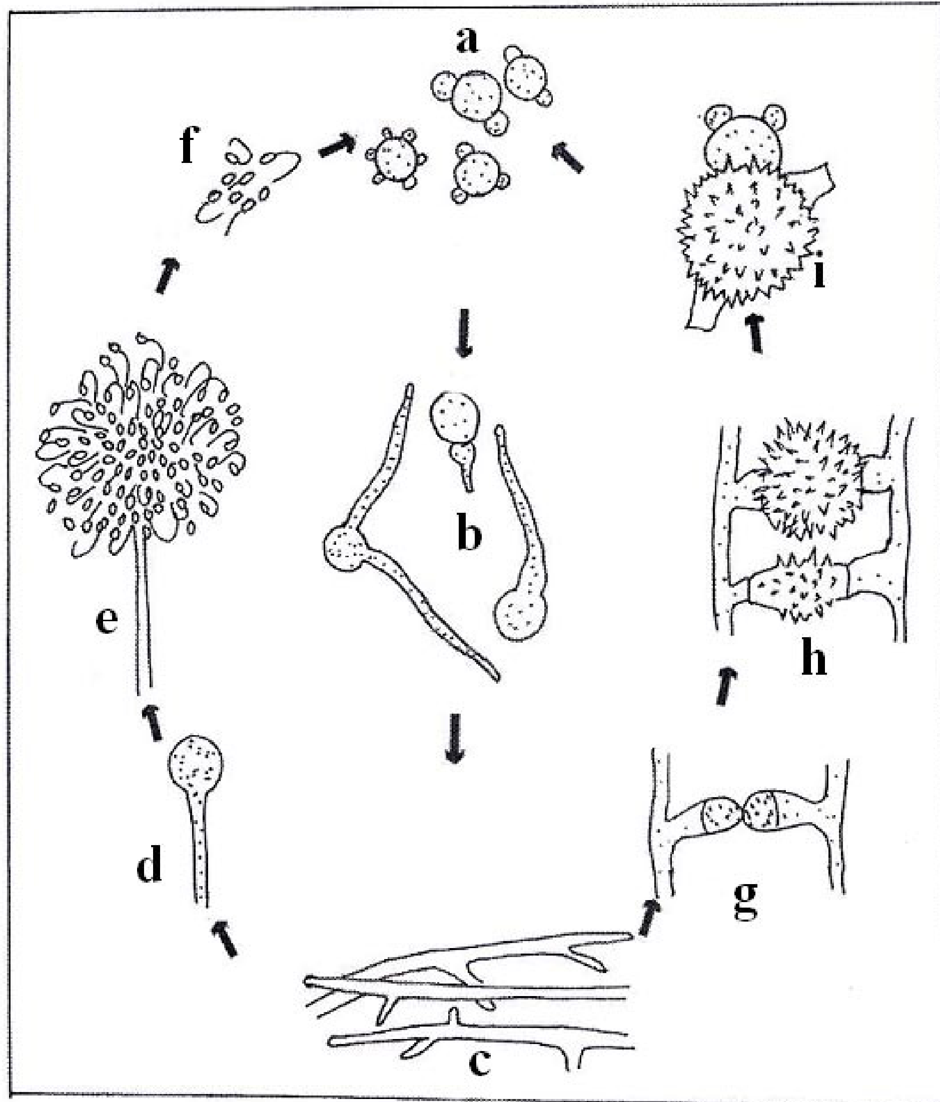

Fig. 1. Life-cycle of *Benjaminiella poitrasii*. a, Budding yeast cells. b, Yeast-hypha transition by germ tube formation. c, Hyphae. d, Formation of apical globose vesicle on the aerial hypha. e, Mature sporangiophore bearing pedicellate sporangiospores. f, Sporangiospores. g, Zygospore formation by gametangial fusion. h, Mature zygospore. i, Bud-type germination of zygospore.

poitrasii. To ascertain the time of the meiotic division before or during germination, the budding yeast cells (Y_z) were also analysed separately for their nuclear and ploidy status. For comparison, the ploidy status was studied in the sporangiospores and the vegetative phase yeast cells (Y) by UV survival, spectrophotometric, flow cytometric methods and DAPI staining. The possible use of *B. poitrasii* as a model to screen anti-fungal agents is discussed.

Materials and Methods

Organism and growth conditions

Benjaminiella poitrasii was assigned to the family *Mycotyphaceae* as described by Benny *et al*. (1985). The parent strain of *B. poitrasii* was maintained and subcultured weekly on YPG (yeast extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l; agar, 20 g/l) slants at 28°C (Ghormade *et al*., 2000). For further studies, the sporangiospores were scraped off the slant and suspended in 0.1% Tween 80. The spore count was taken with a haemocytometer. The yeast-form (Y_z) cells isolated from the budding zygospores were grown on YPG slants at 37°C (Ghormade and Deshpande, 2000). Sporangiospores (2×10^8 /50 ml) were inoculated in YPG at 28°C for 24 hours to obtain parent yeast cells (Y). Y_z cells (2×10^8 /50 ml) were counted on a haemocytometer and inoculated in YPG at 28°C and harvested after 24 hours. The growth in liquid (YPG) as well as on solid agar media was used for further studies after appropriate treatments.

Saccharomyces cerevisiae haploid strain 103X(n) and diploid 103X(2n) was obtained from Tata Institute of Fundamental Research, Mamba. They were maintained on YEPD (yeast extract, 10 g/l; peptone, 20 g/l; glucose, 20 g/l; agar, 20 g/l) slants at 37°C.

Nuclear staining

The sporangiospores, parent yeast cells and Y_z cells were fixed for 30 minutes at 37°C and further for 1 hour at room temperature with 4% (w/v) p-formaldehyde in phosphate buffered saline (PBS pH 7.2) containing 5%(v/v) dimethylsulfoxide (5% DMSO-PBS) according to the modified method of Yokohama *et al*. (1990). The fixed cells were washed in 5% DMSO-PBS twice. The cells were stained with 4,6-diaminidino-2-phenyl-indole (DAPI- 2 μg/ml) and observed immediately. The DAPI-DNA fluorescence was observed on an Axioplan Zeiss microscope with excitation wavelength of 365 nm and

emission wavelength of 397 nm. The images were recorded on a camera (MC 100 Spot).

UV survival method

Benjaminiella poitrasii sporangiospores, parent yeast cells, Y_z cells, were grown in YPG and *S. cerevisiae* 103X n and 2n, grown in YEPD, for 24 hours at 37°C were used for the UV survival method. The cell suspension (100 µl) of sporangiospores or yeast cells containing 2×10^2 cells were spread on the plates (10 cm diam.) and were exposed to UV germicidal lamp (1 year old, 15W, Phillips, Holland) at a distance of 30 cm for 1 minute (15 J/s). The plates were incubated in the dark at 28°C and colony counts were taken after 48 hours. The experiment was repeated three times.

DNA isolation

Benjaminiella poitrasii sporangiospores (60×10^6) were centrifuged, freeze dried in liquid N_2 and crushed lightly. The crushed spores were transferred to extraction buffer (Tris pH 8.0, 10 mM; NaCl,100 mM; SDS, 1%; DTT, 50 mM; β-ME, 1%) and mixed 2-3 times by inversion. Further processing was done according to Chitnis *et al.* (2002). The experiment was repeated three times.

Flow cytometry

For flow cytometry (FC) analysis, *B. poitrasii* sporangiospores and yeast cells were grown as described under organism and growth condition. The sporangiospores and yeast cells at the concentration of 1×10^6 /ml were centrifuged and washed in phosphate-buffered saline (pH 7.2). The pellet was fixed with 1 ml of 70% (v/v) chilled ethanol and kept overnight at 4°C following the protocol of Olaiya and Sogin (1979). The sporangiospores and yeast cells were centrifuged and the pellet was incubated in 50 µl of RNAase (20mg/ml) for 1 hour at room temperature followed by addition of 450 µl of propidium iodide (PI - 50 µg/ml). Following staining 500 µl of 10^6 cells /ml were resuspended in phosphate buffered saline. Samples were vortexed at low speed for 5 seconds to disrupt the cell clumps. The total number of events analysed were 20,000.

Data acquisition

The cells were analysed in a FACS Vantage (Becton-Dickinson, Immunocytometry system, BDIS, CA) machine employing an argon laser tuned to an excitation wavelength of 488 nm. After excitation PI emits signal at 617 nm that was collected by a FL2 band pass filter. The instrument was calibrated with a standard of calf thymus nuclei. The flow rate on the FACS Vantage was on the lowest possible setting of 200-300 cells per second for cells suspended at 10^6/ml.

During data acquisition the cells were gated on the basis of forward scatter- side scatter (FCS-SSC) for uniform population size (gate 1) and for exclusion of any cellular debris that might have been present in the sample. A second gate was to exclude the clumps or aggregates of 2-3 cells on the FL2 A *vs* FL2 W.

Data analysis

Cell quest software (BDIS) was used for analysis and display of flow cytometry data. Data are presented as a histogram of relative number of cells vs PI fluorescence. Estimates of G1, S and G2-M dwell times were based on the assumption that during exponential growth the number of cells in the population is increasingly asynchronous. The correlated arrays of PI induced DNA fluorescence and FL2 A scatter data for each type of yeast cells/ sporangiospores were contoured at levels that allowed identification of the G1, S and G2-M regions. The cells present in the G1 area were taken as the cells having 'n' (haploid) content of DNA whereas the cells in the G2 area could be assigned to the budding cells of a asynchronous population having double DNA content.

Estimation of melanin contents

Benjaminiella poitrasii sporangiospores are dark grey in colour due to the presence of melanin, a substance known to absorb UV radiations and bind to PI causing a shift in the UV survival and flow cytometric values. The presence of melanin in the sporangiospores was confirmed by its extraction and estimation. For the extraction of melanin, the sporangiospores were hydrolysed in the presence of 6N HCl at 100°C for 12 hours and dried under vacuum as described by Bull (1970). Melanin was estimated by measuring the extinction at 540 nm and referring to standard values of synthetic melanin (Sigma). Melanin extracted from *B. poitrasii* sporangiospores was incubated with 450 µl

of propidium iodide (PI - 50 μg/ml) and the fluorescence was observed at excitation wavelength of 488 nm and emission wavelength of 617 nm in a Perkin Elmer LS 50B fluorimeter.

Results

Nuclear staining

The sporangiospores were uninucleate (Fig. 2a). The germination of the asexual sporangiospore (Fig. 2b) to form the yeast cell, showed that the uninucleate spore enlarged in 2 hours and became binucleate (Fig. 2c). This cell showed further enlargement to the size of the yeast cell (12-15 μm) and the nuclei divided till the cell contained about 8-12 nuclei/cell after 4 hours (Fig. 2e). The yeast cells from the parental strain and those from the zygospore (Y_Z) contained 8-12 nuclei per cell (Fig. 2e,f respectively).

The nuclear staining with DAPI for the zygospore was carried out by removal of the dark outer ornamental cover. The nuclear staining showed a sharper contrast for the 2-3 day old immature zygospore (Fig. 3a), while the 7-40 day old zygospores showed a decrease in the staining contrast caused by an increase in background fluorescence due to the degeneration of nuclei, which function in the nourishment of fusion nuclei, and the presence of oil globules (Fig. 3b-e).

DAPI staining of the 2-3 day old immature zygospore showed the presence of numerous nuclei (>50). Few nuclei fused to form dumbbell-shaped structures (Fig. 3a). These dumbbell-shaped structures were not observed further and the 5-7 day old zygospore showed a reduction in the number of the nuclei (20-30/zygospore, Fig. 3b). The number of fused nuclei reduced further to 8-10/zygospore in the 15 day old zygospore (Fig. 3c). The size of the fused nuclei was larger than that of the unfused nuclei (Fig. 3c). In the 20 day old zygospore the meiotic division of the nuclei resulted in groups of 4 or 8 nuclei that appeared at 8-10 places in the zygospore (Fig. 3d). After the reduction division the size of the nuclei was similar to that of the unfused nuclei (Fig.3d). The nuclei remained in the same state till 40 day when the nuclei regrouped in a single cluster (Fig. 3e).

Ploidy determination

Ploidy for *B. poitrasii* sporangiospores, vegetative yeast cells (Y) and yeast cells produced by the budding zygospore (Y_z) was determined using i)

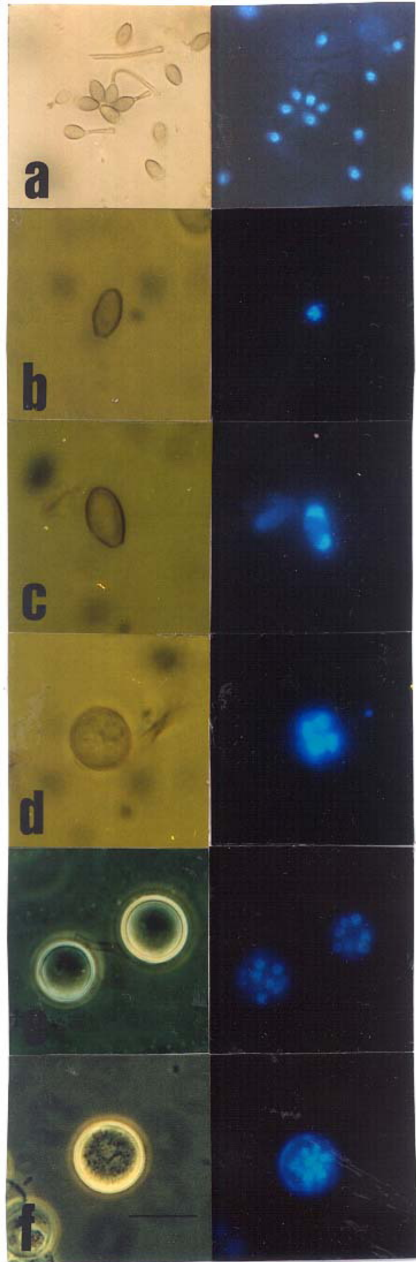

Fig. 2. Nuclear staining with DAPI of sporangiospore germination of *B. poitrasii* and yeast cells**.** a, panel 1- sporangiospores with attached pedicel at 0 h, panel 2, sporangiospores showing uninucleate status. b, panel 1- enlarged spore at 1h, panel 2- uninucleate sporangiospore. c, panel 1- enlarged spore at 2 h, panel 2- binucleate condition. d, panel 1- spherical enlarged cell at 3 h, panel 2- enlarged sporangiospore with 4 nuclei. e, panel 1- yeast-form cell at 4 h, panel 2- yeast-form cell with 8-12 nuclei. f, panel 1- yeast-form cells from the budding zygospore (Y_z), panel 2- Y_z cells with 8-12 nuclei. Panel 1– Phase contrast photographs. Panel 2 – Fluorescence photographs. Magnification 630X. (Scale 1cm =10 µm).

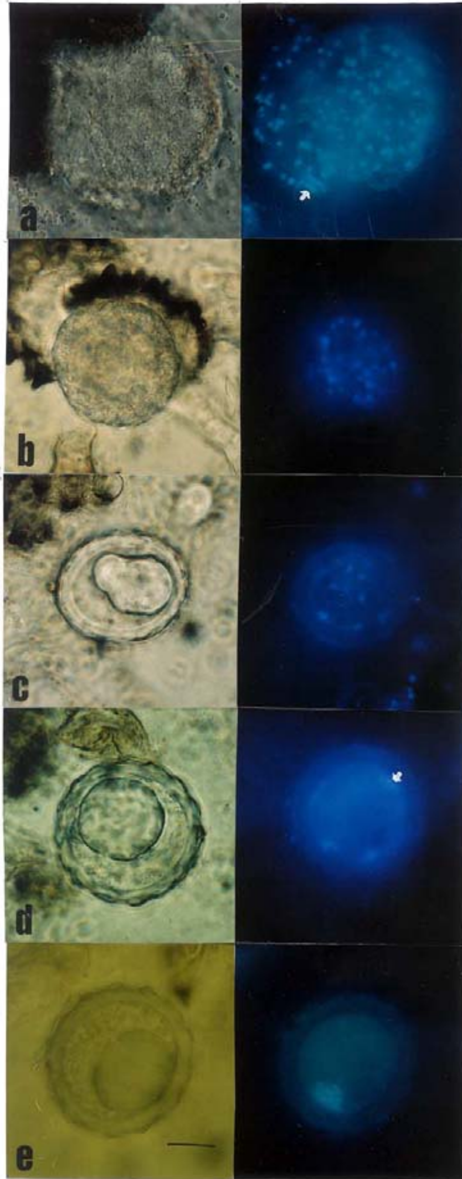

Fig. 3. Nuclear staining of *B. poitrasii* zygospore with DAPI. a, panel 1 - zygospore showing partially removed outer ornamental wall (exospore), panel 2 - endospore with a number of single and a few dumbbell shaped fused nuclei (arrow). b, panel 1 - zygospore without outer ornamental wall, panel 2- endospore with 15-20 nuclei. c, panel 1- endospore containing oil globules, panel 2- endospore with 8-10 nuclei. d, panel 1- endospore, panel 2-endospore after meiotic division with groups of 4 or 8 nuclei (arrow). e, panel 1- endospore, panel 2- endospore showing clustering of nuclei at a single spot. Panel 1 – Phase contrast photograph; Panel 2 – Fluorescence photograph. Magnification 630X. (Scale 1cm = 10µm).

UV survival method, ii) DNA estimation by UV absorption at A_{260} , and iii) Flow cytometry.

UV survival method

The UV survival analysis of sporangiospores and stationary yeast cells *viz* Y and Y_z of *B. poitrasii,* as well as the haploid and diploid *Saccharomyces cerevisiae* strains for comparison was carried out. It can be seen from Table 1 that the percentage survival after 1minute UV exposure of *S. cerevisiae* diploid strain was higher (41%) than the haploid (2%). For *Benjaminiella poitrasii* sporangiospores it was 90% while for the Y and Y_z cells it was 61% and 60% respectively (Table 1).

Measurement of DNA contents

The DNA isolated from *Saccharomyces cerevisiae* haploid and diploid strains and *Benjaminiella poitrasii* sporangiospores, Y and Y_z cells was spectrophotometrically checked for purity using a A_{260}/ A_{280} ratio. For all the samples the ratios were between 1.8-1.9. The DNA content of *Saccharomyces cerevisiae* diploid strain was found to be 1.8 times more than the haploid strain. The yeast cells of *Benjaminiella poitrasii* showed ten times higher DNA content than the sporangiospore (Table 1).

Table 1. The analysis of DNA content of *Benjaminiella poitrasii* and *Saccharomyces cerevisiae*.

Organism	**Survivals after exposure to UV light for 1 minute(%)**	**DNA contents (fg/ cell)**	**Flow cytometry Geometric mean MFI* (AU)**
S. cerevisiae			
Haploid	2 ± 1	21.4 ± 1.1	63.2
Diploid	41 ± 4.3	37 ± 1.9	101.1
B. poitrasii			
Sporangiolum (S)	90 ± 4.3	40 ± 2	100.4
Yeast (Y)	61 ± 13.5	415.9 ± 20.8	115.7
Yeast (Y_Z)	60 ± 17	359.8 ± 18	109.5

MFI, mean (geometric) fluorescent intensity
AU, arbitrary units

Flow cytometry

In flow cytometry for *Saccharomyces cerevisiae* the exponential phase of

growth offered two points of calibration, G-1 and G-2, as compared to stationary phase where only a G1 value was obtained. The difference in the DNA content for cells in G1 and G2 was, two-fold (data not shown). The non-gated populations of stationary haploid and diploid cells of *S. cerevisiae* were analysed on the FL2 W vs the FL2 A for their G1 values. The geometric mean 63.2 AU (arbitrary units) of the haploid strain was 37% less than that of the diploid strain (Table 1).

Similarly, in *Benjaminiella poitrasii* the sporangiospores, stationary Y and Y_z cells were analysed for their G1 values as depicted in Table 1. The asexual sporangiospores showed a single peak with a geometric mean of 100.4 (Table 1). As the yeast cells had a tendency to flocculate they were gated to exclude clumps that appeared with increase in the FL2 W. The Y cells showed a single prominent peak with a geometric mean of 115.7 AU for Y cells (Table 1). The geometric mean for the Y_z cells was 109.5 AU (Table 1).

Estimation of melanin contents of **Benjaminiella poitrasii** *sporangiospores*

The sporangiospores of *B. poitrasii* showed an increased absorption of PI by flow cytometry that gave increased DNA contents. This increased value could be due to the binding of melanin with PI. Sporangiospores (10 mg) were found to contain 12.5 μg of melanin by spectrophotometric estimation. The estimation of adsorption of propidium iodide by solubilised melanin (12.5 μg) from *B. poitrasii* sporangiospores with PI showed an increase in the fluorescence values (3.7 to 4.7) at excitation wavelength of 488 nm and emission wavelength of 617 nm.

Discussion

Among the fungi the asexual spores are uninucleate or in some cases multinucleate (Cole, 1986). Most of the *Mucor* species have a single nucleus/sporangiospore while the multinucleate condition is observed in case of *Mucor mucedo* (Orlowski, 1991).

Cutter (1942) demonstrated 4 types of nuclear behaviour during the formation and the germination of the zygospore. On the basis of the character of nuclear fusion and the time of meiotic division in *Benjaminiella poitrasii* zygospore, this species can be grouped intermediate to the *Absidia glauca* and *Mucor hiemalis* patterns. In *Benjaminiella poitrasii* the time of the nuclear fusion and the reduction division is similar to the *Absidia glauca* pattern while it is similar to *Mucor hiemalis* in that all the fusion nuclei participate in the reduction division (Figs 3a,d). Interestingly, Voigt and Wöstemeyer (2001) have recently shown that the phylogenetic position of *Benjaminiella poitrasii* is

intermediate to *Absidia glauca* and *Mucor hiemalis* on the basis of combined analysis of actin and translation elongation factor EF-1α genes. The 40-day-old zygospore shows clustering of nuclei at what may be at a possible germ pore site in preparation for zygospore germination.

In general, the germination frequency of zygospores beyond 60 day dormancy is very much reduced (Cutter, 1942; Yu and Ko, 1997; Ghormade and Deshpande, 2000). Kayser and Wöstemeyer (1991) suggested that in most mucoraceous fungi the germination of the zygospore have low efficiencies and thus the manifestation of the meiotic process must be a rare event. However, Gauger (1977) stated that meiosis may be induced due to the process of germination in the immature zygospores in *Rhizopus stolonifer*. In *P. blakesleeanus* it was recently suggested that meiosis may not be occurring in the zygospore and the mycelia produced from the germ spores were diploid or partially diploid in nature and the haploid status of the nuclei was restored by gradual loss of chromosomes (Mehta and Cerda-Olmedo, 2001).

Usually the ploidy status of fungi is established by estimating the DNA contents spectrophotometrically, by UV radiation studies and FC analysis (Olaiya and Sogin, 1979; Riggsby *et al*., 1982; Torres-Guerrero, 1999). The *S. cerevisiae* haploid strain did not survive >2% after 1 minute exposure. The survival of *Benjaminiella poitrasii* following UV exposure was >50%. This can be attributed to the multinucleate state of the yeast cells of *B. poitrasii* and ultrastructure of the cell walls (Khale and Deshpande, 1992). In the case of the sporangiospores of *B. poitrasii*, increased survival (90%) might be due to the presence of melanin in the spore walls that provides protection against UV exposure. Fungal spores contain melanin as it helps in their survival under adverse conditions (Fogarty and Tobin, 1994).

The DNA contents show wide variation for fungi belonging to the different taxonomic groups (Clutterbuck, 1995). The DNA contents for the strains of *Saccharomyces cerevisiae* haploid and diploid cells are 24.5 fg/cell and 49.5 fg/cell respectively (Fasman, 1976). In this study, *S. cerevisiae* diploid strain showed 1.8 times more DNA content than the haploid strain (Table 1). In *Aspergillus sojae,* DNA content was 88 fg/cell for haploid and 169 fg/cell for diploid cells (Fasman, 1976). The spectrophotometric analysis of the uninucleate *Benjaminiella poitrasii* sporangiospores revealed that the DNA content/spore was 40 ± 2.00 fg approximately twice than that of the haploid *Saccharomyces cerevisiae*. The DNA content of the parent yeast cells was ten times more than the sporangiospores. This can be attributed to the multinucleate condition of the parent yeast cells. However, DNA contents/nucleus were approximately the same for the sporangiospores (40

fg/nucleus), parental yeast cells (41.5 fg/nucleus) and the Y_z cells (35.9 fg/nucleus).

Flow cytometry has become a reliable method to determine the ploidy of the cells (Dvorak *et al*., 1987). One of the disadvantages, however is that it cannot be used for filamentous cells. Therefore, in the case of the dimorphic fungus *B*. *poitrasii*, it was convenient to carry out the analysis using the yeast-form cells. Flow cytometry utilizes the G1 region of the DNA content axis for comparison (Haase and Lew, 1997). The flow cytometric analysis of the sporangiospores, the parent yeast cells and the Y_z yeast cells gave a geometric mean value that was nearly the same (Table 1). However, the estimation of DNA contents using spectrophotometry showed the appropriate differences in the sporangiospore and yeast cells, that was approximately 10 times more (Table 1). Melanin is known to bind to fluorescent dyes (Saito *et al*., 2002; Ikeda *et al*., 2003). It has been reported that in case of *Cryptococcus neoformans*, the melanised cells showed increased uptake of fluorescent dye during flow cytometry (Ikeda *et al*., 2003). In case of *Benjaminiella poitrasii* melanin from the sporangiospores showed an increase in fluorescence after binding PI. Therefore, the similar geometric mean values of uninucleate sporangiospores and the yeast cells having 8-12 nuclei can be attributed to the adsorption of propidium iodide by the melanin of sporangiospores (Table 1).

The flow cytometric analysis showed that the DNA content values were similar in the parent yeast cells and yeast cells from the budding zygospore (Table 1). The DNA content/nucleus of the uninucleate asexual sporangiospores as estimated by the spectrophotometric method was similar to the DNA content/nucleus of the multinucleate Y and Y_z. Therefore it may be suggested that the haploid condition extends from the zygospore germination until the vegetative and asexual stages in the life cycle of *B. poitrasii;* the diploid condition is probably restricted to the zygospore.

It has been reported that in *Cokeromyces recurvatus,* a dimorphic zygomycete that the germination of asexual sporangiospores in to the yeast-like cells could cause zygomycosis in humans (Kemna *et al*., 1994). While sexual ascospores of the human pathogen *Histoplasma capsulatum*, germinated into yeast-like cells at 37°C (Kwong-chung, 1971). Therefore, the understanding of germination of both sporangiospores and zygospores as well as the time of diploid to haploid change (as the haploid stage is more sensitive) in a non-pathogenic dimorphic fungus *Benjaminiella poitrasii* will help in designing anti-fungal strategy.

Acknowledgements

VG would like to thank the Council of Scientific and Industrial Research, Government of India for the award of a Research Associateship.

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(Received***; accepted 29 March 2005)