DIMORPHISM IN *BENJAMINIELLA POITRASII*: A MODEL SYSTEM TO STUDY THE MORPHOGENESIS AND FOR SCREENING ANTIFUNGAL DRUGS

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DIMORPHISM IN *BENJAMINIELLA POITRASII*: A MODEL SYSTEM TO STUDY THE MORPHOGENESIS AND FOR SCREENING ANTIFUNGAL DRUGS

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Affectionately dedicated to my husband

Satish and daughter Sujya

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DECLARATION

Certified that the work incorporated in the thesis entitled "**Dimorphism in *Benjaminiella poitrasii* : A model to study the morphogenesis and for screening antifungal drugs**" submitted by Vandana Satish Ghormade was carried out under my supervision. Such material as has been obtained from other sources has been acknowledged in the thesis.

(M.V. Deshpande)

Research Guide

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National Chemical Laboratory

Pune, India.

Vandana Ghormade

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List of abbreviations

μg	Micrograms
μl	Microliter
β-ME	β-Mercaptoethanol
μmol	Micromole
AU	Arbitrary units
cAMP	Adenosine 3'5'-cyclic monophosphate
	chitotrioside
DTT	Dithiothreitol
FC	Flow cytometry
FITC-Con A	Fluorescein isothiocyanate concanavalin A
FITC-WGA	Fluorescein isothiocyanate wheat germ agglutinin
GlcNAc	*N*-Acetylglucosamine
h	Hour(s)
LM	Light microscope
4-MU-$(GlcNAc)_3$	4-methyl-umbelliferyl-β-D-*N*,*N'*,*N''*-triacetyl
4-MU-GlcNAc	4-methyl-umbelliferyl-β-D-*N*-acetyl-glucosaminide
min	Minute(s)
PMSF	Phenylmethane sulphonyl fluoride
SEM	Scanning electron microscope
Tris	Tris(hydroxymethyl)aminomethane
w/v	Weight to volume

Glossary of mycological terms used in the thesis
(From Alexopoulos, 1980; Benny *et al*, 1960)

Azygospore, a zygospore that develops parthenogenetically

Budding, the production of a small outgrowth (bud) from a parent cell

Diploid, containing the double (2n) number of chromosomes

Haploid, containing the reduced (n) number of chromosomes

Homothallic, refers to fungi in that sexual reproduction takes place in a single thallus which is, therefore, essentially self-compatible

Hypha, the unit of structure of the fungi; a tubular filament

Meiosis, a pair of nuclear divisions in quick succession, one of which is reductional. Four haploid nuclei are produced as a result of meiosis

Mycelium, mass of hyphae constituting the body (thallus) of a fungus

Sporangiolum, a unisporous ellipsoidal body attached to the vesicle with a pedicel

Sporangiophore, a hypha that bears a sporangium

Sporangium, an aggregation of sporangiola borne on a vesicle

Yeast-hypha transition, unicellular yeast to tubular hypha. In a broader sense, a term yeast-mycelium transition has also been used

Zygophore, a specialized hyphal branch bearing zygospores

Zygospore, a resting spore which results from the fusion two gametangia in the Zygomycetes

CHAPTER I

Introduction

Introduction

"*The nature of things is oft better perceived in small than in great"*

-Francis Bacon

The essential principles of development are most plainly displayed by the simplest creatures as bacteria and fungi. The prokaryotes, bacteria exhibit limited number of morphological forms as cocci, rods, spirals and spores in a few genera as compared to the eukaryotic fungi. On the other hand, fungi produce a variety of morphological structures in their life cycle that broadly include unicellular budding yeasts, coenocytic or septate hyphae, asexual conidia and sporangiola and sexual spores too. Therefore, the knowledge of the mechanism of their formation has been useful to understand developmental processes in higher eukaryotes including embryogenesis.

Differentiation in fungi

Different fungal organisms have been identified and extensively studied to unravel the process of differentiation (Table 1. 1). The vegetative growth in fungi, apart from budding in unicellular yeasts, is generally by elongation of hyphae which is a paradigm of apical growth (Harold, 1999). Hyphae branch at some distance behind the apices i.e. in their sub- apical region where the cell walls may be weakened by enzymes pushed out by cytoplasmic flow to initiate branching (Table 1. 1).

The regulation of cell polarity, kinetics of filamentous growth and the ultrastructural changes in the tip region and biochemical and molecular aspects of hyphal tip growth have

Table1.1. Models for fungal differentiation

Growth pattern	Morphological event	Organism	Reference
Asexual reproduction	Conidia formation	*Neurospora crassa*	Turian and Bianchi (1972)
		Aspergillus nidulans	Martinelli and Clutterbuck (1971)
	Sporangiospore	*Phycomyces blaksleeanus*	Herrera-Estrella and Ruiz–Herrera (1983)
	Conidial discharge	*Conidiobolus coronatus*	Phadtare *et al* (1989)
Sexual reproduction	Fruiting body	*Dictyostelium.mucoroidies*	Garrod and Ashworth (1990)
	Zygospores	*P. blaksleeanus*	O'Donnell (1976)
	Fruiting body	*Schizophyllum commune*	Schwalb (1977)
	Ascospores	*Saccharomyces cerevisiae*	Rendeulez and Wolf (1988)
	Basidial development	*Ustilago maydis*	Staben (1995)
Vegetative growth	Hyphal tip growth	*Saprolegnia monoica*	Fevre *et al* (1990)
		A.nidulans	Grove (1978)
	Branching	*N.crassa*	Mahadevan and Mahadkar (1970)
	Septum formation-filamentous fungi	*Wangiella dermatitidis*	Harris and Szaniszlo (1986)
	Septum formation - budding	*S. cerevisiae*	Herskowitz (1988)
	Sclerotia formation	*Sclerotium rolfsii*	Rawn (1991)
	Arthrospore formation	*Mucor rouxii*	Barrerra (1991)
	Yeast↔Mycelium transition	*Candida albicans*	Odds (1985)
		Histoplasma capsulatum	Kobayashi *et al*(1985)
		Blastomyces dermatitides	Domer (1985)
		Paracoccidioides brasiliensis	San-Blas and San-Blas (1985)
		M.rouxii, M.bacilliformis	Ruiz-Herrera (1985)

Modified from Deshpande (1992).

been studied extensively and reviewed by number of researchers (Bartnicki-Garcia, 1973, Gow 1995;Grove, 1978; Prosser, 1995).

Septum formation occurs in vegetative hyphae of filamentous fungi as well as in yeasts except in most members of the zygomycetes (Gooday and Gow, 1994). However, in *Ballocephala verrucospora*, *Conidiobolus coronatus* and related zygomycetous fungi of Entomophthorales, septum formation was observed during differentiation *viz* conidium development (Latge *et al*, 1989; Deshpande, 1992; Saikawa, 1989). In filamentous fungi septa usually serve to add to the rigidity to the hyphae and can serve to limit any damage that may occur to the tip cell or to the few intercalary compartments. Gull (1978) stated that the main function of the septa is to give hyphae the ability to undergo differentiation such as sporulation.

In budding yeast *Saccaharomyces cerevisiae* a primary chitinous septum bulges centripetally at the region connecting the mother cell to the bud (Cabib *et al*, 1989) The cell division cycle of yeasts is one of the simple morphogenetic pathways intensely studied from the biochemical and genetical perspectives. Budding and apical extension are closely allied and the transitions between them are common. Many fungi switch from apical extension to the generation of a round bud at an early stage of conidiation and the cells of pathogenic yeast *Candida albicans* can grow either in yeast like form or as a hyphae depending on the environmental perturbations (Table 1. 1)

In some fungi, hyphae become interwoven to form small aggregates called sclerotia that have the ability to withstand adverse conditions (Willetts, 1978) *Sclerotium rolfsii* a member of mycelia sterilia group was the most favourable model so far used by the researchers to understand the biochemical changes occuring during the differentiation (Cohen *et al*, 1986; Lachke and Deshpande,1988; Rawn, 1991).

Fungi show a remarkable diversity in their asexual and sexual spores that are adapted for their particular roles in dispersal as well as survival by using different growth mechanisms such as blastic, holoblastic and thallic development (Cole, 1986).

The conidial hyphomycetes like *Aspergillus nidulans,* and the ascomycete like *Neurospora crassa* are still popular models to study differentiation (Table 1. 1). Blastic conidiation was studied extensively in *N. crassa* and *A. nidulans* with the help of developmental mutants. The contrast between the relative simplicity of a zygomycete, *Phycomyces blakesleeanus* and the light induced sporangiospore development has intrigued various researchers. The photophysical response of *Phycomyces* sporangiospore and of the regulation of cell wall chitin synthesis was studied extensively (Herrera-Estrella and Ruiz-Herrera,1983; Miyazaki and Ootaki, 1997). One of the fascinating events of conidial formation and release from the conidiophore and the biochemical changes in *Conidiobolus coronatus* was well-documented (Table 1. 1) (Phadtare *et al* 1989).

Fungi exhibit many variations in their sexual cycles i.e. in the extent of differentiation of sex organs and in the types of spores formed. The well-known processes such as fruiting body formation in *Dictyostelium mucoroides,* zygospore formation in *Phycomyces* and *Mucor*, ascospore formation in *S.cerevisiae* and basidial development in *Schizophyllum commune* , *Poria latemarginata*, *Ustilago maydis* and *Coprinus cinereus* were documented in the literature (Bolker *et al*,1995; Staben,1995).

However among all the morphological transitions being studied to unravel the enigma of eukaryotic development, the study of dimorphism in fungi stands out distinctly, primarily because of its reversible nature (Table 1.1)

Dimorphism, an intriguing phenomenon

Dimorphism is the ability of cells to grow yeast like or mycelium like in a reversible manner that is observed in fungi belonging to different taxonomic groups e.g. Zygomycetes like *Mucor* and *Mycotypha, Benjaminiella,* Ascomycetes such as *Candida* and *Yarrowia and* Basidiomycetes like *Paecilomyces, Ustilago* and *Ophiostoma* to name a few (Barathova *et al*, 1977; Bartnicki-Garcia and Nickerson, 1962b; Bolker *et al*, 1995; Deshpande,1996; Gow, 1995; Muthukumar and Nickerson, 1984; Scherer and Magee, 1990; Schulz *et al*, 1978).

A number of medically important dimorphic fungi examplified by *Histoplasma capsulatum*, *Paracoccidiodes brasiliensis* and *Sporothix shenkii* have been correlated with the saprophytic and parasitic modes of growth. Some researchers restrict the term dimorphism to pathogens such as *Coccidioides immitis* and *Blastomyces dermatitidis* that grow in filamentous form at room temperature and as a budding yeast or as spherules in human body or at 37° C. Others use dimorphism for a fungus that can exist as two different morphological forms, regardless of its nature.

The study of dimorphism in dimorphic fungi possesses distinct advantages as follows:

- It is a reversible process that can be manipulated by the subtle change in the environment that presents an advantage for developmental studies over more popular systems such as *N. crassa* where development is unidirectional.
- It is a phenomenon prevalent among the medically important human pathogenic fungi and can be used as a system to study the cause and management of disease.
- It can be studied to understand the evolutionary relatedness among the different taxonomic groups of fungi.

Factors triggering dimorphism

Different dimorphic fungi respond differently to a number of environmental changes. Among these, the change in growth temperature is the most important factor, especially for human pathogenic fungi. The carbon dioxide concentration, which probably affe cts the oxidation-reduction potential, enhances the morphological conversion in *C. immitis, Y. lipolytica, M. ruoxii* and *S. schenckii*. The pH, glucose concentration, the presence of serum etc, in the growth medium affect the morphological outcome in most of these organisms singly or in combination (Barth and Gaillardin, 1997; Cole and Sun, 1985; Ruiz-Herrera, 1985; Travassos, 1985).

Temperature

Most of the human pathogenic fungi grow in filamentous form at 25°C, and after they enter the human body i.e. at 37° C they change their morphology to the spherical or yeast form. *H.capsulatum*, the causal agent of histoplasmosis grew in a mycelial form (saprophytic) at 28° C and in a yeast form (parasitic) at 37° C. In *Candida* the yeast and mycelial forms are triggered by temperature in conjuntion with other factors such as blood serum, blood glucose,etc (Shepherd *et al*,1979). In *B. dermatitides, S. shenkii, P. brasiliensis* and *Coccidioides immitis* the elevation in the temperature caused the parasitic spherical or yeast form to prevail (Cole and Sun, 1985; Domer, 1985; San-Blas and San-Blas, 1985; Travassos, 1985;). *Benjaminiella poitrasii* a dimorphic, non-pathogenic, zygomycete studied in the present investigations showed yeast form at 37° C and mycelial form at 28° C (Khale *et al*, 1990).

Anaerobiosis

The dimorphic fungi such as *M.rouxii*, *Mycotypha microspora* and *Mycotypha africana* exhibited mycelial growth under aerobic conditions, while the yeast form was favoured by anaerobiosis (Bartnicki-Garcia and Nickerson, 1962a; Schulz *et al*, 1972). Unlike *Mucor* however *S.cerevisiae* and *Y.lipolytica* showed mycelium formation under anaerobic conditions (Viard and Kuriyama, 1997; Zinjarde *et al* , 1996). Interestingly, in case of *Mucor* merely anaerobiosis was not important but the presence of CO_2 or N_2 that created anaerobiosis was significant too (Bartnicki-Garcia and Nickerson, 1962a).

pH

The pH of the medium is known to exert an effect on the form of the fungus. Interestingly, in *Mycotypha* the pH range between 4.5 to 7.5 favoured the yeast form, while the pH below or above this range compeled the fungus to grow in a mycelial form (Schulz *et al*, 1974). Stewart *et al* (1988) reported that in *C.albicans* when the external pH was in the range of 6-8 germ tube formation took place and at or below 4.5 the yeast type growth occurred. They correlated this change to the cytoplasmic alkalinization.

Sugar

The kind and concentration of hexoses in the medium is an important factor in inducing dimorphism. Hexoses such as fructose, mannose and galactose stimulated yeast development in *M.rouxii* in a decreasing order of efficiency (Bartnicki-Garcia, 1968a). In *M.rouxii* hyphal development was favoured in low glucose media while in high glucose media purely yeast growth appeared (Bartnicki- Garcia, 1968a; Friedenthal *et al*, 1974). Again in *B.poitrasii* the response was similar to as seen in the case of *M.rouxii* (Khale *et al*,1990).

Other factors

In *H.capsulatum* the formation of mycelium is promoted by the presence of sulphydryl containing compounds (Maresca and Kobayashi, 1989). In *C.albicans* the yeast mycelium transition is favoured by a medium containing aminoacids such as proline, glutamine and arginine and aminosugars such as *N*-acetylglucosamine and *N*-acetylmannosamine (Gow, 1995; Leija *et al*, 1986; Shepherd and Sullivan, 1976). Serum and serum derivatives also promote germ tube production in the *C.albicans* (Odds, 1985). Metal ions such as zinc have been implicated in the dimorphism of *H.capsulatum* (Pine and Peacock, 1957), *Mucor rouxii* (Bartnicki-Garcia and Nickerson, 1962 a) and *C.albicans* (Sabie and Gadd, 1990). In all three the addition of Zn^{2+} favoured the yeast growth.

Second Messengers

The signal transduced from various environmental factors is transferred via second messengers such as Ca^{2+}, cAMP and inositol lipids and gets translated into specific intracellular responses, important in metabolism, growth and differentiation (Gadd, 1995). The role of Ca^{2+} as a second messenger in fungal growth and differentiation has been investigated in several yeasts and fungi. Calcium can transduce external stimuli such as physical, chemical or electrical signals into specific intracellular effects. Many effects of Ca^{2+} are mediated by calcium calmodulin (CaM) that can also in some cases affect the phosphorylation and dephosphorylation of the proteins (Anraku *et al*, 1991). The role of CaM in morphogenesis has been reviewed in the literature (Gadd, 1995; Paranjape and Datta, 1997).

Apart from calcium, as the second messenger, many aspects of fungal differentiation involve cAMP that binds to cAMP dependent protein kinases (Gancedo *et al*, 1985). Cohen

(1992) reported that after activation, protein kinaseA phosphorylated regulatory proteins that resulted in conformational changes and altered biochemical properties. Larsen and Sypherd (1974) showed that in *Mucor* high intracellular levels of cAMP were associated with yeast cells and low levels were characteristic of hyphal cells. In *M.rouxii* the yeast - mycelium transition was preceded by a decrease in cAMP levels (Paveto *et al*, 1975). In *C.albicans* Niimi *et al* (1980) reported that a rise in the intracellular cAMP accompanying the germ tube formation and addition of its lipophyllic derivative, dibutyryl cAMP (dbcAMP) promoted mycelium formation. However, some strains of *C.albicans* exhibited decreased germ tube formation with increase in cAMP levels. The exact role and contribution of cAMP in the yeast-mycelium transition is not clear.

Cell wall

Fungal cell walls play a major role in determining the shape of the fungal cell (Peberdy, 1990). The mechanical strength of the wall enables the fungi to assume a variety of forms such as penetrative, ramifying hyphae, proliferating yeast cells and spores of many shapes and sizes (Harold, 1997). The cell wall is a dynamic and multifunctional entity. The physical make-up of the cell wall protects the protoplast against environmental stresses like osmotic pressure (Kollar *et al*,1997). While the spore walls provide protection against ultravoilet radiation, enzyme lysis, organic solvents toxic chemicals and dessication. In addition to protection, the outer surface of the cell wall acts as the recognition site for a number of interactions with the outside environment.

During the morphogenetic change of the fungal cell it is the modulation in its cell wall pattern that gives final shape to its physical entity. The change in the synthesis of cell wall components and their organisation governs the final shape of the fungal cell (Bartnicki-Garcia, 1968b; Chaffin *et al*, 1998). The many different forms of fungi result from different

patterns of polarized deposition of cell wall materials. The yeast cells display isotropic pattern of cell wall deposition while hypha follows regulated polarized pattern. Intermediate forms show a deregulated type of growth motif (Szaniszlo, 1985). One of the ways the fungal pathogen protects itself is by changing its cell wall deposition pattern.

Chemical composition

The analysis of the cell walls of fungi belonging to different taxonomic groups have shown a wide range of polymers chiefly polysaccharides (Bartnicki-Garcia, 1968a; Peberdy, 1990; Wessels *et al*, 1983). The shape determining structural or skeletal polysaccharides *viz* chitin, chitosan, chitin- glucan complexes and cellulose make up the inner layer of the wall. These are embedded in more matrix-like polymers such as glucans and glycoproteins which extend outwards to make the outer layer of the wall. The major polysaccharides of the cell wall matrix consist of chitin (polymer of *N*-acetylglucosamine) glucans (polymers of glucose), mannans (polymers of mannose), chitosan (polymer of glucosamine), and galactans (polymers of galactose). Small amounts of fucose, rhamnose, xylose, and uronic acids may be present. The strength and resilience of the fungal cell wall depends on the linkages between chitin and β1-3 glucan as well as among glycoproteins, β1-3 and β1-6 glucans (Chaffin *et al*, 1998; Kapteyn *et al*, 1999).The most common glucans composing cell walls have the β-configuration. Insoluble β-glucans are apparently amorphous in the cell wall. Polymers with (β1-3)- and (β1-6)-linked glucosyl units with various proportions of 1-3 and 1-6 linkages are common. However α-glucans are also common in dimorphic fungi like *P.brasiliensis* (San-Blas and San-Blas, 1985).

In *P. brasiliensis*, the hyphal cell wall consists of chitin and β-glucan. In contrast, the thick yeast cell wall has the inner chitinous surface, containing some α-glucan, and the outer layer containing α-glucan. It has been suggested that the (α1-3)-glucan occurs in a

microfibrillar form in *P. brasiliensis* (San-Blas and San-Blas, 1982), *H. capsulatum* (Kobayashi *et al*, 1985) and *B. dermatitidis* (Domer, 1985). The increase in (α1-3)-glucan content of the cell wall was related to the virulence for the strain too (Klimpel and Goldman, 1988; San-Blas *et al*, 1984).

Many fungi, especially the yeasts, have soluble peptidomannans as a component of their outer cell wall in a matrix of α- and β-glucans. Mannans, galactomannans, and, less frequently, rhamnomannans were also reported to be present in medically important fungi (Gil *et al*, 1994; Travassos,1985).

In addition to chitin, glucan, and mannan, cell walls may contain lipid, protein, melanin, and inorganic ions such as phosphorus, calcium, and magnesium. A number of dermatophytes contain glycopeptides are present and the proportions of these components vary greatly from fungus to fungus. Table 1. 2 summarizes the relationship between cell wall composition and taxonomic grouping of the fungi.

Chitin, a key structural polymer

The rigid cell wall of fungi is composed of a stratified structure consisting of chitinous microfibrils embedded in a matrix of polysaccharides, proteins, lipids, inorganic salts, and pigments that provides skeletal support and shape to the enclosed protoplast. Chitin is the β(1-4) linked polymer of *N*-acetylglucosamine (GlcNAc), its deacetylated form is known as chitosan, the β(1-4) linked polymer of glucosamine. Chitosan is formed by progressive deacetylation of chitin. It is present in the cell walls of zygomycetous fungi. It has been reported to be present in the ascospore wall of *Saccharomyces cerevisiae* (Briza *et al*, 1988).

Chitin is produced in the cytosol by the transfer of GlcNAc from uridine diphosphate GlcNAc (UDP-GlcNAc) into chains of chitin by chitin synthase, which is located in the

Table 1. 2 Cell wall composition in the different taxonomic groups.

Structural polymers	Matrix components	Taxonomic group	Representative genera
Chitin, chitosan	Glucan	Zygomycetes	*Rhizopus arrhizus*
Chitin, β-(1-3), β-(1-6) glucan	Galacto-mannoproteins, α-(1-3)-glucan	Ascomycetes Basidiomycetes Fungi Imperfectii	*Pseudellescheria boydii* *Schizophyllum commune* *Phialophora verrucosa*
Chitin, β-(1-3), β-(1-6) glucan	Xylomanno-proteins, α-(1-3) -glucan	Ascomycetes Fungi Imperfectii	*Saccharomyces cerevisiae* *Candida albicans*

Modified from Peberdy (1990)

cytosol in organelles called chitosomes (Bartnicki-Garcia *et al*, 1978). The chitin microfibrils are transported to the plasmalemma and subsequently integrated into the new cell wall.

Although the wall polymer is synthesized within the cytoplasm or at the plasma membrane level a degree of cross-linking as well as assembly takes place within the cell wall. The chitin molecules have great mechanical strength. Individual polysaccharide chains are hydrogen bonded between adjacent GlcNAc units, giving them rigidity.

Biosynthesis

For the biosynthesis of chitin, both carbon and nitrogen metabolism pathways are essential. The enzyme glucosamine-6 PO_4 synthase (glutamine-D-fructose-6-PO_4 amido transferase, EC 2.6.1.16) brings together these two pathways for chitin synthesis. However, the key enzyme for chitin synthesis is chitin synthase (EC 2.4.1.16). The chitin synthesis in fungi are well documented in the literature (Bulawa, 1993; Cabib *et al*, 1990; Gooday, 1990).

However, the research into the regulation of chitin synthase (CS) has been hampered by the lack of progress in purification studies. In fact, the activity of CS must be under precise control so that chitin is deposited at the right time at the right location.

In most of the fungi, chitin synthases are chiefly zymogenic, being activated often manifold, by proteolysis (Cabib *et al*, 1990). Activation by proteases though appears to be a general mechanism for regulation of fungal CS other possible regulatory processes have not been discarded.

CS zymogen

↓

Active CS

↓

$$\text{UDP-GlcNAc} + (\text{GlcNAc})_n \rightarrow (\text{GlcNAc})_{n+1} + \text{UDP}$$

Different hypothesis of chitin regulation

The alternate concept that the chitinous wall must be continuously loosened by lytic enzymes in order to expand has been favoured by Bartinicki-Garcia (1973). The growth of the wall at the hyphal apex requires that the wall in this region has plastic properties which contrast with the requirement of rigidity elsewhere in the hyphae. The concept presumes a delicate balance between wall synthesis and wall lysis that involves the participation of the wall lytic enzymes to plasticize the wall at the apex and to allow new wall material to be inserted. Evidence for the association of chitinase a wall degrading enzyme, with CS comes from paralled behaviour of the two activities during spore germination in *M.mucedo* (Gooday *et al*, 1986) and during exponential growth in *M rouxii* (Rast *et al*, 1991) and *C.albicans* (Barrett-Bee and Hamilton, 1989). The enzymatic hydrolysis of chitin to GlcNAc is performed by different enzymes working sequentially. These include endo-chitinase (EC 3.2.1.14) and β-*N*-Acetylglucosaminidase (EC 3.2.1.52) (Patil *et al*, 2000).

The third hypothesis regarding the regulation of cell wall chitin synthesis states the contribution of membrane stress or local stretching in activating chitin synthase. The activities of membrane bound enzymes can be affected significantly by the changes in membrane fluidity. It has been suggested that CS could be stretched locally by membrane stress or local stretching. In *C. albicans* and in *B.poitrasii* cells that were subjected to hypo-osmotic stress had increased native chitin synthase specific activities (Deshpande *et al*, 1997; Gooday and Schofield, 1995).

Number of fungi are found to produce competitive inhibitors of chitin synthase. For instance, Wenke *et al* (1993) reported CS inhibiting activities in the culture filtrate of an osmophilic strain *Aspergillus fumigatus*. Two compounds namely pseurotin A and 8-O-demethylpseurotin A have been identified. The production of such type of CS inhibitors by fungi can be correlated to the regulation of chitin synthase, directly or indirectly.

Other biochemical correlates

It has been suggested that proteolysis is one of the essential processes for the post-translational control of cellular functions. Proteinases are found to play a significant role in the morphological event in fungi (Deshpande, 1992). They are important in activation of zymogenic cell wall building enzymes, removal of interfering protein during differentiation, processing of precursor proteins and the hydrolysis of cell wall that in turn affects fungal differentiation. In *Saprolegnia monoica* the cellulosic cell wall is overlaid by an glucan layer, and the enzyme (β1-3) glucan synthase involved in the synthesis of glucans, probably exists in the inactive form and can be activated by moderate proteolysis (Fevre *et al*, 1990). In contrast, (β 1-4) glucan synthases from *S. monoica* can be inactivated by trpysin under identical experimental conditions. Fevre *et al* (1990) suggested that both of them must be having different regulatory processes with respect to time and location in the cell wall synthesis.

In case of most of the dimorphic fungi such as *M. rouxii* and *B. poitrasii* the life span of active chitin synthase may be regulated by proteinases as in the yeast form cells, zymogenic form of the enzyme predominates, while in the mycelial form cells, the reverse was true (Deshpande *et al*, 1997; Zou *et al* 1990). While Cohen *et al* (1986) reported that *in vivo*, endogenous acid proteinases might function to activate *S.rolfsii* chitin synthase, that was important in the formation of large number of hyphal branches during sclerotia formation.

The involvement of a proteinase, especially serine proteinase, in the growth cycle of *C.coronatus*, in particular in the conidial discharge , has been suggested by Phadtare *et al* (1989). The possible role of serine proteinase could be in the activation of chitin synthae involved in the formation of chitin septum to separate conidium from the conidiophore.

Other examples, reporting correlation of intracellular proteinase activity and asexual sporulation are *Blastocladiella emersonii, Phycomyces blakesleeanus*

Botryodiplodia theobromae, Microsporium gypseum and *Dictyostelium sp.* In all these fungi, proteinases are involved in either activation of chitin synthase, processing of spore specific precursor protein like muiridin, or in the removal of unwanted proteins (Deshpande, 1992). Terashita *et al* (1981) reported that during fruit body formation in *Lentinus edodes* the level of proteinase that is important in fruit body formation is controlled by serine proteinases.

Glutamate dehydrogenase / glutamine synthetase/ glutamate synthase

The nutritional requirements of yeast form of dimorphic fungi like *Mucor* and *B.poitrasii* are in general more specific than those of mycelial form. The optimal yeast growth required the presence of a hexose as a carbon source as well as an organic amino compound as a nitrogen source (Khale *et al*, 1990). In contrast, the mycelial growth occured on a variety of carbon sources and with ammonium salts as the sole nitrogen source. These observations suggested differential expression of some key enzymes of carbon and nitrogen metabolism in the two morphologial forms. Generally in fungi the carbon and nitrogen metabolisms are connected via ammonia assimilation and the participating enzymes are NAD- dependent (EC 1.4.1.2) and NADP- dependent (EC1.4.1.4) glutamate dehydrogenase (GDH) glutamate synthase (GOGAT EC 1.4.1.13) and glutamine synthase (GS, EC 6.3.1.2).

In *M.racemosus*, both NAD- and NADP- dependent glutamate dehydrogenase were reported (Peters and Sypherd, 1979). However only NAD-GDH showed the form specific difference in the level of activity. The levels of this enzyme were 10- fold lower in yeast cells than mycelia. Exogenous addition of cAMP, that induces the yeast morphology under otherwise the mycelial form favouring condition (i.e. an aerobic atmosphere) repressed the NAD-GDH activity to levels found in anaerobic yeast, suggesting a possible correlation of enzyme activity and cell morphology. The NADP-GDH activity remained practically unchanged during yeast mycelium transition.

In case of *B.poitrasii* studies on the levels of GDH, GS, GOGAT are performed as a function of temperature, nutritional conditions and the morphological form of *B.poitrasii* (Khale and Deshpande, 1993; Khale *et al*, 1992). The changes in GS and GOGAT levels did not show any appreciable correlation with the morphological outcome. Since both NAD- and NADP- dependent GDH activities were found in *B.poitrasii,* the quantitative relation between these two enzymes expressed as the NADP-GDH/ NAD-GDH activity ratio (GDH ratio) was reported to evaluate its probable role in morphogenesis (Khale *et al*, 1992). It has been observed that an increase in the GDH ratio led to a change in the morphology from mycelial to the yeast form .

Polyamine synthesis

Polyamines, putrescine, spermidine and spermine are present in all living forms and are reported to be significant contributers in growth essentially by interacting with nucleic acids and membranes (Deshpande, 1998; Shapira *et al*, 1989). In fungi ornithine decarboxylase (ODC) has been associated with a variety of morphological changes. The ODC is a key enzyme that regulates polyamine level in the cell (Martinez-Pachico and Ruiz-Herrera, 1993). During dimorphic transitions in *Mucor* species (Orlowski, 1991) changes in both putrescine levels and ODC activity have been correlated to the morphogenesis, The rate of translation during yeast to mycelial transition in *M.racemosus, M.rouxii* and *M.bacilliformis* may be facilitated by the increase in putrescine levels. Cano *et al* (1987) checked the levels of methylation , during spore germination in the presence and absence of 1,4 diamino-2-butanone (DAB, an ODC inhibitor), using restriction enzymes *MspI* and *HpaII.* When ODC was inhibited in the spores by DAB, the polyamine levels are reduced causing hypermethylation of DNA, preventing germ tube formation from the spores. The

addition of 5-azacytidine , an inhibitor of DNA methylase reversed the effect of DAB on the mycelial growth (Cano *et al*, 1987).

Genetic studies of dimorphic fungi

Genetics in the present day sense deals with the origin, evolution and transmission of the nuclear contents with interactions with the environment with its physical and chemical constitution and its influence on development. The understanding of cellular differentiation in *S. cerevisiae* because of the integration of information on physiological and molecular aspects is most impressive (Herskowitz, 1988). To understand the molecular basis of any phenotypic change in the living organism three different approaches have been used i) the isolation of mutant by treating the parent strain with different mutagenic agents ii) protoplast fusion and iii) molecular cloning.

Isolation of monomorphic mutants of a dimorphic fungus is an excellent tool to examine the regulatory role of genetic machinery in the morphogenesis. In case of *Y.lipolytica* formerly known as *Candida lipolytica*, morphological mutants lacking the ability to change into hyphae were isolated (Barth and Gaillardin, 1997). The mutations in the genes *SEC14*, *CDR1* and deletion of *XPR 6* affect the yeast-hypha transition. However, the role of proteins encoded by these genes in the transition is not well understood (Barth and Gaillardin, 1997). In case of *B.poitrasii* isolation of stable yeast form mutants Y-2 and Y-5 has also been reported (Khale *et al*, 1990). The mutants remained in the yeast form when grown under dimorphism triggering conditions. However, the presence of ethanol (0.5% w/v) both the mutants showed germ tube formation and subsequent mycelial growth (Khale *et al*, 1990).

In the case of human commensal, one of the well studied organisms, *C. albicans*, early studies on genetics involved mutations using ultraviolet light and chemical mutagens such as nitrosoguanidine and ethyl methane sulphonate (Olaiya and Sogin, 1979). The UV

survival curves of *C. albicans* were similar to that of diploid *S. cerevisiae* indicating its diploid nature. This explained the refractory nature of *C. albicans* to mutant manipulations. However, the mycelial mutant, that required further characterization was reported by Cannon (1986). *Candida* is considered as one of the dreadful fungi in immunocompromised patients. The modern era of *Candida* genetics has moved rapidly from mutation to molecular cloning and electrophoretic karyotyping. In case of another human pathogen, *H.capsulatum* similar studies have been reported (Maresca and Kobayashi, 1989). The morphogenesis in *M.bacilliformis* was studied using nitrosoguanidine mutagenesis (Lasker and Borgia, 1980; Orlowski, 1991). Electrophoretic karyotyping was used in *Absidia glauca* to estimate the genome size and the number of chromosomes (Kayser and Wostemeyer, 1991).

Morphogenes and morphogenesis

The switch in the growth form has been studied extensively at the gene level which offered a model for the regulation of morphogenesis (Bossche *et al*, 1992). The genetic approach directs attention to individual genes and gene products that play a role at different stages of the process of differentiation (Cannon *et al*, 1994). In the case of *S. cerevisiae CDC* mutants were isolated, which showed arrest in the cell cycle at particular stage related to abnormal morphology (Harold, 1995). A similar approach was used to construct mutants for the analysis of roles played by different genes in the morphologenesis, particularly in the dimorphic transition in *C. albicans, H. capsulatum* and *M. racemosus* (Gow *et al*, 1994; Maresca and Kobayashi, 1989; Cano-Canchola *et al*, 1992). These mutants can be classified into three groups (Table 1. 3). First group comprised of mutants for genes coding for the structure or production of primary cellular building blocks such as, actin, tubulin and cell wall chitin. In the second group, the mutants of house keeping genes for cell division cycle

Table 1. 3. Key genes in the regulation of cellular processes suggested to be involved in morphogenesis.

Genes encoding -	*S. cerevisiae*	*C. albicans*	*H.capsulatum*	*M. racemosus*	*U maydis*	*P. brasiliensis*	*Y.lipolytica*
Cellular regulators	*BEM1, PKC1, CDC24,STE7, STE11,12,20, CUP*	*CST20, TUP1, ACPR,CPH1, EFG1,RBF1, SAP1-7, HST 7*	*HSP70,HSP83*	*CUP*		*HSP70*	*XPR2*
House-keeping, genes	*CDC3,CDC30 CDC10-12*						
Cellular building blocks	*CAP2,MYO2, TPM1,PFY2, CHS2,3,5*	*CHS 1, CHS 2*	*TUB1, TUB2*				
Product unknown		*HYR1*	*YPS3*		*EG11*		

S. cerevisiae- BEM ,bud emergence (Cabib *et al*,1998); *CAP2*, capping protein; *CHS 2,3,5,* chitin synthase; *MYO2*, myosin; *PFY2*, profilin (Harold,1995); *STE* 7,11,20, kinases ; *STE 12* transcriptional factor (Liu *et al*,1994); *TPM*, tropomyosin (Harold,1995)

C.albicans -CHS1,2, chitin synthase (Gow *et al*,1993*); EFG1,* enhanced filamentous growth (Stoldt *et al*, 1997)*; HYR* , hyphally regulated gene (Bailey *et al*,1996*); HST 7* mitogen activated protein kinase components (Gow *et al*,1995*); RBI 1,* RPG box binding factor 1 (Magee, 1997); *SAP 1-7*, aspartyl proteinase gene (Hube *et al*, 1994); *TUP1*, transcriptional regulator (Magee, 1997).

H.capsualtum- HSP 70 and *83*, heat shock protein*; TUB 1,* α tubulin*; TUB 2,* β tubulin*;YPS 3* , yeast phase specific gene (Maresca and Kobayashi, 1989*).*

M.racemosus-CUP, Copper metallothein gene (Cano-Canchola *et al*,1992)

U.maydis- EG 1 filamentous growth gene (Bolker *et al*,1995).

P.brasiliensis - HSP 70, heat shock protein (Da Silva, 1999).

Y.lipolytica - XRR 2, alkaline protease (Madzak *et al*, 1999).

are classified. While in the third group genes for cellular regulators like protein kinases, calcium binding proteins, etc. are present. Interestingly, two examples of form-specific genes, the products of which are not well understood can constitute, if necessary a fourth group (Table 1. 3). The genes related to various functions have been studied in different dimorphic systems (Table 1. 3). The protein kinases STE11, STE12, STE20 and STE 7 were found to be essential during pseudohyphal growth in *S. cerevisiae* (Liu *et al*, 1994). Transcriptional activators like TUP1 and EFG 1 were reported to be important for the dimorphic transition in *C. albicans* (Stoldt *et al*, 1997; Magee, 1997). The *CHS1* and *CHS2* genes were differentially expressed during yeast and hyphal growth (Gow *et al*, 1993). In case of *C.albicans* nine acid proteinases (SAP1-9) were reported which showed differential expression (Hube *et al*, 1994; Gow, 1996). Aspartyl proteinase genes *SAP2* and *SAP3* were expressed in the hyphae and *SAP1, SAP 2, SAP 3* in the opaque form of phenotypic switching strain 1, while *SAP* 4, *SAP5* and *SAP6* were reported to be hypha specific.

In *H. capsulatum*, α-(*TUB1*) and β-(*TUB2*) tubulin genes were reported from yeast-form cells while two additional isoforms α_2- and β_2- tubulin were reported in the mycelial form (Maresca and Kobayashi, 1989). In *H. capsulatum* heat shock proteins HSP70 and HSP83 were thought to effect the mycelium to yeast transition too. In *P. brasiliensis*, a causative agent for paracoccidiodis in S. America, *HSP70*
gene was expressed with a much higher level in the yeast form than the mycelial (Da Silva *et al*, 1999).

The *YPS3*, a yeast form specific gene in *H. capsulatum* is developmentally regulated and the gene product has been localized to the cell wall and the culture supernatants (Weaver *et al*, 1996). In *C. albicans* the *HYR1* gene associated with its pathogenic form-hyphae was not reported to encode any identifiable product (Gow, 1996). The *EG11* gene associated with the hyphal form in *U.maydis* was suggested to have a speculative role (Bolker *et al*, 1995).

In the dimorphic zygomycete, *M. racemosus* the *CUP* gene encoding a metallothein, was reported to be developmentally regulated (Cano-Canchola *et al*, 1992). The *CUP* genes were not transcribed in the spores, while germ tube emergence was correlated with its active transcription.

The studies on genetics have helped in probing the cellular structure and function at the molecular level. Genetic analyses of dimorphic fungi by estimating the genome size, DNA contents, electrophoretic karyotyping revealed information about the genome and the genetic map of these fungi.

Many genes involved in important physiological processes have been isolated enabling the analysis of their relationships to the process of morphological change. Differential regulation through processes like DNA methylation may play a role in the form specific expression of some of the genes as revealed by the methylation pattern in *U. maydis* and *Y. lipolytica* (Reyna-Lopez *et al*, 1997). The molecular genetic approach combined with cell biological and biochemical approaches will enable an understanding of the morphogenetic phenomenon.

In *M.racemosus* a dimorphic zygomycete, genetic analysis has been hampered by the long dormancy of the sexual zygospore, scarcity of markers and the exacting conditions required for mating. Lasker and Borgia (1980) suggested the use of protoplast fusion in the analysis of the mutants of *M.racemosus*, by the study of complementation and dominance studies.

DNA methylation

Modification of DNA in its bases has been proposed as method for modulating access to genetic information. Differential DNA modification has been demonstrated to be an important level of genetic regulation in prokaryotes (Arber, 1979). In bacterial methylated

residues such as m^6A, m^5C or m^7G residues function in the restriction modification systems (Arber, 1979). In case of eukaryotes the methylation of DNA, particularly at the 5-position of the cytosine residues in CpG and CXG sequences is known to affect the expression of a variety of genes (Doerfler, 1983; Li *et al*, 1993; Razin and Cedar, 1991).

The only naturally occurring modified residue found in the DNA from various vertebrates and higher plants is m^5C. DNA of the higher organisms contain a significant amount of methylated cytosines 2-7% of total cytosines in mammals (Vanyushin *et al*, 1979) and upto 30% in higher plants (Thomas and Sherratt, 1956). This modified base is very scarce among insects (Wyatt, 1951; Adams *et al*, 1979) and undetectable as in *Drosophila* (Urieli-Shoval *et al*, 1982) but it is present in the genome of the lower invertebrates like the sea-urchin (Chargaff *et al*, 1952).

Modification of different bases

The DNA of lower eukaryotes often contains m^5C as a minor base and in addition m^6A . DNAs of several types of protozoa contain m^6A (0.2- 2.5 mole %) rather than m^5C (Ehrlich and Wang, 1981). The nuclear DNA in the unicellular alga *Chlamydomonas reinhardii* contains both m^6A (0.5 mole %) and m^5C (0.5 mole %) just as DNAs of most studied bacterial species contain both modified bases (Ehrlich and Wang, 1981).

Biological importance /functions of DNA methylation

The significance of methylation as a post-replicative modification has been well documented. DNA methylation has a characteristic pattern in each organism that protects its own DNA from endonuclease degradation (Kumpatta *et al*, 1998). Rountree and Selker (1997) demonstrated that in *N.crassa* DNA methylation inhibited the process of transcription

elongation rather than initiation due to chromatin modifications caused by DNA methylation which inhibited the RNA polymerase II activity.

In fungi the presence of m^5C has been demonstrated in a few species (Antiquera *et al*, 1984; Jupe *et al*, 1986; Reyna-Lopez *et al*, 1997). With respect to methylation the fungi appeared to be a heterogenous group (Antiquera *et al*, 1984). The m^5C contents range from undetectable levels (≤ 0.1% in more than 20 species tested) to 0.2-0.5% in case of *Sporotrichum dimorphosporum* and *P.blackesleeanus*. In the species endowed with cytosine methylation, the modified base is located mostly in CpG sequences like higher eukaryotes (Antiquera *et al*, 1984; Dörfler, 1983).

Zolan and Pukilla (1986) reported that the levels of methylation were higher at a centromere linked locus in the basidiomycete *C.cinereus* which was transmitted through meiosis. Gene inactivation by DNA modifications in *N. crassa* was RIP, in which the duplicated sequences detected in the pre-meiotic phase were subject to point mutations (Cogoni and Macino, 1997). DNA methylation induced premeiotically has been shown to play a role in transcriptional gene activity in *Ascobolus immersus* (Goyan and Faugeron, 1989). This process was similar to *N. crassa* except methylation was reversible in *A. immersus* (Goyan and Faugeron, 1989).

Differential DNA methylation in life cycle of fungi

The level of cytosine methylation was found to change during the life cycle of *N.crassa.* The highest levels were found in conidia (mol % m^5C, 0.36), conidial germlings and stationary phase mycelial cells (mol % m^5C, 0.40), while the exponentially growing cells showed lowest levels (mol % m^5C, 0.24) (Russell *et al*, 1987). In the plant pathogen *Phymatotrichum omnivarum*, approximately three fold more m^5C residues were found in DNA from the dormant sclerotia than the DNA from metabolically active mycelia (Jupe *et al*,

1986). Higher levels of methylation were associated with dormancy or low transcriptional activity while high level of transcriptional activity was correlated with the low methylation level and active growth. Cano *et al* (1987) obtained evidence that *M. rouxii* sporangiospore DNA had more residues of m^5C than the growing hyphae. Cano-Canchola *et al* (1992) reported that methylation was involved in selective gene expression during spore germination. For example, the *CUP* genes from *Mucor* were present in an hypermethylated DNA region in non-growing and isodimetrically growing spores and were not transcribed at these stages. After germ-tube emergence, *CUP* genes became demethylated and transcriptionally active.

The relationship between DNA methylation and differentiation was noted and studied in dimorphic fungi by Reyna-Lopez *et al* (1997). The representative genera studied were: *M. rouxii, Y. lipolytica* and *U. maydis*. The methylation status was found to be different in yeast- and mycelial-form cells. The studies on methylation pattern will be useful to understand the interrelationships among the dimorphic fungi and their position in the phylogenetic tree. The methylation contributes significantly in regulating gene expression during the development process. Therefore, these studies can be extended to know the phenomenon of dimorphism and subsequently the antifungal strategies.

Dimorphism as a model to study antifungal agents

Different targets have been reported for the action of antifungal agents (Fig 1. 1). However, there is a continuous need to identify novel antifungal drugs.

Several of the human fungal pathogens show the phenomenon of dimorphism. The yeast-mycelium transition in different pathogenic fungi as *Histoplasma, Candida* is important in proliferation of the fungi in the host. Frost *et al* (1998) used *Candida* as a screen to identify novel antifungal inhibitors interacting with the synthesis, assembly and regulation of

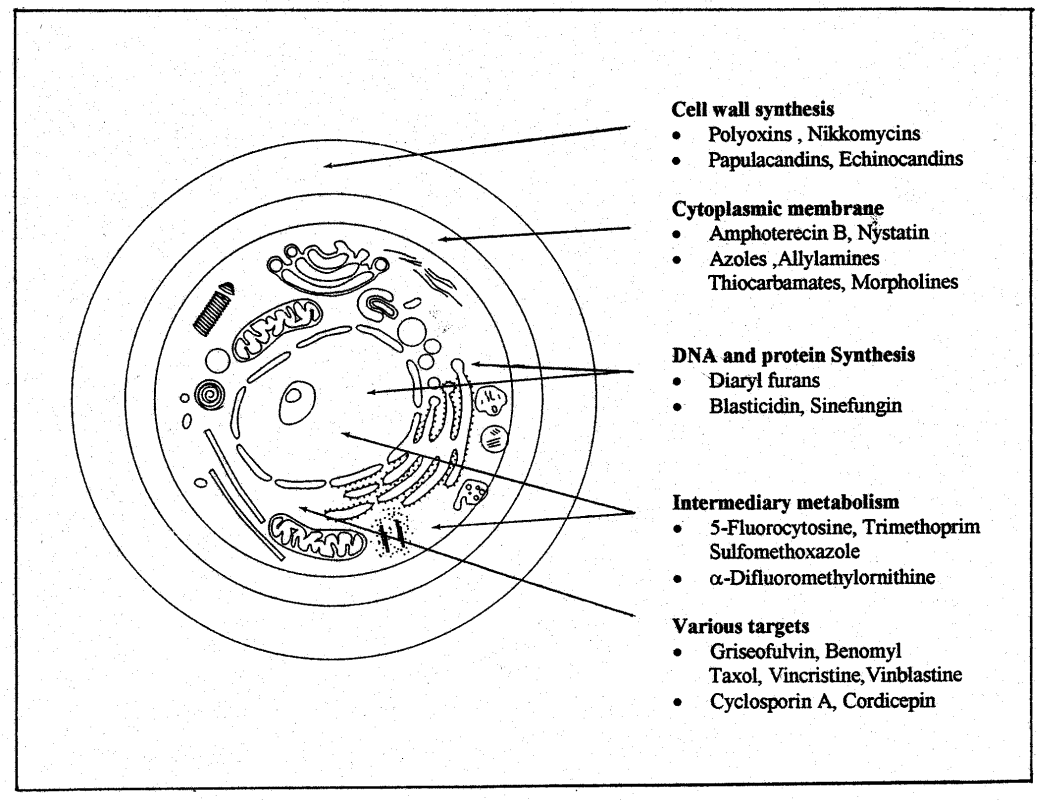


Fig 1.1: Sites of action of some antifungals (modified from Groll *et al*, 1998)

fungal cell wall. Depending on species, pathogenesis is dependent or at least favoured by the switch from one growth form to another so this process itself, may be a target for antifungal chemotherapy. Dimorphism serves as a model for the investigation of the effect of antifungal agents.

Models to screen cell wall inhibitors

In the continuous search for new antifungals, various screening systems have been used to evaluate the effect of the antifungal antibiotics. Hutter *et al* (1965) based the screening test in the morphological changes of the hyphae of *Botrytris cinerea.* Later the same group reported the inhibition of zygospore formation using *Mucor hiemalis* to screen for antifungal antibiotics. Benitez *et al* (1976) studied the germination of spores, mycelial growth and the cell wall biosynthesis of *Trichoderma viride.* The effect of cell wall inhibitors was tested on the mycelia of *B.cinerea* and *M.ruoxii* by Zhu and Gooday (1992). Sukurai *et al* (1999) employed *S.cerevisiae* and its CS I defective mutant to screen for new antifungal antibiotics. Dimorphic fungi have been used to test the efficacy of antifungal drugs such as *P.brasiliensis*, *H.capsulatum* and *C.albicans.* Among them the yeast-mycelium transition in *Candida* has been used as a test for the antifungals to halt the transition as the hyphal form is required for the invasion of tissues (Frost *et al*, 1998).

Dimorphism, a model process to understand fungal differentiation and control of fungal infections

The dimorphic process is an example of fungal differentiation where the morphology is realized in response to the environmental conditons. Dimorphic fungi express this morphological change by altering the pattern of biosynthesis of the cell wall, the final shape determinant. The environmental signals transduced on the cell surface are transmitted to final

realization in the morphological change due to the exquisite interplay of various metabolic processes. The study of the biochemical and morphological changes occuring due to environmental changes in dimorphic fungi can serve as a model system to explore basic regulatory mechanisms that are important in eukaryotic differentiation.

Dimorphism has a dangerous dimension when seen in terms of the ability of pathogenic fungi to change their form to cause infection. Dimorphic pathogens such as C*andida, Histoplasma, Wangiella*, etc can be used *per se* to understand the cause, cure and control of various systemic fungal diseases. However their use as a model faces a number of challenges because of slow growth rate, difficulties in generating clones from single cells, the induction of synchronous growth (Deshpande, 1996; Gow 1995). Several pathogenic fungi are require 24-48h for the complete yeast-mycelium transtion (Domer, 1985). The transition of yeast cells to mycelium in *P.brasiliensis* was within 18h. In case of *C.albicans* as a model the multiple triggering factors such as temperature, pH and serum must be taken into account (Gow, 1995). *W.dermatitidis*, a causative agent of phacohyphomycosis in humans, in addition to the long term incubation, displayed polymorphic forms during transition from thin walled and thick walled yeast, multicellular form, moniliform hyphae and true hyphae (Kester and Garrett, 1995). Therefore, the use of *W.dermatitidis* as a model is restricted. The genus *Mucor* has been an object for medical and developmental studies for many years and responds to glucose and anaerobiosis during morphogenesis. It has received limited success as a model system that may be correlated to the strain dependent variations in the morphological changes. *B.poitrasii*, the fungus identified for the present investigations is a saprophyte and shows glucose and te mperature dependent morphogenesis. The yeast-mycelium transition is rapid and without formation of any intermediate forms. The Chapters III-V will discuss the importance of *B.poitrasii* as a model system.

Present investigations

Based on the literature survey the studies on *B.poitrasii* as a model system to study the morphogenesis and for the screening of antifungals were carried out as follows.

- To investigate the presence of the dimorphic character in the different phases in the life cycle of *B.poitrasii.*
- To study the relative importance of dimorphism triggering environmental changes in the yeast-mycelium transition in *B.poitrasii.*
- To carry out studies for determination of ploidy status of the organism.
- To determine the significance of the chitinolytic enzymes in the morphological transition.
- To test the effect of various inhibitors using *B.poitrasii* as a screen and to understand the significance of morphogenesis in pathogenic fungi.

CHAPTER II

Materials and Methods

Materials

The materials and chemicals used in the studies were purchased from suppliers as shown in Table 2. 1.

Methods

Organisms and culture conditions

The parent strain of *Benjaminiella poitrasii* , a homothallic, zygomycetous dimorphic fungus and its monomorphic mutant (yeast-form, Y-5) were maintained and subcultured weekly on YPG (yeast extract, 0.3%; peptone, 0.5%; glucose, 1%; agar, 2%) slants at 28°C. The stable yeast-form mutant (Y-5) was isolated after mutagenesis using nitrosoguanidine of the spores of parent strain (Khale *et al*, 1990). The growth in liquid as well as on agar media was used for further studies after appropriate treatments as discussed in subsequent sections.

The haploid (Strain 103X n) and diploid (Strain 103X 2n), of *Saccharomyces cerevisiae*, a kind gift from Dr.Zita.Lobo of Tata Institute of Fundamental Research, Mumbai was maintained on YEPD (1% yeast extract, 2% peptone, 2% dextrose) slopes at 37°C.

The bacterial isolates *Bacillus* 101, *Bacillus* 102, *Streptomyces sp* and fungal cultures *Chaetomium* and *Volutella* screened for anti-fungal activity were maintained on MGYP (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3% and peptone, 0.5%) slants.

The mycolytic enzyme producing fungus, *Myrothecium verrucaria* NCIM 903, and a root infecting plant pathogen, *Sclerotium rolfsii*, NCIM 1084 were maintained on potato dextrose (2% PDA) slants.

Yeast-mycelium and* vice versa *transition

The environmental factors such as temperature, glucose concentration of the media and the pH affected the dimorphic behaviour of *B.poitrasii*. The effect of change in glucose

Table 2.1 Source of chemicals and materials

Chemicals, Materials	Supplier
Chitin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-GlcNAc), 4-methyl-umbelliferyl-β-D-*N*-acetyl -glucosaminide (4-MU-GlcNAc), 4-MU-β-D-*N*, *N*, *N*-triacetyl chitotrioside (4-MU-$(GlcNAc)_3$), glucono-δ-lactone, 1,4 diamino-2-butanone, phalloidin, tunicamycin, nikkomycin, FITC-WGA, FITC-ConA, calcofluor white, propidium iodide, digitonin, glutaraldehyde	Sigma Chemical Co. St.Louis,MO,USA
Pharmalytes (pH 3-10)	Pharmacia,Chemicals, Uppsala, Sweden.
Yeast extract, Bacto-peptone	Difco Industries, Detriot, MI, USA
Osmium tetroxide	Fluka,
Azacytidine	Hi-media, India
Glucose oxidase kit	Ranbaxy, India
Sodium cacodylate	S.D.Fine.Ltd, India

All other chemicals used were of analytical grade

concentration, pH and incubation temperature on the yeast-mycelium transition was studied for 6-8 h as described earlier (Table 2.2). The mycelium-yeast transition was followed by incubating mycelial cells as indicated in Table 2.3. Morphology and counting of the cells on a haemocytometer were carried out as described earlier (Khale *et al,* 1992). Single or budding cells were counted as one yeast morphological unit and cells with one or more germ tubes were counted as one hyphal morphological unit. During the mycelium-yeast transition the mycelial strands were counted once, for each haemocytometer square in which they appeared. During transition to the yeast-form cells, the number of mycelial cells remaining was expressed as a percentage of the total cell count (Khale *et al,* 1992).

Asexual sporangiolum germination

The germination of sporangiolum was studied on different solidified (agar, 2%) media, such as YP, YPG (1 %, glucose) and modified Czapek Dox medium (glucose, 0.5%; $NaNO_3$, 0.3%; K_2HPO_4, 0.1%; $MgSO_4$, 0.05%; KCl, 0.05% and $FeSO_4$, 0.001%; pH 7.0) on a slide culture at 28° C and 37° C. The variations in carbon and nitrogen sources and pH have been mentioned appropriately in the Results Section.

Sporangiolum germination for antifungal testing

A spore suspension (8×10^4 sporangiola /ml) was made by suspending sporangiospores from sporulating YPG (1% glucose) agar slants of *B. poitrasii* in distilled water was prepared. The suspension (100 µl) was spread in 10 cm plates. Small filter paper discs were placed on the plates and the inhibitor solution (as indicated) was pipetted on them. About 50 spores/ field were observed at 10X.

Table 2. 2 Conditions for the yeast-mycelium transition

Inoculum	Transition	Chapter
YPG 0.5%, 37°C	YPG 0.1%, 28°C	III
YPG 0.5%, 28°C	YPG 0.1%, 28°C	
YPG 0.1%, 37°C	YPG 0.1%, 37°C	
YPG 0.1%, 37°C	YPG 0.1% (20-37°C)	IV
YPG 0.1%, 28°C	YPG (0- 1.0%) 28°C	
YPG 0.1%, 37°C	YPG 0.1%, 28°C	
YPG 0.5%, 28°C	YPG 0.1%, 28°C	
YPG 0.1%, 28°C	YPG 0.1 %, 28°C	
YPG 0.1%, 28°C	YPG 0.1%,pH 4.0/8.0, 28°C	
YPG 0.5%, 28°C	YPG 0.1%, 28°C	
YPG 0.5%, 37°C	YPG 0.1%, 28°C	Va
(10^7/ 200 ml)		
YPG 0.1%, 28°C	YPG 0.1%, 28°C	Vb

For transition (8×10^6/ 50 ml) were used unless otherwise mentioned.

Glucose % as indicated.

Table 2.3 Conditions for mycelium-yeast transition

Inoculum	Transition	Chapter
YPG 0.1%, pH 8.0, 28°C	YPG 0.5%, 28°C	III
YPG 0.1%, 28°C (10^7/ 200 ml)	YPG 1.0%, pH 4.0, 28°C	V

Glucose % as indicated

Hyphal tip bursting test for cell wall synthesis inhibitors

B. poitrasii spores were inoculated on 1% YPG agar plates incubated at 28°C for 16-18 h to obtain actively growing hyphal tips. The hyphal tip elongation of *B. poitrasii* was approximately 1.5 divisions/ 2 min. The culture filtrate (10 µl) of the potential antifungal organism in presence of sorbitol (0.6 M) was added to the plates. The bursting of the hyphal tips was monitored microscopically up to 3 h. The hyphal tips (15-20) per field were counted and the number of tips that burst in 10 fields were counted to find HTB (%) in a stipulated time as indicated.

Isolation of zygospores

The zygospores were isolated by the method of Yu and Ko (1997). The 5 days old growth from the slant was scrapped off in the sterile distilled water. To obtain zygospores free from other growth forms, the suspension was filtered through the muslin cloth and centrifuged (500 g, 15 sec). Repeated washing and centrifuging resulted in a clean suspension of zygospores. About 100-150 zygospores in 50 µl suspension were pipetted on a sterile filter disc and dried for a day. The discs were then inverted and zygospores transferred on a petri plate containing 3% water agar with and without glucose for germination as described earlier (Ghormade and Deshpande, 2000). The plates were incubated and observed for germination. The areas showing germination were isolated by cutting the agar blocks and were processed for SEM studies.

Fluorescence microscopy

Fluorescent marker, fluorescein isothiocyanate (FITC) tagged with lectins specific to cell wall polymers was used to ascertain the difference in the wall architecture of the yeast, mycelium, and the endospore. FITC - ConA and FITC-WGA (0.5 mg/ml in 1 mM $CaCl_2$,

$MgCl_2$ and $MnCl_2$ in 20 mM Tris-HCl buffer, pH 7.0) and Calcofluor White (0.05 mg/ml in 150 mM NaCl in 20 mM Tris-HCl buffer, pH 7.0) were used for staining as described by Mormeneo *et al* (1996). The stained cells were incubated in dark for 30 min and then washed with buffer to remove excess stain. The cells stained with FITC-labelled lectin were observed under the I 3 filter with an excitation range of 450-490 nm. A-Filter was used to observe cells stained with Calcofluor White with an excitation range of 340-380 nm. The fluorescence was observed by epifluorescence microscope (Leitz Labor Lux) and fluorescence as well as LM photographs were recorded by Wild MPS 32 camera unit

Samples preparation for Scanning Electron Microscopy (SEM)

The mycelium and sporangiola samples were treated using three different methods: A) The samples were critical point dried using a critical point drying apparatus (Polaron, UK) as described by Ginman and Young (1989). B) The specimen agar blocks were quickly immersed for 20-30 min at 22°C in an aqueous solution of 2.5% glutaraldehyde and 1% *p*-formaldehyde buffered by 0.05 M sodium cacodylate at pH 7.2 and osmotically balanced with 1% sucrose (Edelmann and Klomparens, 1995). Primary fixation was followed by 2-3 h rinse with several complete changes of the same balanced buffer.

Secondary fixation was carried out using 1 % osmium tetra-oxide in the same balanced buffer for 4-5 h at room temperature followed by a rinse for 1h in several changes of distilled water. Specimens were dehydrated using a graded ethanol series (w/v, 25%, 50%, 75%, 85%, 95%, 100%, 100%, 100%, for 30 min each). The samples were critically point dried. C) The method consisted of dehydration through a graded acetone (25%, 50%, 75%, 15 min each and 100%, 15 min and 30 min) followed by drying in a critical point apparatus (Jeffries and Young, 1975).

The untreated and treated samples were mounted with silver paste or double sided tape on aluminium stubs and sputter coated with gold-palladium approximately (5 nm) in Polaron coating unit E5000 and examined using Leica Stereoscan 440 (M/s Leica Cambridge Ltd. UK). The micrographs of the samples with 5KV/10KV EHT and 25pA beam current were recorded by 35 mm camera attached on the high resolution recording unit.

UV treatment

B.poitrasii yeast cells, the cells isolated from zygospores and the Y-5 mutant grown in YPG (0.5% glucose) and *S.cerevisiae*. (Strain 103X n and 103X 2n) grown in YEPD for 24h at 37°C were used for UV kill method. The cells suspension(100 µl) of 2×10^3/ml were spread on the plates that were exposed to UV at a distance of 30 cm for 1 min and incubated in the dark at 28°C. The colony counts were taken after 48h.

Flow Cytometry

The cells for FC analysis were grown as mentioned earlier. The cells at 10^6 concentration were centrifuged and washed in PBS (pH 7.2). The pellet was fixed with 1 ml of 70% chilled ethanol and kept at 4°C overnight. Cells were centrifuged and the pellet was incubated in 50 µl of RNAase (20mg/ml) for 1h followed by addition of 450 µl of propidium iodide (PI - 50 µg/ml). Following staining 500 µl of 10^6 cell were placed in 6 ml polystyrene tubes (Falcon) containing PBS buffer. Samples were sonicated on low power for 5 sec to separate the cell clumps. The number of events analyzed for the *B.poitrasii* cells and *S.cerevisiae* cells were 20,000.

Data acquistion

The cells were analysed in a FACS Vantage (Becton-Dickinson, Immunocytometry system, BDIS, CA). The machine employed an argon laser tuned to an excitation wavelength

of 488nm. After excitation PI emits wavelength of 617 nm. The PI signal was collected by FL2 band pass filter. The instrument was calibrated with a fluorescent standard of calf thymus nuclei before analysis each of the cell lines.

During data acquisition a threshold trigger is used to gate out the cellular debris that may be present in the samples. For the DNA content analysis, linear scales of all parameters were appropriate. On basis of side scatter cells of similar size were gated out and further analysis was done by a second gate to screen out populations of equal size on the FL2 W *vs* FL2 A. The flow rate was set on the lowest possible setting and on the FACS Vantage this translates to 200-300 cells per second for cells suspended at 10^6/ml. The fluorescence profiles were allowed stabilize before collection of data.

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. To compare the data from sample to sample the same axis was used in each graph. Estimates of G1, S and G2-M dwell times were based in the assumption that during exponential growth the number of cells in the population is increasingly asynchronous. Correlated arrays of PI induced DNA fluorescence and FL2 A scatter data for each cell line 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 as the cells in the G2 area could be assigned to the dividing cells of a asynchronous population having double DNA content

Isolation of different cell fractions for localization of chitinolytic enzymes The mycelial- and yeast-form cells grown for 24 h in respective media were separated by centrifugation (2 000 x g, 5 min) and the supernatant was used to estimate extracellular

chitinolytic enzyme activities. All further operations were carried out at 4°C, unless otherwise stated. The cells (2.5 g, wet weight) were homogenised in 10 ml 0.05 M sodium acetate buffer, pH 5.0 using cell homogeniser (Braun) with glass beads as described previously (Khale *et al*, 1992). The homogenate was centrifuged (2 000 x g, 5 min) to remove cell wall fraction. The cell wall pellet was suspended in 5 ml of the same buffer and used for the estimation of enzyme activities. The cell wall-free homogenate was centrifuged at 100 000 x g for 40 min to separate the mixed membrane fraction (mmf) and cytosol. The membrane pellet was resuspended in 0.05 M sodium acetate buffer, pH 5.0 and homogenous suspension was used for enzyme assays unless otherwise mentioned (Deshpande *et al.*1997). However, for solubilization, the mixed membrane fraction was re-suspended in 1% digitonin in the same buffer, incubated at 0°C for 30 min and centrifuged (160 000 x g , 1 h) and the supernatant was used for the estimation of enzyme activities.

Chitinase and *N*-acetylglucosaminidase assays

Acid swollen-chitin (ASC) for total chitinase activity was prepared using phosphoric acid as described earlier (Vyas and Deshpande 1989). The 0.3 ml of 1% (w/v) ASC or EGC (for endo-chitinase) was incubated with 0.2 ml of enzyme preparation at 37° C for 1 h and the hexosamine liberated was estimated using the method of Reissing *et al.* (1955). One unit of specific enzyme activity was defined as 1 nmol of *N*-acetylglucosamine/ mg protein / min.

The endo-chitinase and *N*-acetylglucosaminidase assays were performed using fluorogenic glycosides, 4-MU-$(GlcNAc)_{1 \text{ and } 3}$ prepared in 50% (v/v) ethanol. The activities measured on 4-MU- $(GlcNAc)_3$ and on 4-MU-(GlcNAc) were designated as endo-chitinase and *N*-acetylglucosaminidase, respectively (Jackson *et al.* 1996). The 20 µl of 700 µM substrate was incubated with 50 µl of suspended mmf in 130 µl of 0.05 M sodium acetate buffer, pH 5.0 or 5.5 for mycelium or yeast cell preparations, respectively at 37°C for 30 min.

The reaction was then stopped by adding 2.3 ml 0.2 M Na_2 CO_3. The fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer by excitation at 360 nm and reading the emission at 445 nm. One unit of specific enzyme activity was defined as one nmol of 4-methylumbelliferone / mg protein /min.

N-Acetylglucosaminidase activity was also estimated by incubating 0.3 ml of 4% (w/v) *p*NP-(GlcNAc) with 0.2 ml of mmf at 37° C for 1 h. The reaction was terminated by adding 2 ml of 0.2 M Na_2CO_3. The *p*-nitrophenol liberated was measured spectrophotometrically at 405 nm. One unit of specific enzyme activity was defined as one nmol of *p*-nitrophenol / mg protein / min.

The whole cell, *in situ* chitinase activity estimation was carried out as described by O'Donnell (1991). The reaction mixture contained 5 mg (wet weight) of whole cells suspended in 300 µl 0.05 M sodium acetate buffer, pH 5.0 and 20 µl of 700 µM fluorogenic substrate. After incubation at 37 °C for 30 min the reaction was terminated by the addition of 2.2 ml 0.2 M Na_2CO_3. After centrifugation (2 000 x g, 10 min) the supernatant was collected and the fluorescence was measured as described above. One unit of enzyme activity was defined as one nmol of 4-methylumbelliferone/ mg wet weight of whole cells/ min. All the experiments were carried out three times in triplicate unless otherwise mentioned.

Isoelectricfocussing

The membrane pellet was suspended in 0.05 M sodium acetate buffer, pH 5.0 and the fraction of mmf free of particulate matter was used for isoelectricfocussing which was carried out in a mini scale IEF unit using Pharmalytes in the pH range of 3-10 for rapid (8-10 h) separation as described by Sathivel *et al* (1995). The fractions were collected and the pH and activity on 4-MU-(GlcNAc) and 4-MU-$(GlcNAc)_3$ were estimated.

Glucose utilization

Inoculum was grown as mentioned in the preceding section and transition was carried out at 28°C and 37°C. Samples were taken at 0, 3, 6, 9, 12 and 24 h for estimation of unutilized glucose by glucose oxidase method. The glucose oxidase kit was obtained from Ranbaxy Laboratories, India. The working solution was prepared by dissolving 0.4 gm of enzyme powder in 100 ml of distilled water and adding 1.7 ml of phenol before use. The assay was run by taking 1.0 ml of working solution and adding 10 µl of either blank, standard or test samples to it. The same was mixed and incubated at room temperature for 30 min. The absorbance was read at 505 nm.

Protein estimation

Protein was estimated according to Lowry *et al* (1951) using bovine serum albumin as standard.

DNA isolation

B.poitrasii yeast cells, for DNA isolation were obtained by filtration through Whatman No 1 paper and washed extensively with sterile distilled water. These cells (1gm) were crushed in liq. N_2 with mortar and pestle. The crushed cells were transferred to extraction buffer (Tris pH 8.0, 10 mM; NaCl ,100 mM; SDS, 1%; DTT, 50 mM; BME, 1%) and mixed by inversion 2-3 times. Extraction with phenol:chloroform was carried out thrice followed by two chloroform extractions.

The DNA was precipitated using 0.8 volumes of iso-propanol. The pellet was then washed with absolute alcohol and was air dried. The pellet was re-suspended in TE (Tris pH 8.0, 10 mM; Na - EDTA, 1mM).

DNA estimation

The DNA was estimated by the UV absorption ratio at $A_{260} : A_{280}$ on a Perkin-Elmer Spectrophotometer and samples in the range of 1.8 - 1.9 were used.

CHAPTER III

Study of dimorphism in the life cycle *Benjaminiella poitrasii*

A) Light, scanning and fluorescence microscopy studies of different phases in the life cycle of *Benjaminiella poitrasii*

B) Ploidy determination of parent and monomorphic mutant of *Benjaminiella poitrasii*

A) Light, scanning and fluorescence microscopy studies of different phases in the life cycle of *Benjaminiella poitrasii*

Introduction

Benjaminiella poitrasii, a dimorphic mucoralean fungus has been classified in a family Mycotyphaceae because it produces unicellular yeast-like cells and possesses the similar mode of sporangiolum secession as *Mycotypha* (Benny *et al*, 1985). Previously, most of the studies were focussed on the formation of different vegetative and reproductive structures to clarify systematic position of *B. poitrasii*. They were also used to elucidate interspecific and intergeneric relationship among the members of Mycotyphaceae such as *B. poitrasii, B. multispora, B.youngii, Mycotypha africana, M. indica* and *M. microspora* (Benny *et al*, 1985; Kirk, 1989). *Benjaminiella* exhibits a reversible yeast-mycelium transition in response to the change in glucose concentration or pH of the nutritional medium and incubation temperature (Ghormade & Deshpande, 2000; Khale *et al*., 1990). The biochemical changes correlated with the dimorphic transition in *B. poitrasii* parent and its yeast-form (Y-5) mutant are well documented (Deshpande, 1998; Khale-Kumar and Deshpande, 1993; Khale *et al*, 1990).

Until now, the studies of the dimorphic fungi have been limited to the vegetative phase, i.e. the yeast-mycelium transition (Gow, 1995). Recently, studies on the pattern of asexual and sexual spore germination in *B. poitrasii* under dimorphism triggering conditions using fluorescence microscopy were reported (Ghormade and Deshpande, 2000). As the spores are the etiological agents of human as well as plant pathogens, the study on their formation is also important. Here we have used light and scanning electron microscopy to elucidate the effect of dimorphism triggering conditions, *viz*. glucose, pH and temperature change, and ethanol on the vegetative forms of *B. poitrasii* parent and mutant strain, formation and germination of asexual as well as sexual spores. The light microscopy revealed that yeast cells produced uni-, bi- and multi-polar budding while during yeast-mycelium transition, initially bud like structures emerged that eventually produced germ tubes. The

yeast-form mutant Y-5, in the presence of ethanol (0.5% w/v) exhibited germ tube formation either directly or emerging through bud-like structure. The mycelium - yeast transition was observed to proceed by producing terminal and lateral budding. The zygospore formation was in the zig-zag manner. Scanning electron microscopy (SEM) revealed that asexual spore formation started with the swelling of the hyphal tip followed by emergence of stub like outgrowth on the surface of the vesicle that later formed the sporangiola on their tips. Sexual spore formation took place by the fusion of two opposing gametangia during zygospore formation. The ornamentation formed by the fusion of 6-8 verrucae on the surface of the zygospore could be seen by SEM. For the first time the presence of dimorphic response during asexual and sexual spore germination has been reported under the dimorphism triggering conditions in *B. poitrasii*. The zygospores germinated into budding yeast when subjected to yeast- form supporting conditions. The mycelium-form favoring conditions gave rise to true mycelium. The supporting evidence for the dimorphic response of the zygospore was obtained from the autofluorescence and cell wall staining studies.

Similarly, the asexual spores displayed a dimorphic response during germination. Our observations suggested that dimorphism is an intrinsic ability present in the vegetative, asexual and sexual forms of the fungus. It was evident from the SEM studies that the asexual as well as the sexual spores directly produced yeast cell under yeast favouring condition. As dimorphic fungi are intermediate to the unicellular yeast and the filamentous forms, understanding of the dimorphic character could be useful to trace the evolutionary relationships among taxonomically different fungi. Moreover, the implication of the spore germination during the onset of pathogenesis and in drug development for human health care has been discussed. The importance of these studies to understand its systematic position and phylogenetic relationships has been discussed.

Results

Life cycle of *Benjaminiella poitrasii*

B. poitrasii, dimorphic, homothallic fungus produced yeast and mycelium during its vegetative phase, sporangiospores (sporangiola) in its asexual phase and sexual phase comprised of zygospores. The size of yeast cells was 12 ± 2 µ, and the non-septate mycelium was 3.5 ± 1 µ thick (Fig. 3A. 1a). In *B. poitrasii*, the yeast-mycelium transition and *vice versa* in response to the change in glucose concentration and/or temperature has been well documented (Khale *et al.*, 1990). The lowering of glucose concentration and/or change in incubation temperature from 37° C to 28° C triggered yeast-mycelium transition. The yeast cells produced bud-like structure which further produced germ tubes (Fig. 3A. 1b; Fig 3A. 12 a, b). The mycelium-yeast transition carried out as described under materials and methods proceeded by the formation of terminal buds (Fig. 3A. 1c; Fig. 3A. 12d) as well as lateral buds (Fig. 3A. 1d; Fig. 3A. 12d).

The monomorphic (yeast- form) mutant (Y-5) cells were of similar size as the parent yeast cells but slow growing and appeared to be more vacuolated (Fig. 3A. 1e) The mutant grew as yeast under fore-mentioned dimorphism triggering conditions. However, it reverted and produced germ tubes in presence of 0.5% (w/v) ethanol (Fig. 3A. 1f). Unlike yeast-mycelium transition exhibited by parent strain, in large number of cells the ethanol induced germ- tube formation in Y-5 was direct without formation of bud-like structure (Fig. 3A. 1f).

On YPG (1% glucose) medium the grayish mat of asexual ellipsoidal, unicellular sporangiola (3 ± 1µ) appeared within 72 h while the profuse development of dark brown zygospores (40 ± 5 µ; 200-300 / field at 100X) occurred after 5-7 days at 28°C (Fig. 3A. 1g,h; Fig. 3A. 12e-j). In the presence of 0.1% glucose in YP medium also, the asexual and sexual spores appeared within 5 and 7 days, respectively. On YP medium without glucose, the asexual sporulation was dense, and the zygospore development was scanty (10-15/field

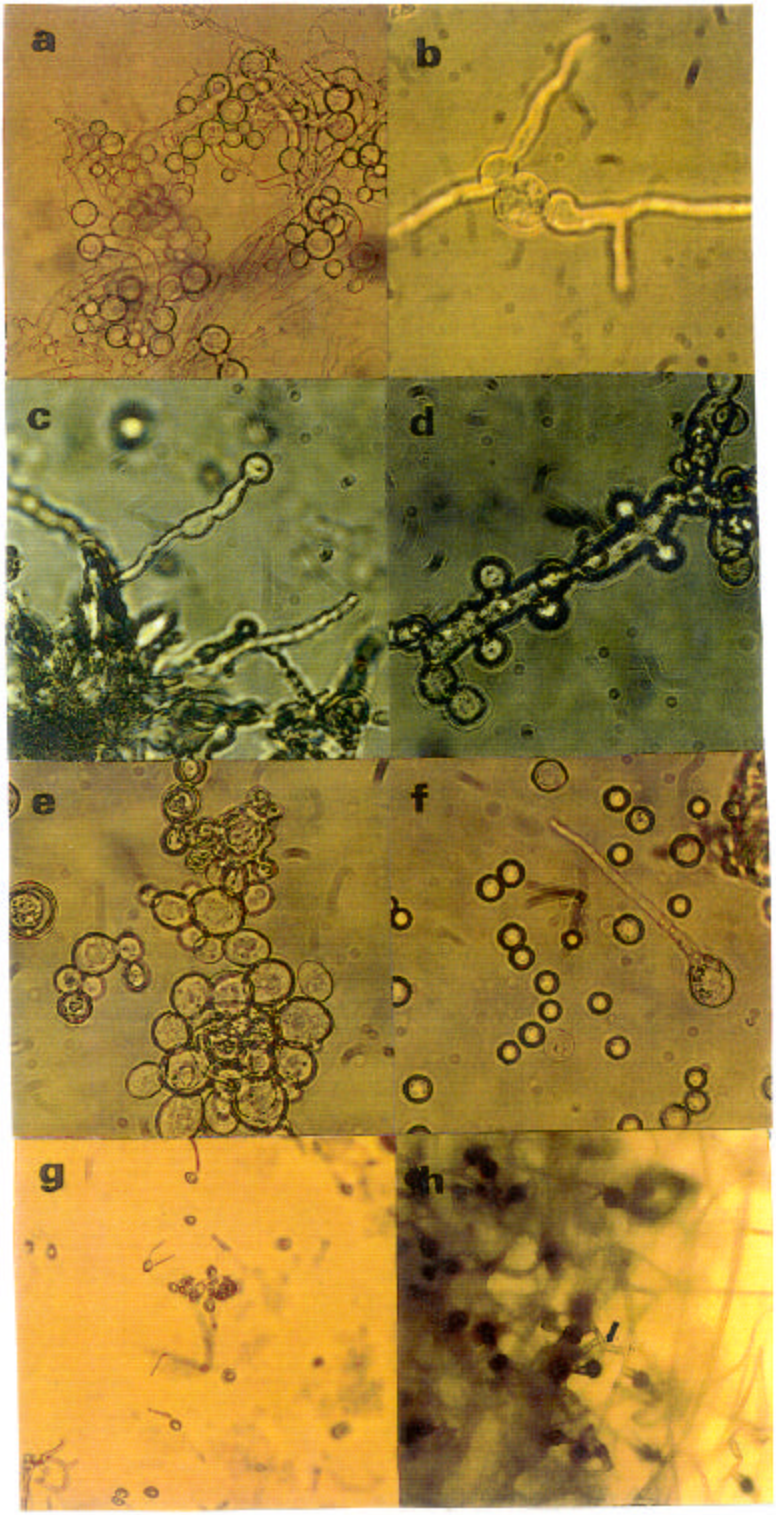


Figure.3A. 1: Different phases in the life cycle of *Benjaminiella poitrasii* parent and mutant strain. Light microscopy (400 X). a) Yeast- and mycelial-form cells; b) Yeast-mycelium transition in the parent strain; c) Mycelium-yeast transition, terminal budding; d) Mycelium -yeast transition, lateral budding; e) Yeast-form (Y-5) mutant; f) Germ-tube formation in Y-5 mutant; g) Asexual sporangiola; h) Zygospore formation in ladder like manner indicated by the arrow.

100X). At 37° C, on YPG (1% glucose) only yeast-form cells were observed up to 8 days. On the other hand, sporangiola appeared within 7 days on YP and YPG (0.1% glucose) media. The zygospore formation was rarely seen up to 10 days in all three media at 37°C.

Sample preparation for Scanning Electron Microscopy

As mentioned under Materials and Methods, along with untreated sample, three different methods (A, B, C) were used for the treatment of *B. poitrasii* cells (Fig. 3A. 2a-d). The high percentage of collapsed spores and mycelium was observed in samples, which were either untreated or fixed with protocols A and C used (Fig. 3A. 2a,b,d). The critical point drying was found to be essential to reduce the dehydration of the specimen and to maintain the structure. In protocol B, the fixation of the samples was done with glutaraldehyde/ formaldehyde as well as with osmium tetroxide and drying was carried out using washing with 25-100% ethanol followed by critical point drying. Due to osmium tetroxide treatment the sporangiola were found to be darkened. As the structural integrity of the samples was found to be preserved with the protocol B (Fig. 3A. 2c), it was used for further studies unless otherwise mentioned.

The SEM pictures showed that as compared to the yeast cells the surface of the mycelium had spiny outgrowths (Fig. 3A. 3a,b)

Asexual sporangiolum formation

The scanning electron microscopy studies were carried out to study the development of asexual apparatus (Fig. 3A. 4a-d). As shown in Fig. 3A. 4 (a) *B. poitrasii* produced aerial sporophores consisted of globose vesicles with stub like outgrowths arising to form the pedicellate sporangiolum. The pedicels of different lengths, each with basal swelling and a single sporangiolum at the tip were seen to come out from the vesicle (Fig. 3A. 4b-d).

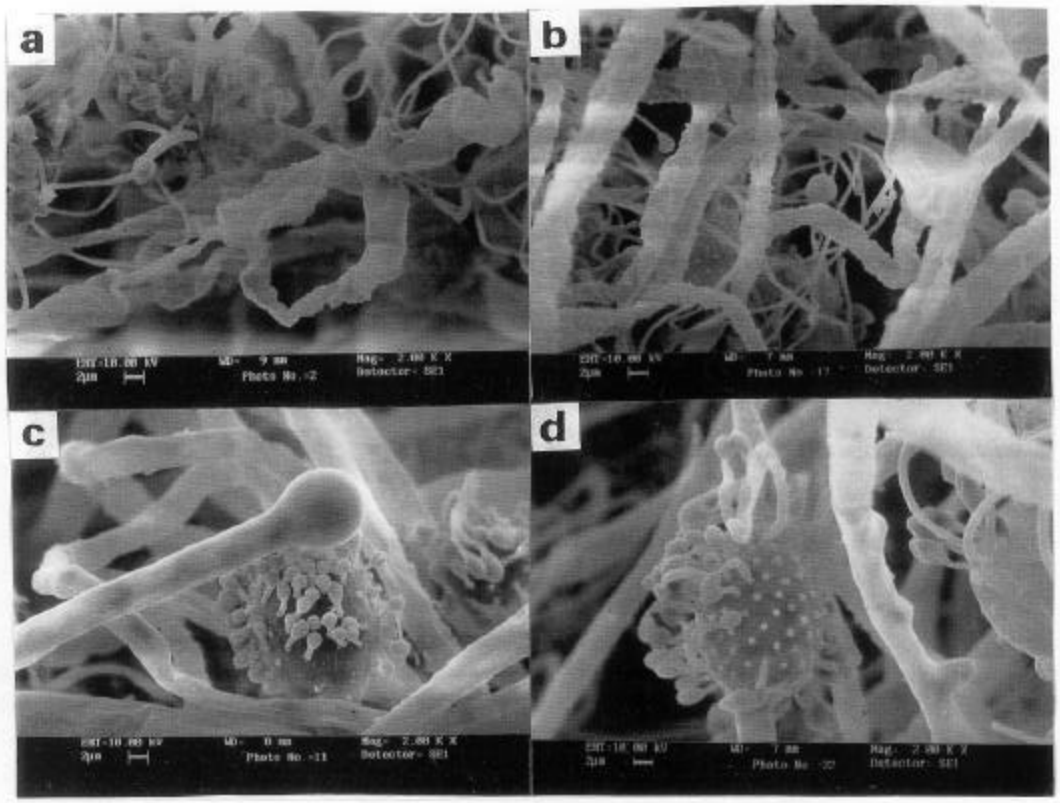


Figure.3A. 2: Different treatments to prepare samples for scanning electron microscopy of *Benjaminiella poitrasii*. Scanning electron microscopy (2000 X). a) Untreated sample; b-d) Treatment with methods A,B,C, respectively as described under Materials and Methods.

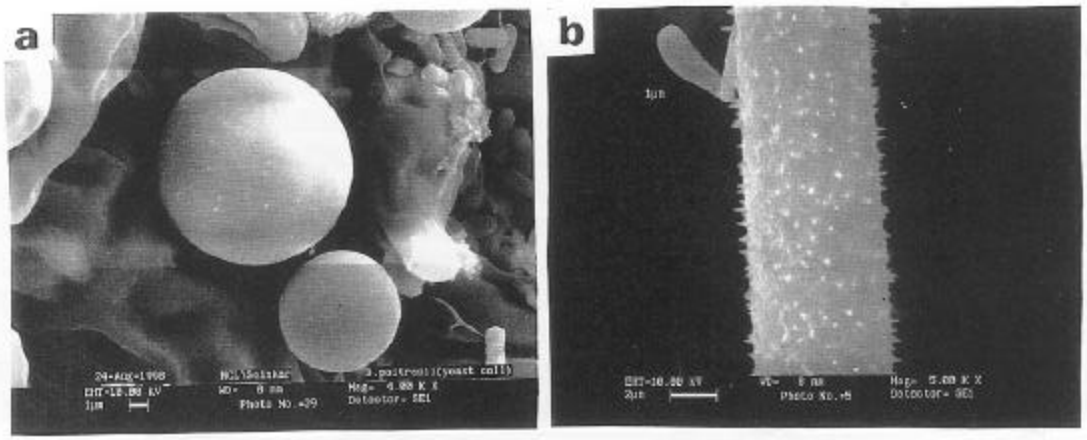


Figure.3A. 3: The yeast and mycelium of *Benjaminiella poitrasii.* Scanning electron microscopy. A) Yeast cells (4000 X); b) Mycelium (5000 X)

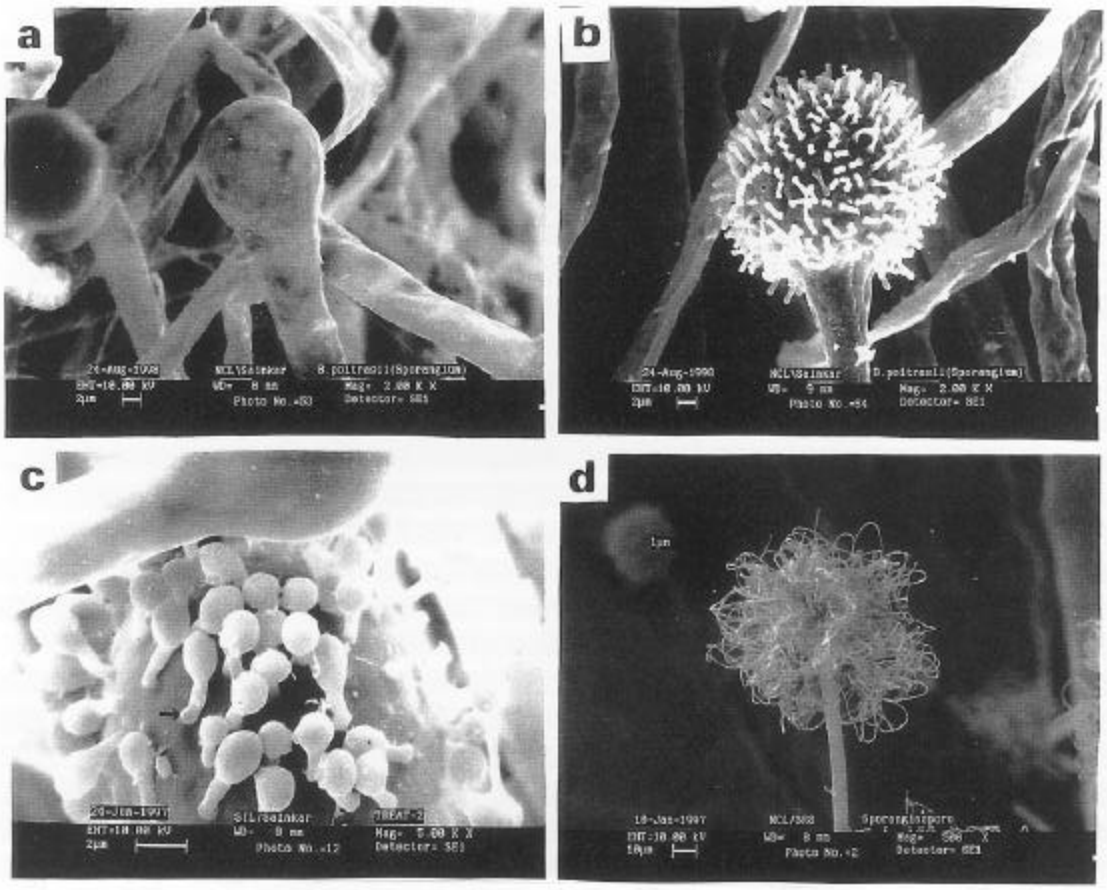


Figure.3A. 4: Development of asexual apparatus in *Benjaminiella poitrasii*. Scanning Electron Microscopy. A) Sporophore (2000 X); b) Sporangiola development (2000 X); c) Sporangiola borne on distal end of pedicels(5000 X); d) Mature sporangiophore (500 X).

Sporangiolum germination

The sporangiospores of *B. poitrasii* displayed a dimorphic response during germination. The spore germination was studied on a modified Czapek Dox medium ($NaNO_3$, 0.3%; K_2HPO_4, 0.1%; $MgSO_4$, 0.05%; KCl, 0.05% and $FeSO_4$, 0.001%; agar, 2%; pH 7.0) on a slide culture. The condition for yeast type germination was Czapek Dox medium containing glucose (0.5%) incubated at 37°C. While in presence of glucose (0.1%) at 28°C germ tube formation was prominent. Same medium containing glucose (0.5%), pH 4.0 at 28°C was used for yeast type growth while for mycelial growth pH was adjusted to 8.0. The spores produced budding yeast cells in 16-18h and germ tubes within 48h under their respective form favoring conditions.

During germination, the sporangiolum initially enlarged (Fig. 3A. 5a) and produced bud like structures in presence of organic nitrogen, viz. peptone and yeast extract. In the synthetic Czepek-Dox medium containing $NaNO_3$ and glucose (0.1%), and pH 8.0 the sporangiolum germinated by producing germ tube (Fig. 3A. 5b) at 28° C in 48-72 h, while in the same medium the spores showed budding at pH 4.0 (Fig. 3A. 5 c). At 37° C, in pH 8.0 medium the irregular growth was observed (Fig. 3A. 5d). The germination was found to be rapid (within 24 h) when $NaNO_3$ was replaced with $(NH_4)_2SO_4$. At times 1-5 germ tubes were seen to emerge. Two to three germ tubes are commonly observed. The glucose was replaced with either N-acetylglucosamine (GlcNAc) or glycerol (0.5% w/v) to examine their germ tube favouring effect, if any. The direct germ tube formation was observed in both the cases. However, the germination was delayed by more than 72 h.

Zygospore formation

The homothallic *B. poitrasii* profusely produced globose, brown zygospores that were covered with conical projections, on YPG medium in 5-7 days at 28° C (Fig. 3A. 1h). As shown in Fig.1h, a zig-zag structure was observed during zygospore formation. Zygospore

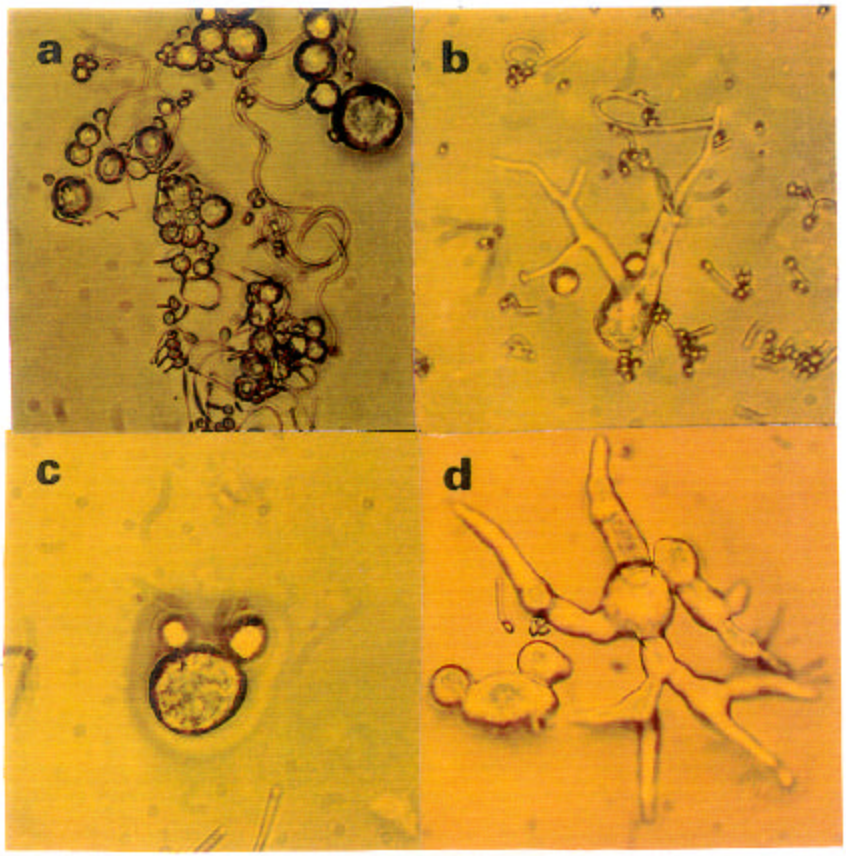


Figure.3A. 5: Germination of asexual sporangiolum in *Benjaminiella poitrasii.* Light microscopy (400 X). A) Enlarged sporangiola; b) Germination in the synthetic medium, pH 8.0 at 28° C; c)Germination in the synthetic medium, pH 4.0 at 28° C; d) Germination in the synthetic medium, pH 8.0 at 37° C.

development started with the progametangia formation, that developed into homogametangia, followed by their subsequent fusion (Fig. 3A. 6a). Both the gametangia were of equal size (Fig. 3A. 6a). The mature zygospore was formed between the opposed suspensors (Fig. 3A. 6b). The remnants of the gametangial wall were seen as a distinct rim on the margins of the zygospore wall near the suspensors (Fig. 3a. 6c). The verrucose wall developed over primary endospore wall and came out as distinct projections with 5-6 ridges (Fig. 3A. 6c,d).

Zygospore germination

The zygospores were isolated using the protocol described by Yu and Ko (1997). The zygospore germination recorded till now consisted of mycelium and germ -sporangial type (Gauger 1965; Guo and Michailedes 1998). In the present investigations, the dimorphism triggering conditions, i.e. 3% water agar at 28°C for mycelial type of growth and 3% water agar containing 1% glucose at 37°C for yeast type of growth, were used for zygospore germination. Fig. 3A 7(a) depicts mycelium formation by zygospores under mycelium favoring conditions. When subjected to yeast-favouring conditions the zygospores of *B. poitrasii,* produced bud-like structures (Fig. 3A. 8a). Zygospore germination through suspensors have also been reported earlier (Blakeslee, 1906). In this regard too, the dimorphism triggering conditions influenced the germination pattern in a similar fashion in *B. poitrasii* (Fig. 3A. 7b & Fig. 3A. 8b).

In the presence of organic nitrogen the sporangiolum germinates initially into bud-like structure, that subsequently either produce yeast colony or mycelium in response to the environmental conditions. Recently, for the first time it was reported that the *B. poitrasii* zygospores too germinated into budding structures under the yeast-form favouring conditions (Ghormade and Deshpande, 2000). The present SEM investigations confirmed the emergence of bud-like structure from the asexual as well as sexual spore (Fig. 3A. 9 a, b).

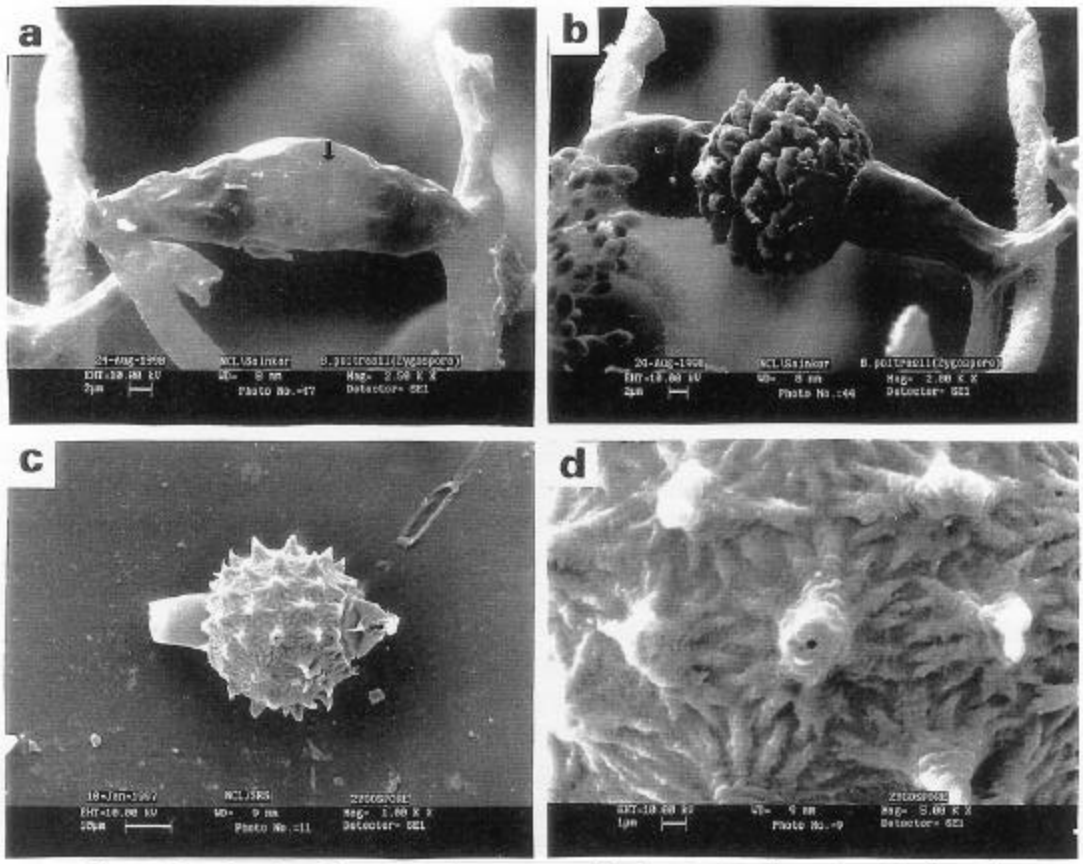


Figure.3A. 6 : Development of zygospore in *Benjaminiella poitrasii.* Scanning electron microscopy. A) Gametangial fusion indicated by the arrow (2500 X); b) Development of zygospore between opposed suspensors c) Mature zygospore with remnants of the gametangial wall indicated by the arrow (2000 X); d) Verrucose wall ornamentation of the zygospore (5000 X).

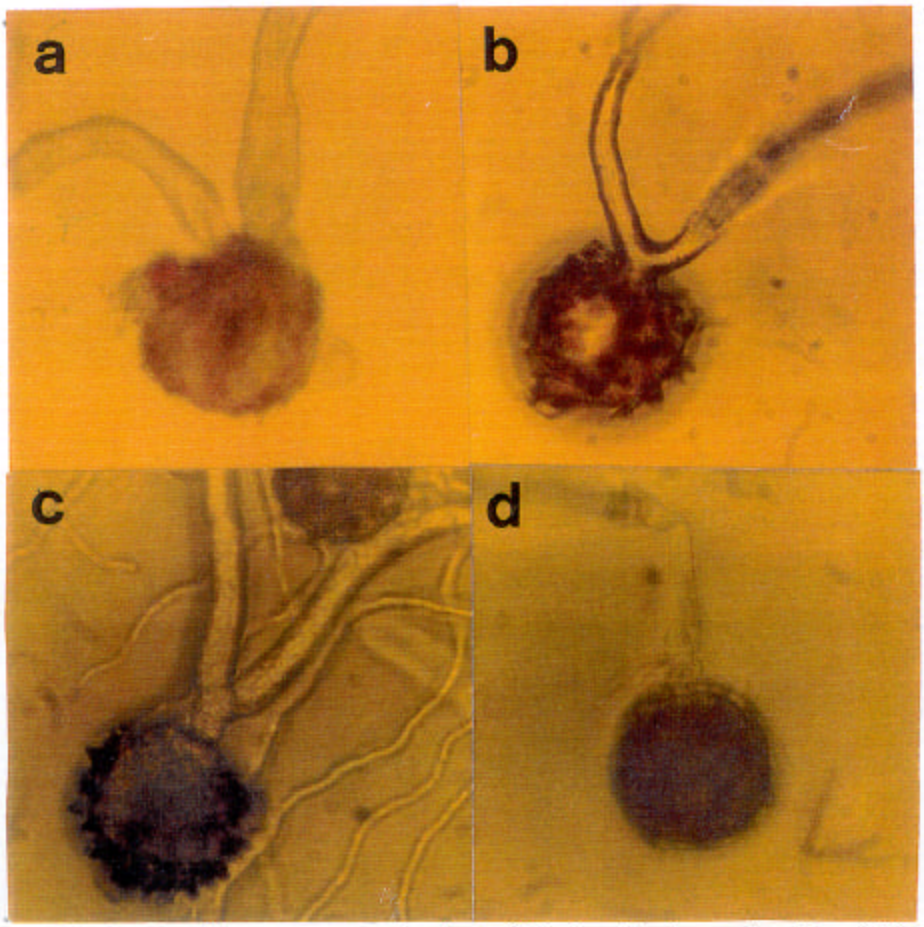


Figure.3A. 7. Mycelium type germination of *Benjaminiella poitrasii* on 3% water agar at 28°C: a, Mycelium formation from zygospore. b, Germination through the suspensor. **c,** Germination of endospore emerging through a crack in the wall. d, Germination of free endospore (Scale bar, 1 cm = 10 μ; Magnification 400X).

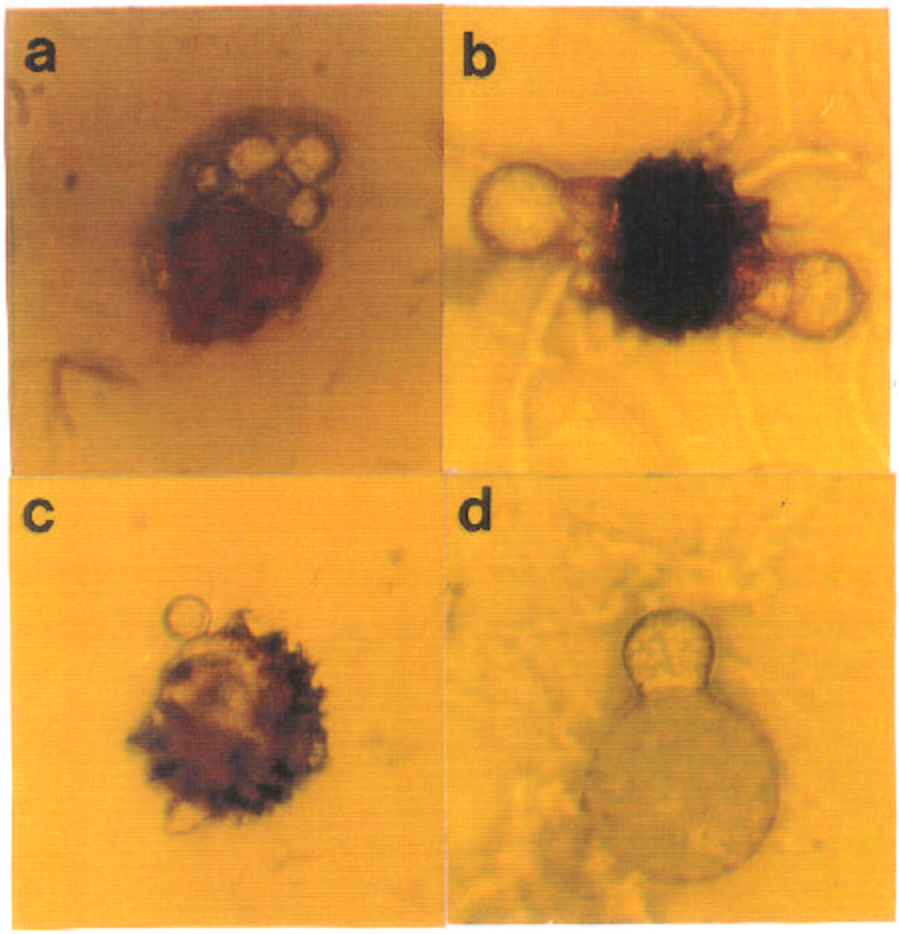


Figure.3A. 8. Yeast type germination of *Benjaminiella poitrasii* on 3% water agar medium containing 5% glucose at 37°C: a, Bud formation from zygospore . b, Budding through the suspensors. **c,** Bud formation from endospore emerging through a crack in the wall. d, Germination of free endospore (Scale bar, 1 cm = 10 μ;Magnification 400X).

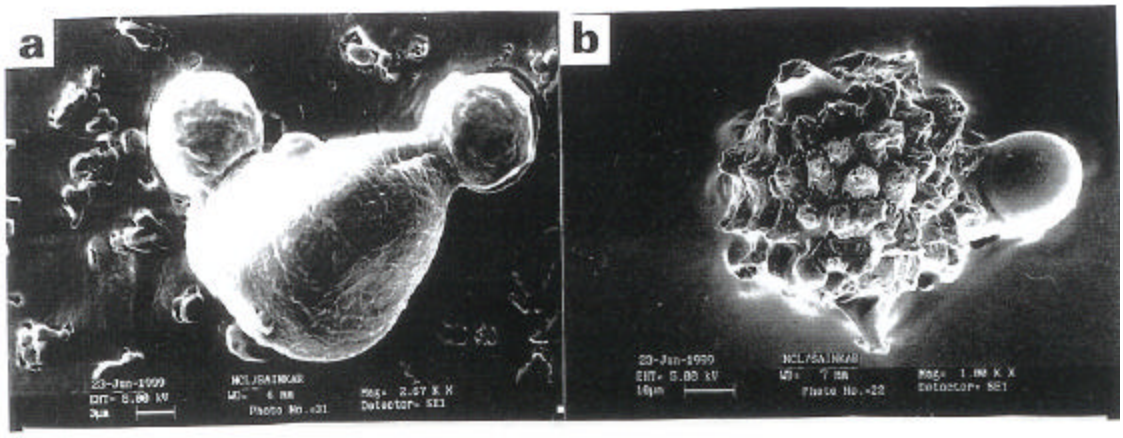


Figure.3A. 9: Yeast type germination of asexual and sexual spore in *Benjaminiella poitrasii*. Scanning electron microscopy. A) Asexual sporangiolum germination (2670 X); b) Zygospore germination (1000 X).

During isolation of the zygospores, on few occasions the outer exosporangial wall was either ruptured or sloughed off revealing the endospore. The partially exposed (Fig. 3A. 7c & Fig. 3a. 8c) as well as free endospores also followed the same norms of germination (Fig. 3A. 7d & Fig. 3a. 8d).

The germination frequency, irrespective of the morphological outcome was 1 and 3% for the 15 and 90d old zygospores, respectively. The maximum germination (40% in 3 d) was shown by 45 -50 d old zygospores. The germination percentage increased from 5 - 40 % in 1 and 3 % water agar medium, respectively.

As mentioned before, to nullify the effect of temperature change, suitable medium of different pHs were used for the zygospore germination at 28°C. There was no change in the pattern of germination on comparison to the one depicted in Fig. 3A. 7 and 8 for the effect of glucose and temperature. The zygospores germinated by bud formation at pH 4.0 and at pH 8.0 by mycelium formation, in 3% water agar. All other usual parameters, such as age of the zygospores (15 d - 90 d), size ($40 \pm 5\mu$) and agar concentration (1-3%) of the medium though affected the germination frequency (either bud or germ tube formation), did not change the dimorphic response.

It was apparent that the cell wall composition as well as the layering of the components was different in the different morphological structures. Fluorescent marker, fluorescein isothiocyanate (FITC) tagged with lectins specific to cell wall polymers was used to ascertain the difference in the wall architecture of the yeast, mycelium, and the endospore.

The zygospore showed a yellow fluorescence while the bud or the mycelium emerging from it displayed a green fluorescence thus suggesting the different metabolic states of the zygospore and the bud or mycelium produced from it (Fig. 3A. 10b,d). The exosporangial wall, sloughed off from the zygospore, did not show any autofluorescence. A distinct yellow fluorescence was observed in the free endospores, while the bud

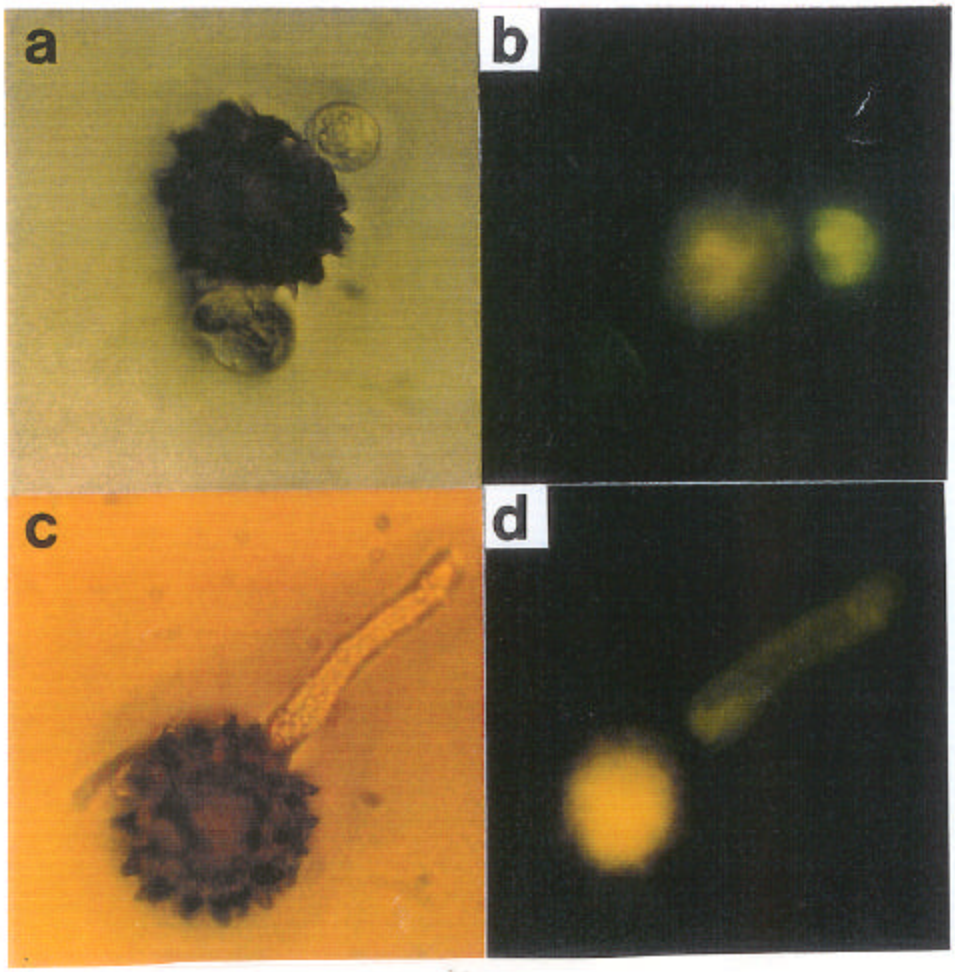


Figure.3A. 10. Autofluorescence of germinating zygospores of *Benjaminiella poitrasii*. Bud and germ tube formation (a,b, respectively) under the conditions as described in Fig.1 legend. Zygospore exhibits yellow and bud and germ tube show green fluorescence (c,d, respectively) (Scale bar, 1 cm = 10 μ; Magnification 400X, autofluorescence at 360-460 nm excitation).

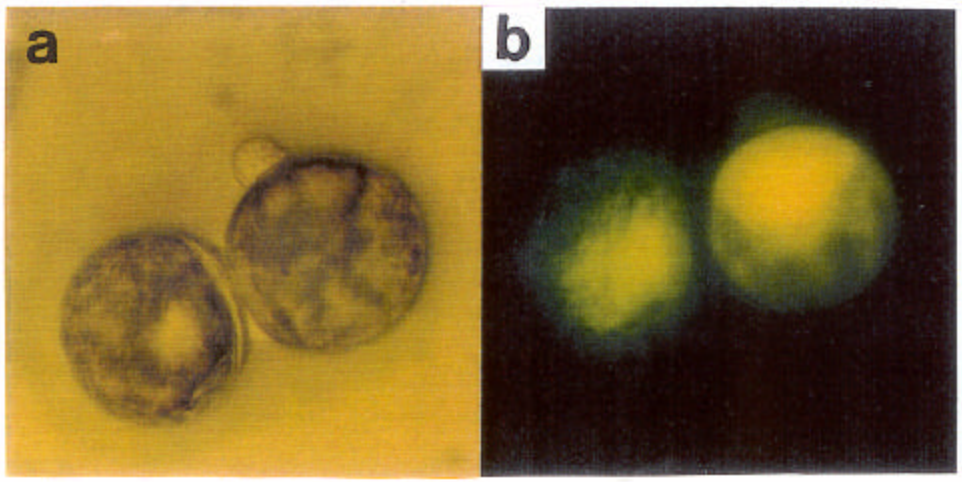


Figure.3A. 11. Autofluorescence of germinating endospore of *Benjaminiella poitrasii*. a, Endospore showing bud formation. b, Endospore showing yellow fluorescence and bud with a distinct green fluorescence (Scale bar, 1 cm = 10 μ; Magnification 400X, autofluorescence at 360-460 nm excitation).

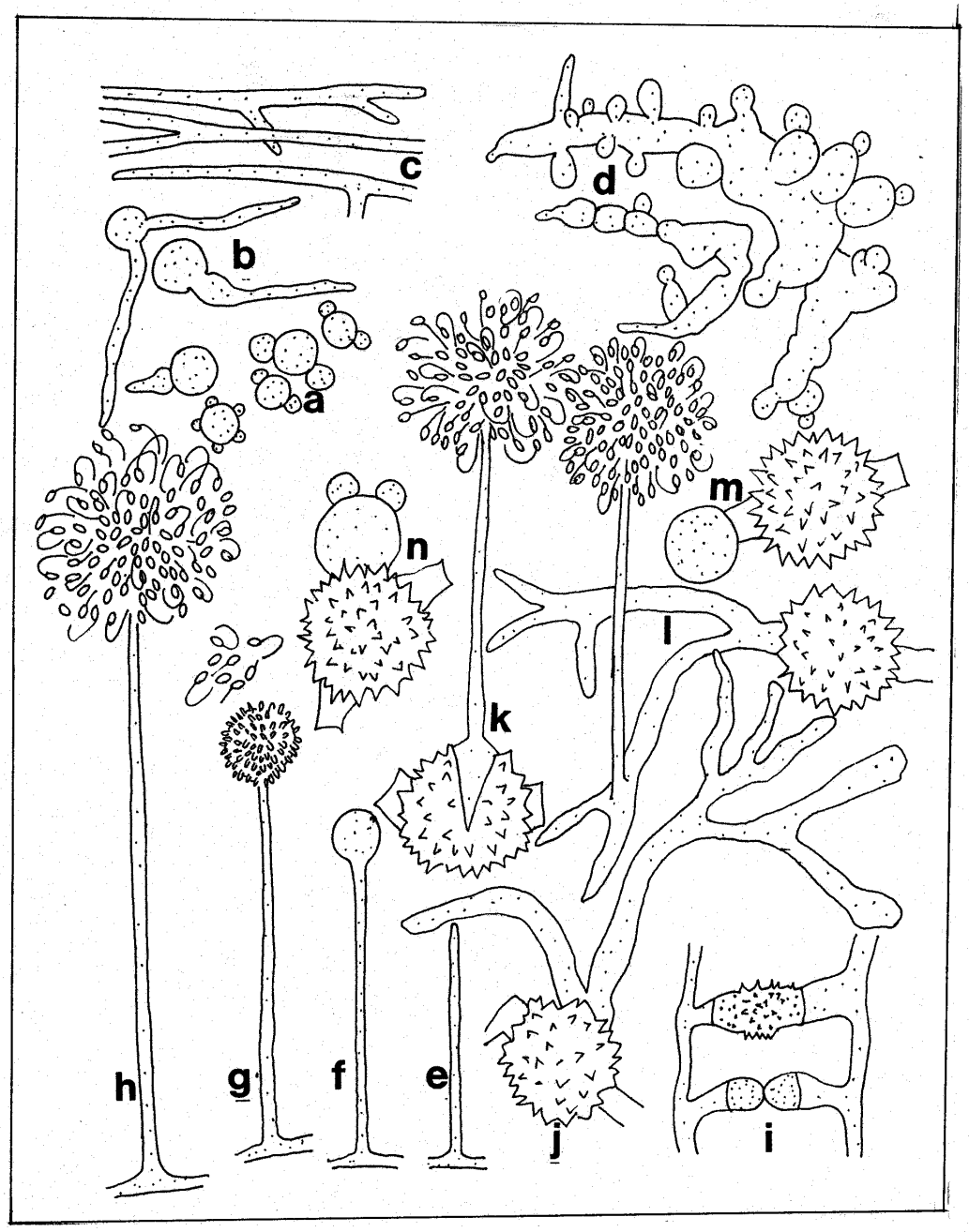


Figure.3A. 12. Life-cycle of *Benjaminiella poitrasii.* a, Budding yeast cells. b,Yeast mycelium transition by germ tube formation. c, Mycelia. d, Mycelium-yeast transition by lateral and terminal budding. e, Young sporophore. f, Formation of globose vesicle apically. g, Emerging stub like outgrowths representing sporangiola. h, Mature sporangiophore bearing pedicellate sporangiola. i, Zygospore formation by gametangial fusion. j, Mycelial germination of zygospore. k, Germ-sporangial germination. l, Mixed germ-sporangial and mycelial germination through suspensor. m, Bud-type germination through suspensor. n, Bud-type germination of zygospore.

emerging from it showed a green fluorescence (Fig. 3A. 11b). Furthermore, the spectral analysis of the intact as well as the homogenized yeast cells and endospores in distilled water at excitation 360 nm and 460 nm supported the microscopic observations (Data not shown).

The yeast cells showed more intense staining with a mannose specific FITC-Con A than the mycelial cells. It can be attributed to the much higher mannan contents in the cell wall of yeast-form than the mycelium (Khale and Deshpande, 1992). The zygospore and the endospore both showed the formation of typical budding yeast cells that was confirmed by the staining with FITC-Con A. The yeast, mycelium and the endospore showed a respective decrease in the intensity of the stain. The bud emerging from the endospore displayed fluorescence intensity similar to that of the yeast cells that can be attributed to the qualitative equality in the cell wall composition between them.

The fluorescence staining with FITC-Wheat Germ Agglutinin (FITC-WGA) which is specific for *N*-acetylglucosamine of chitin and a non-specific stain Calcofluor White did not reveal appreciable difference in the brightness between the bud and the endospores.

Discussion

Dimorphism in fungi has been studied mostly for the reversible change in the vegetative forms, i.e. yeast or mycelium (Gow, 1995). As the spores are etiological agents for dimorphic human pathogenic fungi such as *H. capsulatum*, *P. brasiliensis*, and *Sporothrix schenckii*, we need to improve upon the knowledge of germination of spores into the vegetative form. Therefore, we have studied the life-cycle of a non-pathogenic, dimorphic fungus, *B. poitrasii* as a model to understand the intriguing phenomenon of dimorphism (Fig. 3A.12). Furthermore, there is very less information regarding involvement of the dimorphic event in the evolution of fungi, particularly for the zygomycetous members such as the

entomophthorales (Mitchell *et al* 1995; Nagahama *et al,* 1995). These studies will also be useful to identify its possible role in the evolution of fungi.

B. poitrasii, exhibited a rapid and simple one step process of yeast-mycelium transition in response to the temperature and /or glucose change (Khale *et al,*1992; Deshpande, 1998). High glucose in a complex nitrogen medium (yeast extract, 0.3% and peptone, 0.5%, pH 6.5) and/or 37°C supported the yeast ($12 \pm 2\mu$) form whereas low glucose and 28°C favored the mycelium (non-septate, $3.5 \pm 1\mu$ thick) form (Khale *et al,* 1992). The temperature is known to effect the regulation of metabolic activities at the cell membrane level through the alteration in membrane structure and permeability (Schulz *et al,* 1974) that in turn affects the differentiation in fungi (Anderson 1978; Dennetiere *et al,* 1991). Therefore, the change in pH of the medium as one of the triggers for the alternating morphological forms was identified. Change in pH (from 4.0 to 8.0) of the nutritional medium triggered yeast to mycelium transition irrespective of the glucose concentration of the medium and incubation temperature (Chapter IV). *B. poitrasii* also produced asexual sporangiospores (3 ± 1 μ) and sexual zygospores ($40 \pm 5\mu$) in its life cycle.

The environmental factors usually affect the vegetative growth and the formation of reproductive structures. In the case of *B. poitrasii* the glucose level of the nutrient media and the incubation temperature, either 28°C or 37° C, affected the formation of either yeast or mycelial cells, and the development of sporangiola and zygospores too. At 37°C in the presence of glucose and complex nitrogen (YPG medium containing 1 % glucose) only yeast-form cells were observed up to 8 days. The conditions supporting sexual sporulation are reported to be invariably more exacting than that required for asexual sporulation (Smith, 1978). For instance, in *B. poitrasii* the profuse development of zygospores was observed on the media containing glucose, while sporangiola development was dense in the absence of glucose (YP medium).

Most of the fungi have the ability to use three different cell wall deposition patterns (Szaniszlo, 1985). In case of dimorphic fungi, either simultaneous expression of more than one pattern or change in the deposition pattern in response to the environmental perturbations may contribute in the morphological outcome. In *B. poitrasii* the parent yeast cells produced bud-like structures prior to the germ tube formation (Fig. 3A. 1b) while in case of Y-5 mutant the ethanol-induced germ tube formation in large number of cells was direct (Fig. 3A. 1f). This can be correlated to the different growth rates between parent and mutant cells which in turn affected levels of the switching of one cell wall deposition pattern to another, viz. isotropic enlargement with discontinuous polarized to continuous and polarized with very little isotropism.

Most of the studies to understand the process of dimorphism are carried out using yeast-mycelium transition. The studies with respect to the mycelium-yeast transition have also been carried out in the case of *H. capsulatum* (Maresca and Kobayashi, 1989), *M. racemosus* (Sypherd *et al*, 1978) and *Y. lipolytica* (Zinjarde *et al*, 1998), to name a few. The mycelium- yeast transition was reported to be slower than the reverse process in *H. capsulatum* and *M. racemosus*. However, in the case of *Y. lipolytica* a rapid mycelium-yeast transition was observed. In *B. poitrasii* it was found to be a slower process. Furthermore, all the mycelia did not transit in to yeast type cells. This can be correlated either to the fact that metabolically more active apical and sub-apical regions can support the biochemical changes required for the transition, or to the differentiation within mycelium. Zinjarde *et al* (1998) reported that during mycelium-yeast transition in *Y. lipolytica* on solid medium protoplasm moved towards hyphal tips, yeast cells budded off and eventually yeast colony developed at the tip. In the present study, the beaded structure was observed at the tip region of the hypha in a basipetal sequence (Fig. 3A. 1 c). Additionally, as reported in *M. racemosus* (Sypherd *et*

al, 1978), lateral bud formation was also observed during mycelium-yeast transition (Fig. 3A. 1d).

Three different protocols used for the preparation of the SEM samples from zygomycetous fungi were tried for *B. poitrasii.* The protocol A used for homothallic fungi like *Mucor azygospora* and *Mucor bainieri* was essentially useful to study the development of azygosporangia that had a two layered wall (Ginman and Young, 1989). Therefore, to study the vegetative mycelial and yeast cells of *B. poitrasii* the method was not found to be suitable (Fig. 3A. 2 b). While Jeffries and Young (1975) studied the infection process and the spatial relationship between mucoralean fungi, a mycoparasite, *Piptocephalis unispora* and the host, *Cokeromyces recurvatus,* using surface scanning electron microscopy. The higher concentration (2%) of the agar reduced the shrinkage of the blocks during drying with acetone. However, in case of *B. poitrasii* the mycelium strands appeared flattened (Fig. 3A. 2d). The pre-fixation in formaldehyde/glutaraldehyde as used for *Zygorhynchus* (Edelmann and Klomparens, 1995), was found to be suitable for *B. poitrasii*. Furthermore, the post-fixation treatment with osmium tetra oxide was useful to maintain the morphology of different structures (Fig. 3A. 2c).

Our SEM studies showed that the surfaces of the yeast and mycelial cells were different confirming earlier report on the quantitative difference and layering of the cell wall components between them (Fig. 3A. 3a,b) (Khale and Deshpande, 1992).

The development of asexual apparatus was typical for *B. poitrasii*. In contrast to *Mycotypha,* the fertile vesicle was globose (Fig. 3A. 4a). The other species of *Benjaminiella* such as *B. youngii* and *B. multispora* produce bi or multisporous and rarely unisporous sporangiola (Benny *et al*, 1985; Kirk, 1989). While the sporangiola in *B. poitrasii* were unisporus (Fig. 3A. 4d).

The dimorphic germination was seen for sporangiola of *Mucor rouxii* which showed yeast and mycelial type germination in response to the respective dimorphism triggers (anaerobiosis and change in glucose concentration) (Bartnicki-Garcia and Nickerson, 1962b). The conidia of *H. capsulatum, P. brasiliensis, Blastomyces dermatitidis* and *S. shenkii,* in response to a change in temperature, that is again a dimorphism trigger for them, also exhibited yeast and mycelial type of germination (Anderson, 1978). The asexual spore of *B. poitrasii* germinated into yeast type cells when subjected to conditions of high glucose and high temperature (Khale *et al*, 1992) and pH 4.0 (Fig. 3A. 5c). The presence of organic nitrogen supported yeast type-growth (Khale *et al*, 1990). Therefore, under mycelium favouring conditions, asexual spores germinated by producing bud-like structures initially which subsequently produced germ-tubes. The direct germ tube formation was not observed. However, the asexual spores germinated directly in to germ tube in the absence of organic nitrogen i.e. in synthetic medium (Fig. 3A. 5b). The GlcNAc was found to enhance germ tube formation during yeast-mycelium transition (Ghormade *et al*, 1999). Therefore, the sporangiolum germination was checked in the presence of GlcNAc in synthetic medium. Though the germ tube formation was delayed, direct germ tubes without initial budding was observed. As glucose favours yeast-type growth in the synthetic medium it was replaced with glycerol. There was no added advantage in direct germ tube formation. It can be correlated to the fact that the absence of complex nitrogen was necessary and sufficient to trigger direct germ tube formation. We have successfully used stable yeast-form mutant (Y-5) to study the process of dimorphism in *B. poitrasii* (Khale *et al*, 1992; Ghormade *et al*, 1999). However, our earlier attempt to isolate mycelial monomorphic mutant was not successful because in the presence of complex nitrogen spores germinated first in to budding yeast (Khale *et al*, 1990). The present observations on direct germ tube formation (Fig. 3A. 5b) will now be useful to isolate and maintain monomorphic (mycelial-form) mutant.

In *C. recurvatus*, a homothallic member of Thamnidiaceae profuse formation of zygospores at and just below the agar surface was observed (Jeffries and Young, 1983). While in *M. indica, B. multispora* and *B. youngii* the zygospores were observed in the aerial hyphae (Benny *et al*, 1985; Kirk, 1989). In the present study, we found *B. poitrasii* produced zygospores near the agar surface. The typical zig-zag formation of zygospores was also noted (Fig. 3A.1f).

In other homothallic species, as *Zygorynchus heterogamus* the process of zygospore formation started with the appearance of a septum in an erect aerial hypha, and the gametangia as well as the suspensors were unequal in size. A single sexual hypha formed the zygospore by means of upward macrogametangia fusion with microgametangia (Edelmann and Klomparens, 1995). While in *B. poitrasii* septum formation was not observed and the gametangia were found to be isomorphic (Fig. 3A. 6).

In case of *Phycomyces* species, a heterothallic fungus it was suggested that the zygospore germination was not influenced by the incubation conditions (Bergman *et al*, 1969). While Guo and Michailedes (1998) reported that in case of *Mucor piriformis* the mode of germination was affected by the nutritional and incubation conditions. For instance, germ sporangial type germination was observed on water agar at pH 4, while potato dextrose agar supported more of mycelial-type germination. According to Orlowski (1991) *Mucor* yeast were known to be developed from sporangiospores, arthrospores and by subjecting mycelium to the yeast favouring condition, and not directly from zygospores. The supporting evidence for the dimorphic response of the zygospore was obtained from the autofluorescence and cell wall staining studies. The autofluorescence, a property of living cells, can be a true reflection of physiological state of the cell (Preece, 1971; Chance *et al,* 1962). The autofluorescence was observed by epifluorescence microscope (LaborLux) using G - Filter with an excitation

range of 340-460 nm. At this range of excitation wavelength, NADH and flavoproteins were reported to be the main contributors for the fluorescence (Franke *et al*, 1980).

These investigations suggest that *B. poitrasii* exhibits dimorphism as a constitutive ability, where its sexual, asexual and vegetative phases respond to the dimorphism triggering condition. This makes dimorphism a commitment of the organism during the entire life cycle rather than restricting it to a particular phase in *B. poitrasii*. This will be useful to understand the position of dimorphic fungi *per se* in the fungal kingdom.

The significance of yeasts in the phylogeny of the fungal kingdom has been reviewed extensively (Hoog de, 1987; Prillinger, 1987; Sterflinger et al,1999). The induction of yeast-like vegetative growth in the zygomycetous dimorphic fungi such as *Mucor* and *Rhizopus* was reported to be significant in the phylogeny of Eumycota (Prillinger, 1987). Furthermore, the germination of chlamydospores from *Asterophora lycoperdiodes* into yeast was implicated in the process of speciation in homobasidiomycetes (Prillinger, 1987). Therefore, the present observations of asexual sporangiospore and sexual zygospore germination into yeast may also be important in understanding the evolutionary relatedness among the fungi.

Fungal phylogeny can also be traced using the differences in the cell wall polysaccharides (Bartnicki-Garcia, 1987; Dörfler *et al*, 1986; Dow and Rubery, 1977; Prillinger *et al*, 1993). In the case of *B. poitrasii* the chitin and mannan contents of the yeast were significantly different than the filamentous cells (Khale and Deshpande, 1992). In this context, dimorphic fungi showing varying mannan contents in the yeast and mycelial cells, can be the intermediate group of organisms in the evolution of fungi.

Lastly, number of medically important fungi, especially species causing systemic infections are dimorphic in nature. To beat the physiological and cellular defenses of the host, most of them change to a convenient morphological form such as unicellular yeast or filamentous mycelium (Deshpande, 1996; Khale *et al*, 1992; Ryley and Ryley 1990). This

change is reversible and is suggested to be the target for antifungal drugs (Georgopapadakou and Walsh, 1994). In case of human pathogenic fungi the spores/conidia are the etiological agent which spread the disease. It has also been reported that the sexual ascospores of pathogenic *H. capsulatum* germinated into yeast like cells at 37°C (Kwon-chung, 1971). Furthermore, in the dimorphic zygomycetous fungus *Cokeromyces recurvatus* the germination of spores into the yeast cell could possibly have led to pathogenesis (Kemna *et al*, 1994). Thus, a sexual spore that has the ability to germinate into yeast cells may have a comparable potentiality for invading a susceptible host. Therefore, halting the germination of the spores can constitute an important approach in the antifungal strategy to stem the spread of pathogenic fungi in the human body. In view of this, the knowledge of sexual and asexual spore germination, especially for the dimorphic fungi would give the useful lead in the study of fungi with regard to the pathogenesis and their evolution.

C) Ploidy determination of parent and monomorphic mutant of

Benjaminiella poitrasii

Introduction

The determination of the ploidy holds an important position in the genetic characterization of eucaryotic organisms. The fungal systems like *N. crassa* , *A. nidulans* , *S. cerevisiae* and *C.albicans* have a well characterized genomic constitution (Brody and Carbon, 1989; Carle and Olson, 1985; Orbach *et al*, 1988; Shepherd *et al*, 1985). The complete genomic analysis of *S. cerevisiae* chromosomal complement and the genetic interpretation have been achieved on the basis of the study of the changes in its ploidy level during the life cycle. Its life cycle goes through the haploid (n) and diploid (2n) alternation of generation in form of the a/α and aα cells, respectively (Herskowitz, 1988). The ploidy status changes during the asexual phase and sexual phase of the fungal life-cycle and this would be useful to understand the alternation of generation.

The methods used for ploidy determination are UV kill curve and the quantification of DNA (Olaiya and Sogin, 1979) and flow cytometry (Watson, 1992). The flow cytometry (FC) is a recently developed technique which allows individual cells that have been stained with a fluorescent stain to pass through a laser beam (Deere *et al*, 1998). Thus using specific fluorescent markers, measurement of size and shape in every single cell of a population as well functional and structural parameters such as enzyme activities, respiration, protein and DNA contents can be made with the FC. In case of fungi, rapid measurements of DNA contents of single yeast cells have been reported (Dvorak *et al*, 1987). The technique enables the analysis of large amount of data *i.e.* 300-1000 events/sec. Therefore, statistically significant determinations on large samples are easily obtained in a few minutes time. In case of ploidy determination of monokaryotic cells, the intensity of the fluorescent signal is proportional to the cellular content of DNA being measured. Now it has become a reliable method to determine the ploidy of the cells. One of the disadvantages, however is its failure

in characterizing filamentous cells. Therefore, in case of dimorphic fungi, the analysis of yeast form cells has been carried out.

To understand the process of dimorphism, one of the advantages in selecting *B. poitrasii*, was the availability of monomorphic (yeast form Y-5) stable mutant (Khale *et al*, 1990). Though the physiological studies with the mutants proved supportive in gaining knowledge of yeast-mycelium transition, the genetic characterization of the parent and the mutant strains will be useful in confirming our observations on the biochemical correlates of morphogenesis. Here the DNA contents of vegetative yeast cells, yeast cells produced from the zygospores under yeast-favouring conditions, the asexual sporangiola and the monomorphic mutant Y-5 have been analyzed.

Results

Ploidy determination using UV kill curve

The UV kill analysis of the vegetative yeast cells (Y), the cells produced from the zygospore (Y_Z) under yeast favouring conditions (details in Chapter IIIa), Y-5 mutant and sporangiola (S) of *B. poitrasii,* of haploid and diploid *S. cerevisiae* strains was carried out. As shown in Table 3B.1, the significant difference in the % survival, 2 and 41% after 1min UV treatment was seen between haploid and diploid strains of *S. cerevisiae*, respectively. Both the strains did not survive under the UV exposures longer than 1 min.

In case of *B. poitrasii,* Y and Y_Z cells exhibited 61 and 60%, respectively survival indicating the similar ploidy status. The Y-5 mutant cells and sporangiola showed 50% and 90% survival after 1 min, respectively (Table 3B. 1). The higher % survival in case of sporangiola, of course can be correlated to the nature of spore coat and melanization. As compared to *S. cerevisiae,* the *B. poitrasii* cells were found to be more resistant to the UV exposures. The % survival was > 10% after 5min (data not shown).

Table 3B.1. The analysis of DNA contents of *Saccharomyces cerevisiae* and *Benjaminiella poitrasii*.

Organism	Survivals after exposure to UV light for 1 min (AU)	DNA contents (fg/ cell)	Flow cytometry Geo mean (%)
S. cerevisiae			
Haploid	2	21.39	63.2
Diploid	41	37.00	101.08
B. poitrasii			
Yeast (Y)	61	310.03	115.69
Yeast (Y_Z)	60	268.25	109.52
Y-5 mutant	50	ND	118.99
Sporangiolum (S)	90	ND	100.37

ND, not determined due to extensive shearing of DNA during isolation procedure.

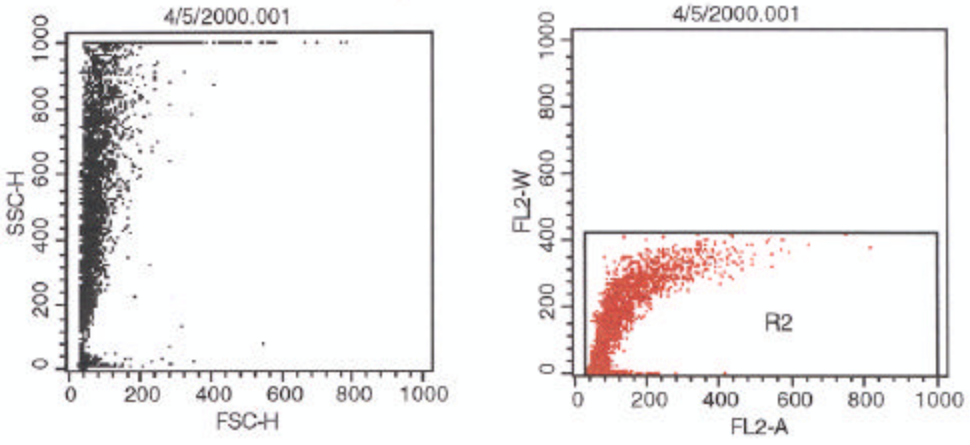
Measurement of DNA contents

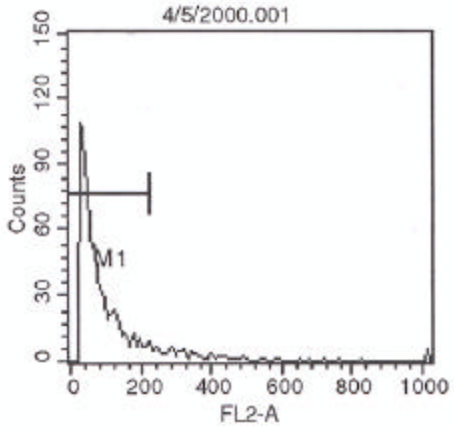
The DNA was isolated from *S. cerevisiae* and *B. poitrasii* parent strain (Y and Y_Z) cells was isolated. The purity of DNA was checked spectrophotometrically using A_{260}/ A_{280} ratio. For all the samples the ratios were between 1.8-1.9. The DNA contents of *S. cerevisiae* haploid and diploid strains found to be 21.39 and 37 fg/cell, respectively. The yeast cells of *B. poitrasii* showed higher DNA contents *viz* 310.03 and 268.25 fg/cell in Y and Y_Z, respectively (Table 3B.1).

Flow cytometry

The flow cytometry allo wed individual cells that were stained with a fluorescent stain, propidium iodide to pass through a laser beam. The intensity of the fluorescence measured was proportional to the cellular content of DNA and was assigned to one of a predetermined number of channels. In case of *S. 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, twofold. The non-gated populations of haploid and diploid *S. cerevisiae* were analyzed on the FL2 W vs the FL2 A(Fig.3B. 1 and 2 respectively). The geometric mean 63.2 AU of the haploid strain was almost half of that of the diploid strain (101.08AU) (Table 3B. 1).

Similarly, *B.poitrasii* parent yeast (Y), Y_Z, Y-5 mutant cells and sporangiola were analyzed (Fig 3-6). As heavy flocculation was observed in case of Y cells of the FL2 W *vs* the FL2 A profile was gated to avoid clumps that appeared with increase in the FL2 W (Fig.3B. 3). The histogram showed a single prominent peak with a geometric mean of 115.69 for Y cells (Table 3B. 1), while the asexual sporangiola showed a single peak (Fig.3B 6a) with a geometric mean of 100.37(Table 3B. 1). The Y_Z cells (Fig.3B. 4) had a geometric mean of 109.52 (Table 3B.1) while that for Y-5 cells (Fig.3B. 5) the geometric mean was





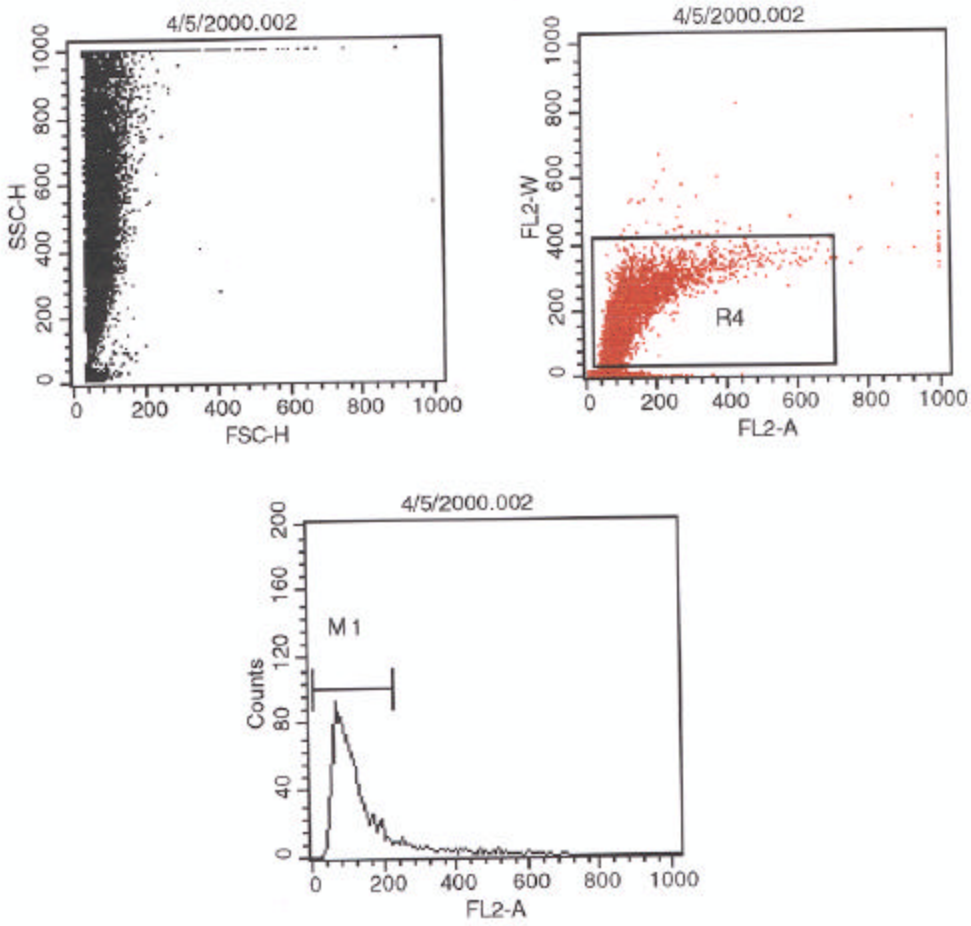
Histogram Statistics

File: 4/5/2000.001
Sample ID: SCN 48HR
Gated Events: 4996
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Gate: G2
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	4996	100.00	24.98	90.80	70.07	28
M1	0, 224	4675	93.57	23.38	74.32	63.20	28

Figure 3B. 1: Flow cytometric analysis of haploid *Saccaharomyes cerevisiae.*



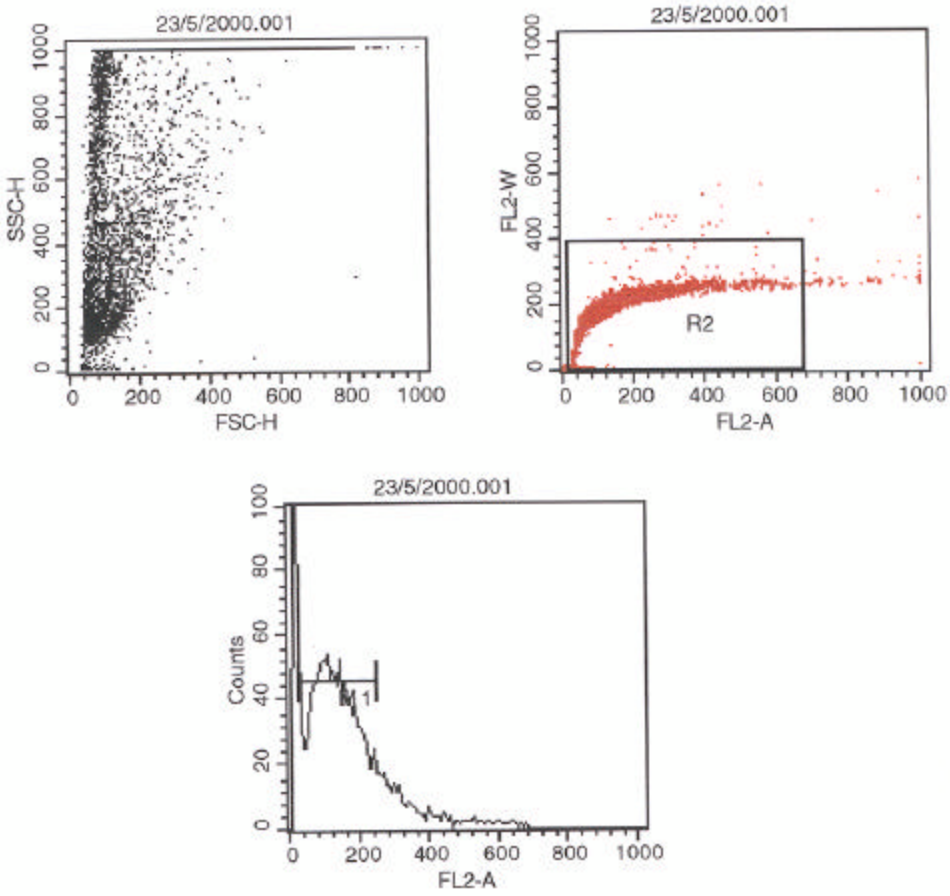
Histogram Statistics

File: 4/5/2000.002
Sample ID: SC 2N 48HR
Gated Events: 6334
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Gate: G4
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	6334	100.00	31.67	126.50	111.07	73
M1	7, 231	5820	91.89	29.10	108.01	101.08	73

Figure 3B. 2: Flow cytometric analysis of diploid *Saccaharomyes cerevisiae*



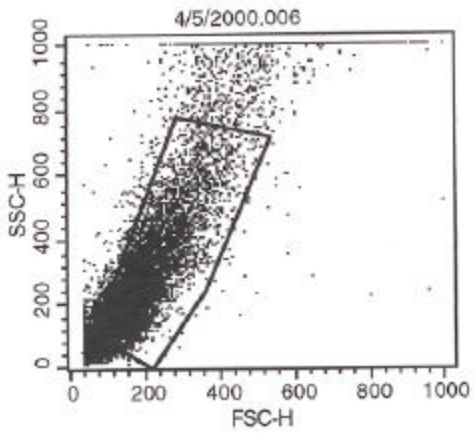
Histogram Statistics

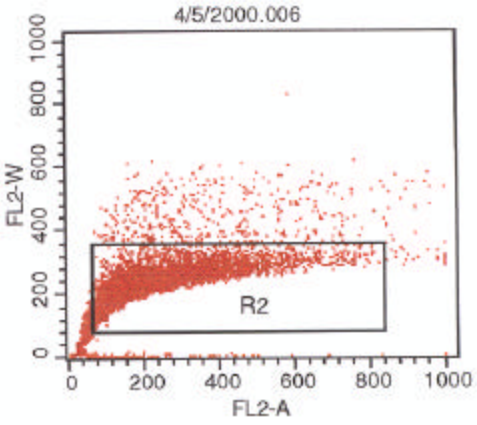
File: 23/5/2000.001
Sample ID: yeast control
Gated Events: 9899
X Parameter: FL2-A (Linear)

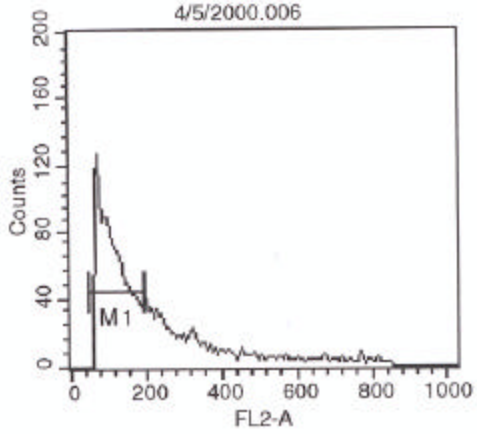
Log Data Units: Linear Values
Gate: G2
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	9899	100.00	49.49	128.58	86.86	14
M1	28, 252	6794	68.63	33.97	130.43	115.69	28

Figure 3B. 3: Flow cytometric analysis of parent yeast cells of *Benjaminiella poitrasii.*







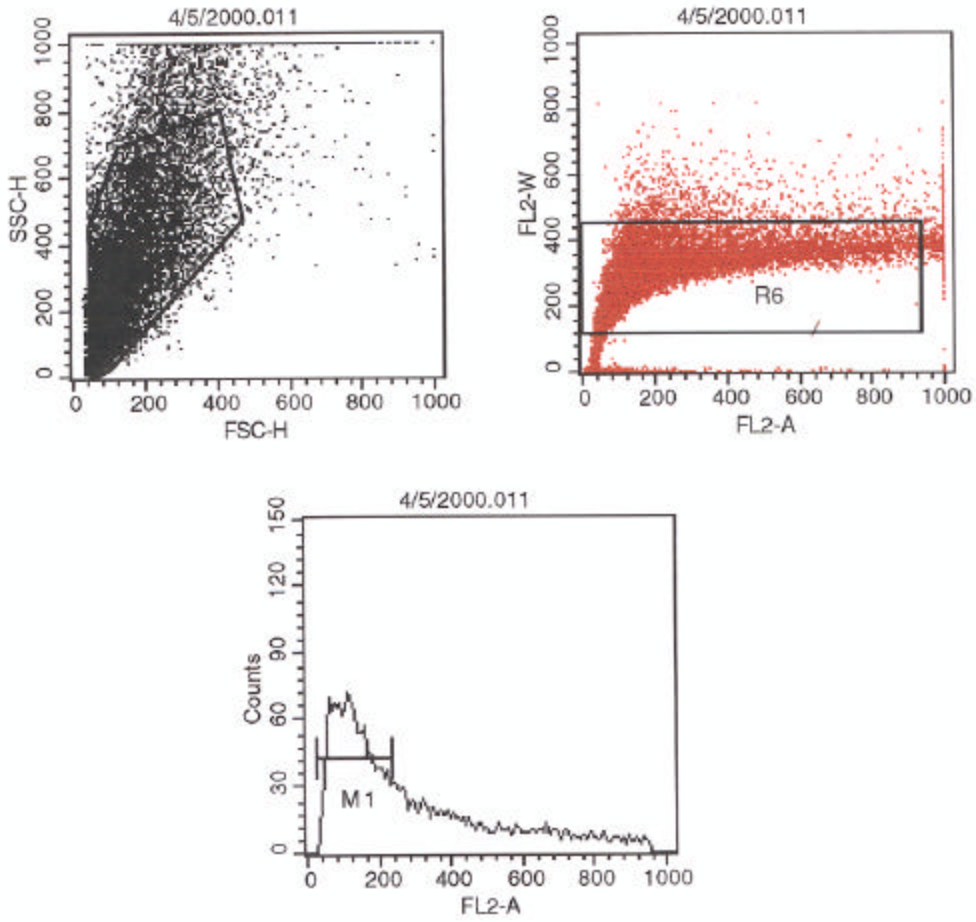
Histogram Statistics

File: 4/5/2000.006
Patient ID:
X Parameter: FL2-A (Linear)

Sample ID: YEAST Z1 24 HR
Gated Events: 11112

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	11112	100.00	55.56	193.60	157.91	71
M1	42, 195	7317	65.85	36.59	115.21	109.52	71

Figure 3B. 4: Flow cytometric analysis of yeast cells from zygospores of *Benjaminiella poitrasii.*



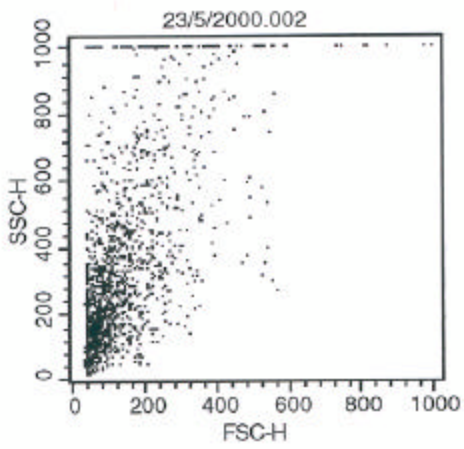
Histogram Statistics

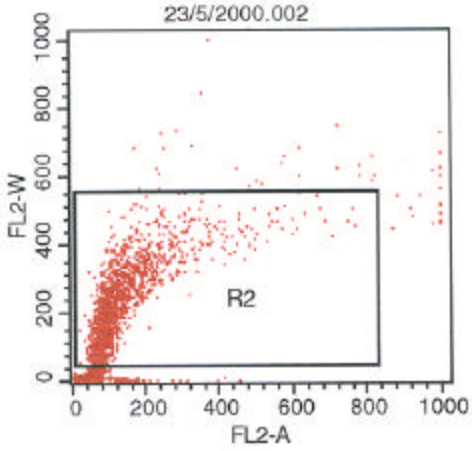
File: 4/5/2000.011
Sample ID: YEAST MUTANT 48 HR
Gated Events: 13541
X Parameter: FL2-A (Linear)

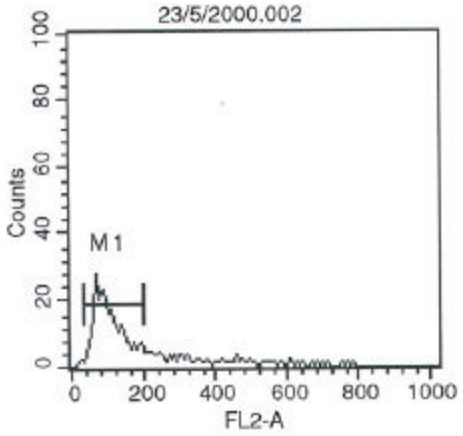
Log Data Units: Linear Values
Gate: G6
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	13541	100.00	67.70	264.27	198.68	113
M1	28, 238	8190	60.48	40.95	129.63	118.99	113

Figure 3B. 5: Flow cytometric analysis of Y-5 mutant of *Benjaminiella poitrasii*.







Histogram Statistics

File: 23/5/2000.002
Sample ID: spores
Gated Events: 1569
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Gate: G2
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	Peak Ch
All	0, 1023	1569	100.00	7.85	138.07	117.79	67
M1	35, 203	1333	84.96	6.67	106.45	100.37	67

Figure 3B. 6: Flow cytometric analysis of sporangiola of *Benjaminiella poitrasii.*

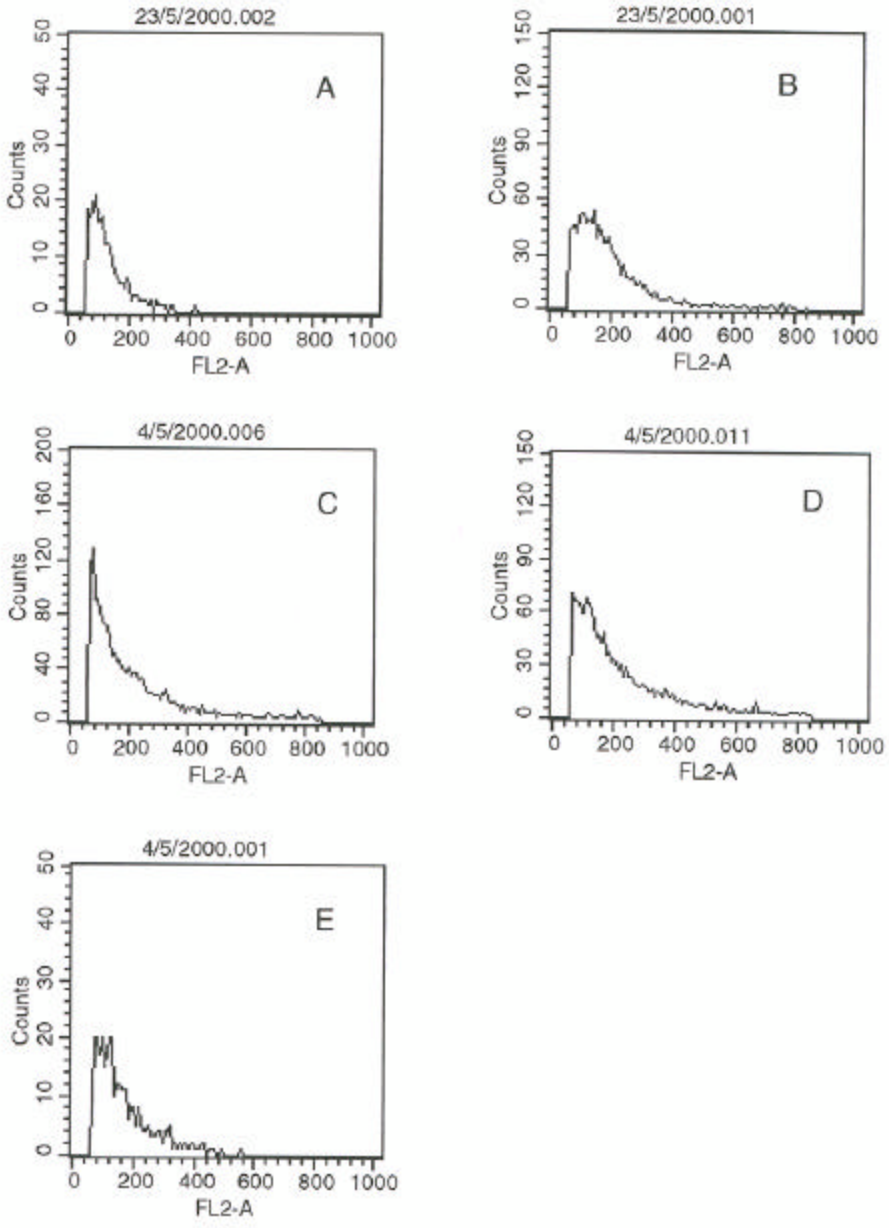


Figure 3B. 7: Comparative DNA content of *Benjaminiella poitrasii* and *Saccharomyces* cerevisiae by flow cytometric analysis. *B.poitrasii* a, Parent yeast cells. b, Yeast cells from zygospore. c, Y-5 mutant cells. d, Sporangiola. *S.cerevisiae* e, Haploid cells

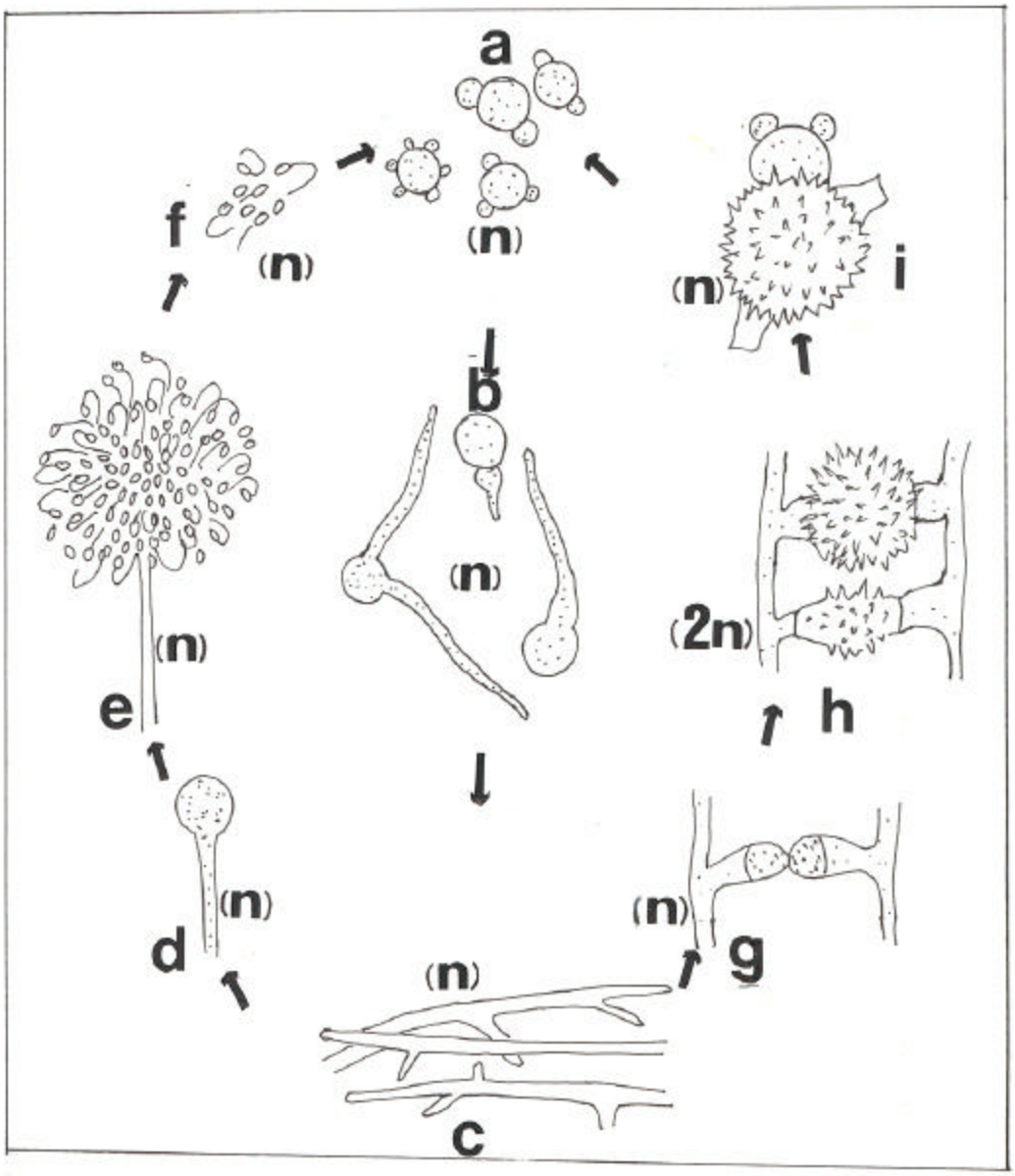


Figure 3B. 8: Alternation of generation in the life-cycle of *Benjaminiella poitrasii*. a, Budding yeast cells. b, Yeast-mycelium transition by germ tube formation. c, Mycelia. d, Formation of globose vesicle apically. e, Mature sporangiophore bearing pedicellate sporangiola. f, sporangiola. g, Zygospore formation by gametangial fusion. h, Mature zygospore. i, Bud-type germination of zygospore.

118.99(Table 3B. 1).

Discussion

For the first time it was shown that zygospores of *B. poitrasii* germinated into yeast cells under yeast favouring conditions *viz.* presence of glucose and 37°C or pH 4.0 of the medium (Chapter IIIa) (Ghormade and Deshpande, 2000). Therefore, the main interests in carrying out these studies were to understand the genetic nature of these cells and the time of meiosis occurring during germination. As the stable yeast- form mutant (Y-5) was being used to understand the biochemical basis of morphological change in *B. poitrasii*, it was interesting to note its ploidy status too.

S. cerevisiae, one of the very well studied fungus was used for comparitive genetic analysis (Haase and Lew, 1997). Olaiya and Sogin(1979) used UV radiation for the ploidy analysis of *S.cerevisiae* and *C.albicans*. In the present study, both the strains of *S.cerevisiae* used did not survive under the UV exposures longer than 1 min. While in case of *B. poitrasii* the survival was 50% after 2 min and >10% after 5 min. This can be correlated to the difference in the chemical composition and ultrastructure of the cell walls in both the organisms (Khale and Deshpande, 1992).

The DNA contents show wide variation for fungi belonging to the different taxonomic groups. The lower fungi have 3-20 times DNA content than that of *S.cerevisiae* (Clutterbuck,1995).The known DNA contents for the standard strains of *S.cerevisiae* haploid and diploid cells are 24.5 fg/cell and 49.5 fg/cell (Table 3B. 1) respectively (Fasman, 1976). In *Aspergillus sojae*, DNA content was 88 fg/cell for haploid and 169 fg/cell for diploid cells (Fasman, 1976). The spectrophotometric analysis revealed that the DNA content/cell of the

B.poitrasii parent yeast cells were >10 times higher than that of haploid *S.cerevisiae* (Table 3B.1).

Generally for the flow cytometric analysis the G1 region was used (Haase and Lew, 1997). The cells that were in the stationary non-dividing phase were in the G1 area and provided a single point of calibration for comparison of the DNA content of the Y, Y_Z cells and sporangiola. These were further compared to the G1 region of *S. cerevisiae*. (Fig.3B. 7).

The geometric mean was a more indicative value than the peak channel value as it indicated the average value for all the cells considered in the mean coinciding with the G1 area (Fig.3B. 1-6). The peak channel value only symbolized the highest number of cells possessing the same DNA content within the mean value.

The flow cytometric analysis of the parent yeast cells and sporangiola gave a geometric mean value of 115.69 AU and 100.37 AU, respectively (Table 3B. 1) The yeast cells (Y_z) from the zygospore had a geometric mean value of 109.52 AU and mutant (Y-5) 118.99 AU (Table 3B. 1). As the DNA content values (Fig.3b. 7) were similar in the parent yeast cells, yeast cells from the zygospore and sporangiola the ploidy would be the same in the major part of the life cycle of *B.poitrasii*.

In *Candida* the long standing question of ploidy was answered using three different lines of evidence. The UV radiation study and FC analysis helped to determine the diploid nature of *Candida.* Kwon-Chung *et al* (1987) employed UV radiation studies and biochemical estimation of DNA content to demonstrate that *C. stellatoidea,* was a diploid. Here we have three different lines of evidence to show that *B.poitrasii* is haploid fungus and has a genome size almost >10 times as compared to that of *S.cerevisiae* haploid cells. The Y-5 cells too had a DNA content and UV profile similar to the parent yeast cells. In case of *C. albicans*, mutant isolation was not possible and later it was confirmed to be a diploid, that explained its refractory nature (Riggsby *et al*, 1982, Sarachek *et al*, 1981). Isolation of the

stable monomorphic yeast like Y-5 mutant of *B.poitrasii* indicated its haploid nature (Khale *et al*, 1990). The Y and Y_Z cells showed a similar survival pattern for the UV exposure. The DNA content analysis also pointed towards the similarity in their ploidy status (Table 3B. 1). The FC analysis showed that the DNA contents were similar in Y, Y_Z cells and sporangiola which further confirmed their haploid status. In case of mucoraceous fungi a major part of the life cycle is in the haploid phase and the diploid phase is confined to the zygospore where the nuclei fuse (Cutter, 1942). Gauger (1977) stated that meiosis may be induced due to the process of germination in the immature zygospores in *Rhizopus stolonifer.* *B.poitrasii* has a typical mucoralean type of life cycle (Fig.3b. 8) that showed an alternation of generation. The diploid state persists till the time the meiotic division takes place and in *B.poitrasii* the yeast cells that developed from the zygospore were haploid in nature indicating that meiosis would have occurred prior to germination. Further work on the karyotyping using pulse field gel electrophoresis will be useful to understand the genetics as well as the process of dimorphism in *B.poitrasii* explicitly.

CHAPTER IV

Yeast-mycelium reversible transition in *Benjaminiella poitrasii*

Introduction

The yeast-mycelium transition in most of the dimorphic fungi is triggered by a variety of environmental perturbations (Gow,1995; Sipiczki *et al*,1998). Broadly, these are divided broadly in to two groups, biophysical such as temperature change and biochemical e.g. nutritional factors such as presence of glucose, metal ions, etc. Most of the dimorphic fungi respond to more than one environmental factors. For example, *C.albicans* exhibits dimorphism in response to temperature change the presence of GlcNAc and serum; while *M. rouxii* shows glucose and anaerobiosis effected by CO_2 environment dependent dimorphism (Gow, 1995; Orlowski, 1991). Interestingly, all *Mucor* species are not dimorphic and all dimorphic *Mucor* species do not respond to the dimorphism triggering conditions identically (Orlowski, 1991). In case of *H. capsulatum*, sulfur containing compounds such as cysteine too play an important role in mycelium-yeast transition (Maresca and Kobayashi, 1989).

Among the all the dimorphism triggers reported in different fungi, temperature and glucose are found to be the crucial ones that directly or indirectly affect differentiation (Deshpande, 1998; Khale *et al* 1990; Maresca and Kobayashi, 1989). For instance the temperature is known to effect the regulation of metabolic activities at the cell membrane through the alteration in its structure and permeability (Schulz *et al*, 1974). Similarly, glucose is the key sugar of the central carbon metabolism of the cell that affects differentiation in most of the fungi (Orlowski, 1991).

In *B. poitrasii,* yeast-mycelium transition triggered by change in glucose concentration and/or temperature has been studied extensively (Khale *et al.,* 1990; Deshpande, 1998). It has also been observed that the change in pH of the medium from 6.5 to 4.0 favours yeast-form growth while at pH 8.0 mycelial form is predominant (Ghormade and Deshpande, 2000). In the present chapter, the relative significance, if any of these factors on triggering morphological change has been evaluated. The yeast-mycelium transition was

studied by i) changing temperature of incubation from 37-28°C; ii) changing glucose concentration of the medium (YPG 0.5% - 0.1% YPG at 28°C); iii) changing pH of the medium (6.5-4.0 or 6.5-8.0);

iv) subjecting cells to a sudden exposure of temperature (22°C,10 min) and/or glucose (10%, 10 min) before transition; v) the reversal effect of these parameters on the pH dependant yeast-mycelium transition in *B.poitrasii*.

Results

As mentioned in the Chapter III A, the sporangiola of *B. poitrasii*, when inoculated in the YP medium initially enlarged and produced bud like structures which on further incubation developed into either mycelial cells or yeast, depending on the temperature. This can be correlated to the presence of complex organic nitrogen in the medium. When the glucose was added in the medium, formation of more number of budding yeast was noted. The yeast-mycelium transition and reverse transition in *B. poitrasii* is a simple one step process. The Y-M transition that takes only 4-6h can be monitored easily by counting the germ tube producing cells. However, mycelium-yeast transition is complicated due to formation of terminal and lateral budding and the quantitation was difficult. Therefore, to understand the relative significance of environmental conditions on growth and differentiation in *B. poitrasii* the yeast-mycelium transition was used.

Effect of incubation temperature on the morphology of *Benjaminiella poitrasii*

The yeast-form cells (8 x 10^6/ 50 ml of the medium) of *B. poitrasii* grown in YPG (0.1% glucose) at 28°C for 24 h were inoculated in the same medium and incubated at different temperatures (20-37°C) under shaking conditions (Table 4. 1). Both mycelial and yeast cells were observed at 28°C and 37°C after 24h. While at 20°C, most of the yeast cells germinated into mycelial cells (Table 4. 1). At 37°C, the yeast cells grew by budding only.

Table 4. 1 The effect of incubation temperature on form-specific growth in *Benjaminiella poitrasii*.

Temperature (°C)	Growth form after 24 h
20	M
28	M ≥ Y
32	M < Y
37	Y

The relative proportion was measured by visual observation. The experiment was carried out using yeast cells as an inoculum in YPG (0.1% glucose) as described under Materials and Methods. M, mycelial-form growth; Y, yeast-form growth.

Effect of addition of glucose on the morphology of *Benjaminiella poitrasii*

The yeast-form cells (8 x10^6/ 50 ml of the medium) of *B. poitrasii* grown in YPG (0.1% glucose) at 28°C for 24 h were inoculated in the YP and YPG containing 0.1 -1.0% glucose and incubated at 28°C and 37°C under shaking conditions (Table 4. 2). In the presence of 0.1% glucose both mycelial and yeast form cells were seen at 28°C as well as 37°C after 24h (Table 4. 2). Higher glucose in the medium (1.0 %) supported yeast form exclusively at 37°C. Interestingly without any addition of glucose, i.e. in YP medium at 28°C only mycelial form cells were seen after 24h while at 37°C, all the inoculated yeast cells did not transit into mycelium (Table 4. 2).

Glucose utilization

The glucose utilization in 0.1% YPG was also monitored at 28°C and 37°C along with the morphology of *B. poitrasii* in 0.1% YPG (5 mM glucose). As seen from the Fig. 4. 1 the glucose utilization was faster at 37°C than at 28°C. The glucose was undetected in the medium after 6h at 37°C while 30% of the utilized glucose was detected at 28°C after 6h. However, at 37°C only 20% cells produced germ tube while >60% germ tube formation was noted at 28°C within 6h. The 50% germ tube formation was observed at 2.75 mM and 5.492 mM glucose utilized at 28°C and 37°C, respectively. This can be correlated to the influence of temperature on differentiation (measured as % germ tube formation) that was more evident than the effect of glucose.

Effect of different triggers in the yeast-mycelium transition

The effect of temperature downscaling from 37°C - 28°C and glucose shift from YPG 0.5% to 0.1% was similar showing 60% germ tube formation in each case. The pH shift from 6.5 to 8.0 evoked a higher response (90% germ tube formation) during the transition. In the shift to pH 4.0 the morphological transition to mycelium was not observed and the cells were

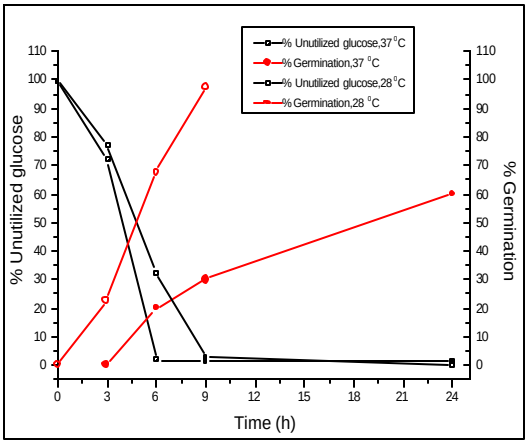
Table 4. 2 The effect of glucose on form-specific growth in *Benjaminiella poitrasii.*

Glucose (%)	Growth form after 24 h	
	28 °C	37 °C
No glucose	M	M < Y
0.1	M > Y	M ≤ Y
0.5	M ≥ Y	M < Y
1.0	M< Y	Y

The relative proportion was measured by visual observation.

The experiment was carried out using yeast cells as an inoculum as described under Materials and Methods.

M,mycelial-form growth; Y,yeast-form growth.


% Unutilized glucose,37°C
% Germination,37 °C
% Unutilized glucose,28°C
% Germination,28 °C
% Unutilized glucose
% Germination
Time (h)
0
3
6
9
12
15
18
21
24
0
10
20
30
40
50
60
70
80
90
100
110

maintained in their original yeast form in 6h. The mycelium formation was very low (10%) when the transition was continued for 24h (Table 4. 3).

Effect of sudden exposure of temperature and/or glucose on the yeast-mycelum transition

To evaluate whether the sudden change in temperature or glucose stimuli traverse the same path to evoke the morphogenetic response during Y-M transition the two triggers were delivered singly and in conjunction to each other. A shock of 10% glucose in YP medium for 10 min was identified for its ability to the to evoke the yeast form while the temperature exposure of 22°C for 10 min was selected to elicit the response in the form of mycelia. The schematic representation of the experiment has been shown in Fig. 4. 2. It can be seen from Fig. 4. 3 that the temperature exposure increased germ tube formation (26.6%) as compared to the control. On the other hand the glucose exposure increased budding by 47.5%. The two stimuli identified were administered in the succession of temperature shock followed by glucose and *vice versa*. In the former the germ tube formation was decreased by 23.7% (Table 4. 4) and in the latter the decrease in budding was 56%(Table 4. 4). It can be suggested that the sudden change in the temperature 22°C, 10 min reversed the influence of 10% glucose for 10 min, on the differentiation significantly.

Effect of sudden exposure of temperature or glucose on the pH mediated yeast-mycelium transition

To evaluate the importance of temperature and glucose as the triggers of yeast - mycelium transition, their effect on pH dependant morphogenesis was studied. The temperature exposure did not evince a change in the transition carried out by change in pH from 6.5 to 4.0 and that was reflected by a marginal increase 10% germ tube formation (Table 4. 5). The glucose shock delivered to cells undergoing transition by change in pH from 6.5 to 8.0 decreased the germ tube formation by 30% (Table 4. 5).

Table 4. 3 Effect of different triggers on the yeast-mycelium transition in *Benjaminiella poitrasii*.

Environmental trigger	Germ tube (%)	Budding (%)
Temperature (37°C - 28°C)	60	40
Glucose (0.5 - 0.1)	62	38
pH (6.5 - 8)	90	10
pH (6.5 - 4)	100	0

The relative proportion was measured by visual observation.

The experiment was carried out using yeast cells as an inoculum as described under Materials and Methods.

Table 4. 4 Effect of sudden temperature and/or glucose exposure on the Y-M transition in *Benjaminiella poitrasii.*

Environmental trigger	Germ-tube (%)	Budding (%)	Increase in Germ tube (%)	Increase in Budding (%)	Decrease in Germ tube (%)	Decrease in Budding (%)
Temperature[a]	76	24	NA	NA	NA	NA
Temperature/ Glucose[c]	58	42	NA	**75**	**23.7**	NA
Glucose[b]	41	59	NA	NA	NA	NA
Glucose/ Temperature[d]	74	26	**80.4**	NA	NA	**56**

The experiment was carried out using yeast cells as an inoculum as described under Materials and Methods.

a, Incubation of cells at 22° C for 10 min.

b, Incubation of cells in YPG (10% glucose) for 10 min.

c, Incubation of cells 22° C for 10 min followed by in YPG (10% glucose) for 10 min.

d, Incubation of cells in YPG (10% glucose) for 10 min followed by incubation at 22° C for 10 min.

Table 4. 5 Effect of sudden temperature or glucose exposure on pH mediated Y-M transition in *Benjaminiella poitrasii.*

Environmental trigger	Germ-tube (%)	Budding (%)	Increase in Germ-tube (%)	Increase in Budding (%)	Decrease in Germtube (%)	Decrease in Budding (%)
pH(6.5 to 8)	90	10	NA	NA	NA	NA
pH (6.5-8)/ Glucose[b]	60	40	NA	ND	30	NA
pH (6.5 -4)	-	100	NA	NA	NA	NA
pH (6.5-4)/ Temperature[a]	10	90	ND	NA	NA	ND

The experiment was carried out using yeast cells as an inoculum.

as described under Materials and Methods.

a, Incubation of cells at 22° C for 10 min.

b, Incubation of cells in YPG (10% glucose) for 10 min.

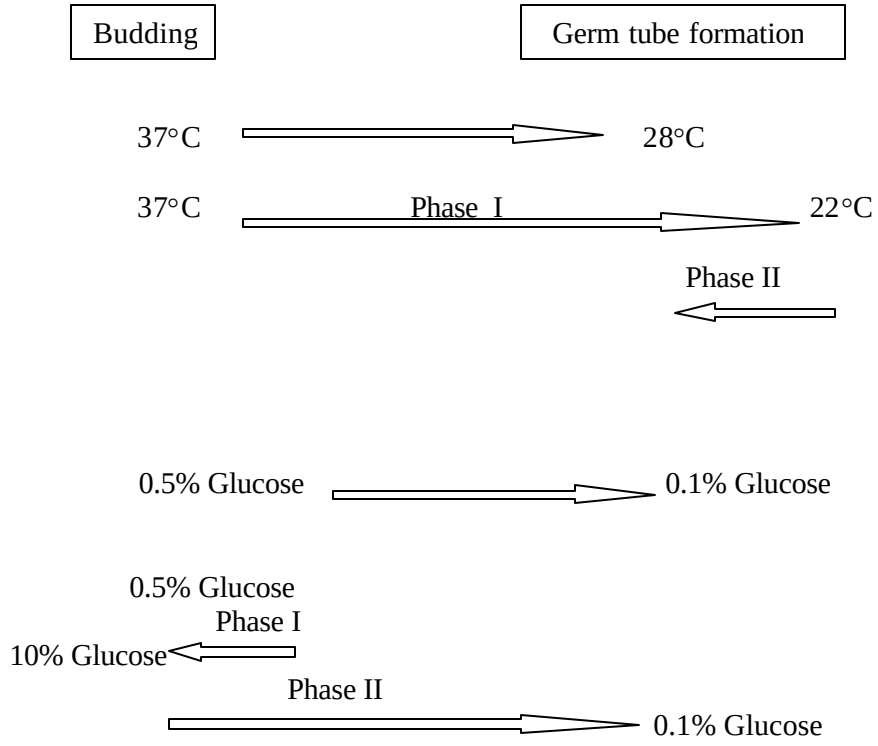
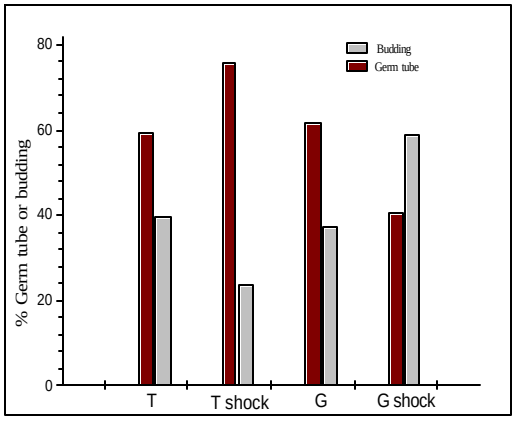


Fig.4. 2 Scheme for the brief exposure of yeast cells to monitor effect on differentiation.


Budding
Germ tube
% Germ tube or budding
0
20
40
60
80
T
T shock
G
G shock

Discussion

The role of environmental triggers in controlling the morphology of dimorphic fungi has been studied intensively but yet remains elusive. *M. rouxii* displays morphogenesis that takes place in response to glucose concentration of the medium and anaerobiosis (Bartnicki-Garcia, 1968a). Sipiczki *et al* (1998) reported that the yeast-mycelium transition in *Schizosaccharomyces japonicus var vaponicus* is temperature dependent. In *Mycotypha africana* the transition was sensitive to temperature and pH change (Hall and Kolankaya, 1974). Stewart *et al* (1988) reported that in case of *C. albicans* external pH of the medium and temperature influence the yeast-mycelium transition. However, it is difficult to assign any single factor as being responsible for the morphological change. In case of *M. rouxii*, the presence of high CO_2 and high glucose favour yeast formation. However, Bartnicki-Garcia (1968a) reported that the presence of low glucose reversed the effect of high CO_2 concentration by producing mycelial cells. Torosantucci *et al* (1984) showed the reversal of the effect of GlcNAc by glucose and serum in *C. albicans.*

The yeast cells used in the study were synchronized so that transition was not observed in all the cells at the same time. Using inoculum grown in 0.1% YPG in 24h for one set of experiment the observations were recorded at the same duration of time (6h). The reproducibility (10-15% SD) was observed in the three sets of experiments.

Tracing the relationship between glucose and temperature by glucose utilization at 28°C and 37°C, drew attention to the effect of temperature on transition. It was evident by the delayed 50% germ tube formation at 5.492 mM glucose utilized in 24 h at 37°C when compared to the 50% germ tube formation at 2.75 mM glucose utilized in 6h at 28°C (Fig. 4. 1). There was no threshold value of glucose that effected the 50% germ tube formation. In spite of the low glucose concentration, due to quickening of the general metabolic rate, maintenance of yeast form at 37°C could be because of the influence of higher temperature.

While at 28°C the 50% germ tube formation took place at much higher glucconcentrations, emphasizing the temperature effect. Bartnicki-Garcia (1968a) also reported the significance of lowering of glucose in mycelial formation in the presence of high CO_2 that favoured yeast formation.

In *B. poitrasii* it has been attempted to investigate the relative importance of temperature and glucose in deciding the morphological outcome. Therefore % germ tube formation was studied by i) altering either temperature or glucose conditions ii) by subjecting cells to a sudden exposure of temperature (22°C,10 min) and/or glucose (10%, 10 min) before transition. iii) The reversal effect of these parameters on the pH dependant yeast-mycelium transition in *B.poitrasii.*

The rationale behind the selection of the conditions of the two factors was in the effect of they impart on the cell morphology. The lowering of the temperature (28-22°C) was selected for the filamentous response elicited while high glucose concentration (10%) for the budding morphology. The effect of these factors in shaping the morphology was evaluated by the ability of either in reversing the effect of the other that was done in effect by exposing the cells to a sudden change (10 min under either condition). The parameters studied were the increased germ tube formation or budding.

For instance, exposure of cells to 22°C increased the % of germ tube forming cells as compared to the respective control (Fig. 4. 3). While immediate exposure of cells to high glucose under identical experimental conditions decreased the germ tube formation and or increased the % budding cell (Fig. 4. 3). Indeed it was easy to monitor the change in during the yeast-mycelium transition. As mentioned earlier (Chapter III) the mycelium- yeast transition follows changes at two distinct sites such as terminal and lateral budding. Therefore, it was difficult to monitor the respective change.

Change in either increase in the % of germ tube or decrease in the % budding was monitored. As the absence of glucose in YP medium also supports mycelium formation, it was difficult to monitor mycelial growth with respect to glucose utilization. Furthermore, glucose supports differentiation (budding in case of mycelium inoculum) in B. poitrasii it was not appropriate to relate mycelial growth with the glucose concentration.

Temperature and glucose both affected the germ tube formation and budding and it was interesting to study whether there was cross-talk in the pathways traversed by the signals of these stimuli. This was done in effect by delivering the stimuli in conjunction with each other and seeing the morphological outcome/consequence. The effect was noted in terms of the reduction of the influence of the primary shock i.e. germ tube formation in case of temperature and budding in case of glucose.

The scheme (Fig.4. 2) for the brief exposure of yeast cells to monitor effect on differentiation shows that the temperature change (22°C) and glucose change (10%) though had different modes, were similar in terms of the effect on the reduction of either budding or germ tube formation (40%).

The exposure to temperature change followed by the glucose change reduced the germ tube formation by 23.7% (Table 4. 4) and increased the budding by 75% (Table 4. 4) with respect to their control. The vice versa motif of exposure increased the germ tube by 80% and decreased by budding by 56% (Table 4. 4). From these observations it can be suggested that temperature had a significant reversal effect over glucose during the yeast-mycelium transition.

The environmental perturbations, glucose and temperature transduce their signals through common intracellular molecules such as cAMP (Gadd, 1995; Pall, 1981; Ronne, 1995; Takezawa, 2000). One of the mechanisms affected by this second messenger is cAMP

dependant phosphorylation and dephosphorylation that may in turn regulate the morphogenesis.

It has been seen in plants, that apart from the membrane fluidizing the cold temperature change effects the Ca^{2+} influx in the plant cells during cold acclamatization. The cold temperature shock causes an increase in the cytosolic Ca^{2+} concentration of the plant cells that is sensed by the calcium binding proteins, like calcium- Calmodulin that alter their conformation to regulate the activity of other proteins (Takezawa, 2000).

Calmodulin (CaM) has been identified in many filamentous fungi and yeasts. Calmodulin is known to interact with protein kinases, phosphatases, adenylate cyclases and phosphodiasterases that directly affect the cAMP mediated pathways. The transduction of the temperature signal may be via Ca-CaM to the regulatory proteins that affect the process of morphogenesis.

On the other hand the increase in the temperature is known to cause a transient dissipation of the cytochemical pH gradient across the plasma membrane leading to decrease in the internal pH of the cell (Coote et al, 1991). Intracellular acidification is also known to be effected by glucose utilization that in turn stimulates cAMP synthesis (Gadd, 1995).

The change in internal pH has been shown to regulate many processes of cellular differentiation. In case of C. albicans the switch from budding yeast to germ tube formation was associated with the alkaline cytoplasmic pH (Robson et al, 1996). Stewart et al (1988) reported that cytoplasmic pH may be controlled by the proton pumping action of the ATPase and that the ATPase inhibitor diethylstilbestrol prevented germ tube formation in C. albicans. The internal pH is influenced by pH of the medium in that the cells are suspended, as well as other factors metabolizable nutrients and temperature. In case of B.poitrasii acidic pH (4.0) favours the yeast-form while the alkaline pH (8.0) promotes the mycelial growth (Table 4. 5).

In case of B.poitrasii the temperature exposure (lower than the normal growth temperature) triggers the germ tube formation and this may be correlated to the cytoplasmic alkalinizatio n. But temperature exposure had a marginal contribution in reversing the effect of acidic pH (Table 4. 5). However the effect of temperature and glucose exposure in the presence of inhibitors like verapamil etc, will be useful to elucidate the course of the signal transduction pathway of both these environmental signals.

Future efforts to unravel the molecular mechanisms underlying the cross talk among these second messengers will provide new insight into the signal transduction pathways in the morphogenetic change.

Most of the pathogenic fungi are able to cause infection due to their ability to change morphologically in response to the host conditions. The relative importance of these environmental conditions in the individual organisms if ascertained, will be useful in deciding the antifungal strategies. In other words, along with the antifungal drugs patients can be treated to improve the specific physiological conditions.

CHAPTER V

The significance of chitin metabolism in the dimorphic transition of *Benjaminiella poitrasii* and its role in development of antifungal agents

A) The involvement of chitinolytic enzymes in the yeast-mycelium transition in *Benjaminiella poitrasii*

B) *Benjaminiella poitrasii* as a model for the screening for antifungal agents as chitinase/ ODC inhibitors

A) The involvement of chitinolytic enzymes in the yeast-mycelium transition in *Benjaminiella poitrasii*

Introduction

Chitin, β-1,4-*N*-acetylglucosamine- linked polymer is the main structural component of most of the fungal cell walls. The chitinase complex comprising of endo-chitinase (EC 3.2.1.14) and *N*-acetylglucosaminidase (EC 3.2.1.52) activities have been implicated in several aspects in the life cycle of fungi, such as hyphal tip growth, spore germination, cell separation, autolysis, etc (Kuranda and Robbins 1991; Rast *et al.* 1991; Sahai and Manocha 1993; Yanai *et al*, 1992). These enzymes are either extracellular or are present intracellularly as cell wall-bound, microsomal or cytosolic (Adams *et al.* 1993; Balasubramanian and Manocha, 1992; Binks *et al*, 1990; Humphreys and Gooday, 1984a; Kuranda and Robbins, 1991). As a function of age, Pedraza-Reyes and Lopez-Romero (1989) reported two distinct cytosolic chitinases in extracts of mycelial cells of *Mucor rouxii*. Later they detected nine chitinases in germinating cells (Pedraza-Reyes and Lopez-Romero, 1991). However, the functional implications of the chitinase multiplicity is still to be understood completely. According to Humphreys and Gooday (1984a) membrane-bound chitinases in *M. mucedo* contributed significantly in the hyphal growth. Similarly, in number of other taxonomically different filamentous and dimorphic fungi, membrane-bound chitinolytic enzymes were reported (Adams *et al.* 1993; Balasubramanian and Manocha 1992; Binks *et al*, 1990; Jackson *et al*, 1996).

Our earlier studies on the cell wall chemistry of a zygomycetous, dimorphic fungus *Benjaminiella poitrasii*, revealed that the mycelial cell walls contain three times more chitin than the yeast cell walls (Khale and Deshpande 1992). The present communication deals with the localization of endo-chitinase and *N*-acetylglucosaminidase activities in the mycelial and yeast-form cells and their possible involvement in yeast-mycelium transition in *B. poitrasii*.

Results

Localization of chitinolytic activities in *Benjaminiella poitrasii*

To find out the biochemical correlation between morphology and chitinolytic enzyme activities, different fractions *viz.* extracellular, cell wall-bound, mixed membrane-fraction and cytosol, were isolated and used for estimation of enzyme activities as described under Materials and Methods. The extracellular endo-chitinase and *N*-acetylglucosaminidase activities were not detected in the respective supernatants. The chitinolytic activities were found to be present in all three fractions *viz.* cell wall, mixed membrane and cytosol (Table 5A. 1).

The mixed membrane fraction (mmf), was the fraction separated from the cytosol and re-suspended in the buffer and used as homogenous suspension for activity determination. The *N*-acetylglucosaminidase activities in cell wall, mmf and cytosol were 41.7%, 37.2% and 21.1% for mycelial cells and 46.5%, 30.8% and 22.7% for yeast cells, respectively. The distribution of endo-chitinase activities of mycelial cells was 53.3%, 23.9% and 22.8% in cell wall, mixed membrane and cytosolic fraction, respectively, while for yeast-form cells it was 39%, 41.4% and 19.6%, respectively. For Y-5 mutant, however, the distribution of chitinolytic enzyme activities was different from the parent strain (Table 5A. 1). The relatively higher *N*-acetylglucosaminidase and endo-chitinase activities (54 and 62%, respectively) was observed in mmf as compared to the parent strain. The cell wall fraction contained 28-30% both the activities. As these activities were found to be distributed in all three fractions, to understand their relationship with the morphological outcome, the differences in the specific activities of both the form cells were studied (Table 5A. 2). It can be seen from Table 5A. 2, *N*-acetylglucosaminidase activities in mmf of mycelial-form cells were 18 fold higher than the yeast-form cells while differences for activities in other 2 fractions were 2-5 fold. Similarly, endo-chitinase activity is 3 fold different in mmf of yeast

Table 5A. 1. Localization of chitinolytic enzymes in *Benjaminiella poitrasii*.

	Total activity (U)*		
	Mycelium	Yeast	Y-5 mutant
***N*-Acetylglucosaminidase activity**			
Cell wall fraction	23.6 ± 3.55 (41.7)	6.51 ± 1.77 (46.5)	1.1 ± 0.18 (28.6)
Mixed membrane fraction	21.05 ± 3.1 (37.2)	4.31 ± 1.56 (30.8)	2.09 ± 0.45 (54.3)
Cytosol	11.85 ± 3.33 (21.1)	3.18 ± 0.33 (22.7)	0.66± 0.17 (17.1)
Endo-chitinase activity			
Cell wall fraction	26.87 ± 3.87 (53.3)	10.3 ± 3.74 (39.0)	11.08 ±1.62 (29.6)
Mixed membrane fraction	12.07 ± 1.38 (23.9)	10.93 ± 2.32 (41.4)	23.26 ±3.35 (62.2)
Cytosol	11.5 ± 1.44 (22.8)	5.18 ± 0.66 (19.6)	3.03 ±0.44 (8.2)

*Total activity (U= one nmol of 4-methylumbelliferone/ min of 2.5 g wet of cells)
The percentage activities has been given in parentheses.

Results are average ± S.D. of 2 sets of duplicate experiments.

Mycelium and yeast cells were grown in YP and YPG(0.5%, glucose), respectively for 24 h at 28°C.

The Y-5 mutant was grown in YPG (0.5%, glucose) for 48 h at 28° C.

Table 5A. 2. Specific activities of chitinolytic enzymes of different fractions in *Benjaminiella poitrasii*.

	Specific activity (U /mg protein)		
	Mycelium	Yeast	Y-5 mutant
***N*-Acetylglucosaminidase activity**			
Cell wall fraction	0.731 ± 0.11	0.261 ± 0.071	0.031 ± 0.005
Mixed membrane fraction	0.761 ± 0.145	0.065 ± 0.012	0.038 ± 0.008
Digitonin solubilized mixed membrane fraction	0.981 ± 0.2	0.073 ± 0.015	0.039 ± 0.011
Cytosol	0.427 ± 0.12	0.048 ± 0.005	0.012 ± 0.003
Endo -chitinase activity			
Cell wall fraction	0.832 ± 0.12	0.413 ± 0.15	0.311 ± 0.045
Mixed membrane fraction	0.436 ± 0.05	0.165 ± 0.035	0.423 ± 0.061
Digitonin solubilized mixed membrane fraction chitinase	0.283 ± 0.05	0.074 ± 0.01	0.385 ± 0.052
Cytosol	0.415 ± 0.052	0.078 ± 0.01	0.055 ± 0.008

Results are average ± S.D. of 3 sets of duplicate experiments.

Mycelium and yeast cells were grown in YP and YPG (0.5%, glucose), respectively for 24 h at 28°C. The Y-5 mutant was grown in YPG (0.5%, glucose) for 48 h at 28° C.

and mycelial cells. Surprisingly, the mutant showed lower *N*-acetylglucosaminidase activity as compared to the parent yeast while the endo-chitinase was 2.5 times higher and comparable to the mycelial form cells. In view of the significant differences in the membrane-bound activities of both the forms, further experiments were carried out using mixed membrane fractions.

After digitonin solubilization, the total *N*-acetylglucosaminidase activity of membrane fraction increased 12% (0.073 ± 0.015 U / mg protein) and 30% (0.98 ± 0.2 U / mg protein) in case of yeast and mycelial cells, respectively as compared to their respective controls (Table 5A. 2). However, the endo-chitinase activity was found to be decreased 45% (0.074 ± 0.01 U / mg protein) for yeast fraction and 65% (0.283 ± 0.05 U / mg protein) for mycelium fraction. For Y-5 mutant, digitonin treatment affected chitinolytic activities marginally (i.e. 2% increase in *N*-acetylglucosaminidase acitivity and 9% decrease in the endo-chitinase activity as compared to the control). The treatment with Triton X-100 (1%) also revealed similar changes in activities (data not shown).

Chitinolytic activities of suspended membrane fractions (mmf) of mycelium and yeast on various substrates were also measured. The total chitinase activity measured on ASC (0.653 ± 0.04 and 0.066 ± 0.04 U/mg protein) EGC (0.544 ± 0.02 and 0.047 ± 0.01 U/mg protein) or *p*NP-(GlcNAc) (4.92 ± 0.1 and 0.84 ± 0.04 U/mg protein) were higher (6-10 times) in mycelium membrane preparation than the yeast, respectively.

The optimum temperature for the activities on fluorogenic substrates was 37°C for all the 3 mmfs. However, pH optima for endo-chitinase and *N*-acetylglucosaminidase activities of mycelium- and yeast- form preparations (both parent and mutant) were 5.0 and 5.5, respectively.

Yeast-mycelium transition

During yeast-mycelium transition, the *N*-acetylglucosaminidase activity of membrane preparation increased steadily throughout the transition (Fig. 5A. 1). The activity (0.05 ± 0.01 U/mg protein) in the yeast cells was increased in 24 h old mycelial cells 17 fold (0.85 ± 0.1 U/mg protein). The activity at 50 % germ tube formation (12 h) was 6 times higher (0.3 ± 0.05 U/mg protein) than the 0 h. The endo-chitinase activity did not show any increase in first 6 h but increased steeply (12 fold) between 6 h (0.05 ± 0.01 U/mg protein) and 12 h (0.59 ± 0.05 U/mg protein) and there after practically remained unchanged up to 24 h.

Mycelium-yeast transition.

As shown in Fig. 5A. 2, the reverse trend in the specific activities of chitinolytic enzymes of membrane fractions was observed during mycelium-yeast transition. As compared to the yeast-mycelium transition, this process was slow. For complete mycelium-yeast transition, the time period required was 30 h.

Isoelectricfocussing

The separation of chitinolytic enzyme activities of soluble portion of the membrane fractions was carried out by isoelectricfocussing. As shown in Fig. 5A. 3, endo-chitinases and *N*-acetylglucosaminidases were resolved into two pH ranges, viz pH 4.3-5.7 and pH 6.1-7.7 (pH 6.1-6.5 and 6.7-7.7), respectively. The endo-chitinases of mycelium- and yeast-form cells (both parent and Y-5) showed relatively similar pattern of resolution. In contrast, for the *N*-acetylglucosaminidase activity a distinct yeast-form specific peak was noted around pH 6.3-6.4 in parent as well as in the mutant. Another peak was observed between 7.3-7.4 for yeast- form cells. While the mycelial fraction showed at pH 7.6 (Fig. 5A. 3). It was interesting to note that the predominant *N*-acetylglucosaminidase activities observed at pHs 6.9 for yeast and 7.1 for mycelium fraction of parent strain could not be detected in the mmf of Y-5 mutant (Fig. 5A. 3).

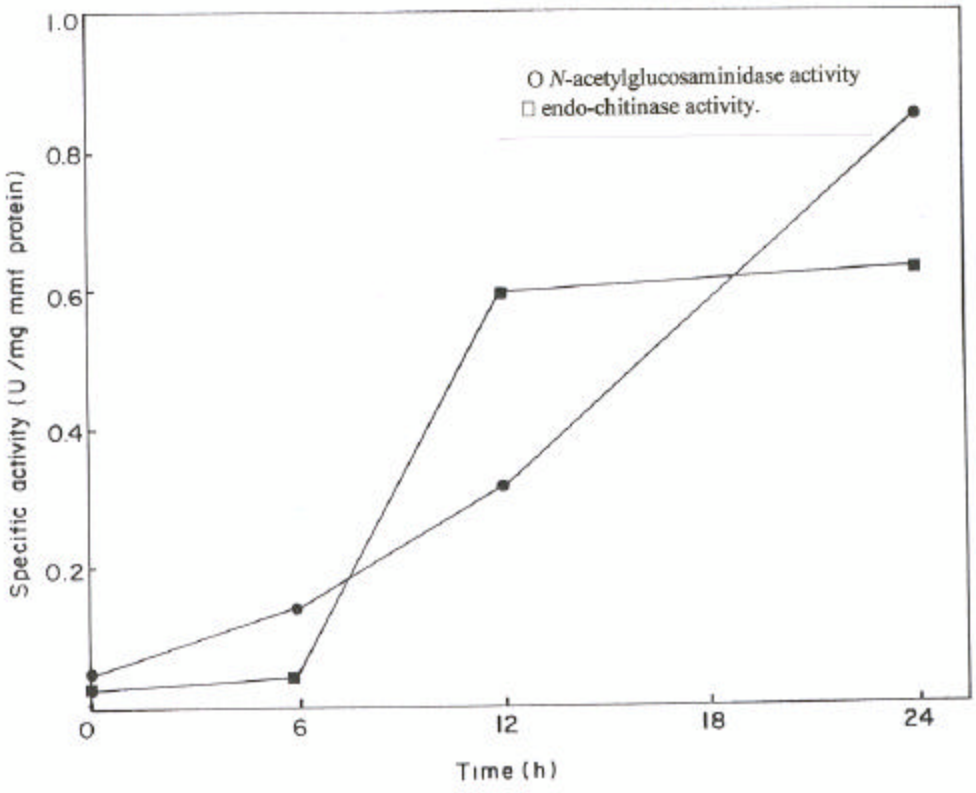


Figure 5A. 1: Changes in the chitinolytic activities of soluble mixed membrane fraction during yeast-mycelium transition in *Benjaminiella poitrasii*.

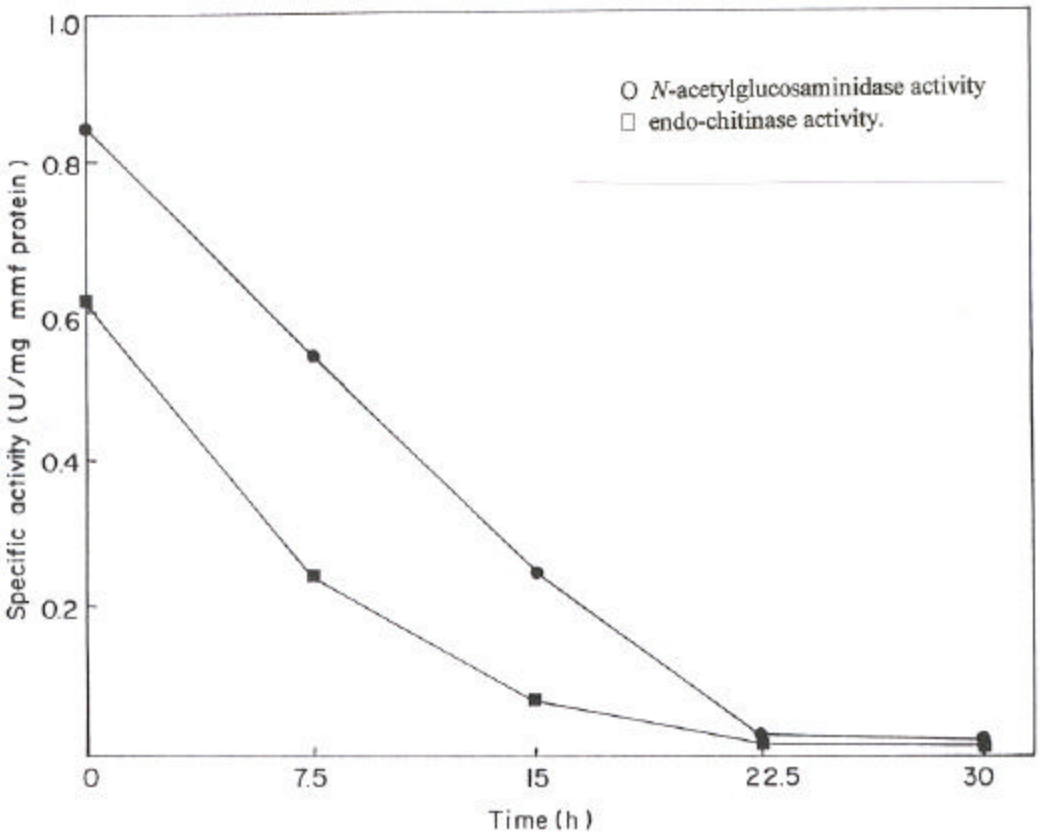


Figure 5A. 2: Changes in the chitinolytic activities of soluble mixed membrane fraction during mycelium-yeast transition in *Benjaminiella poitrasii*.

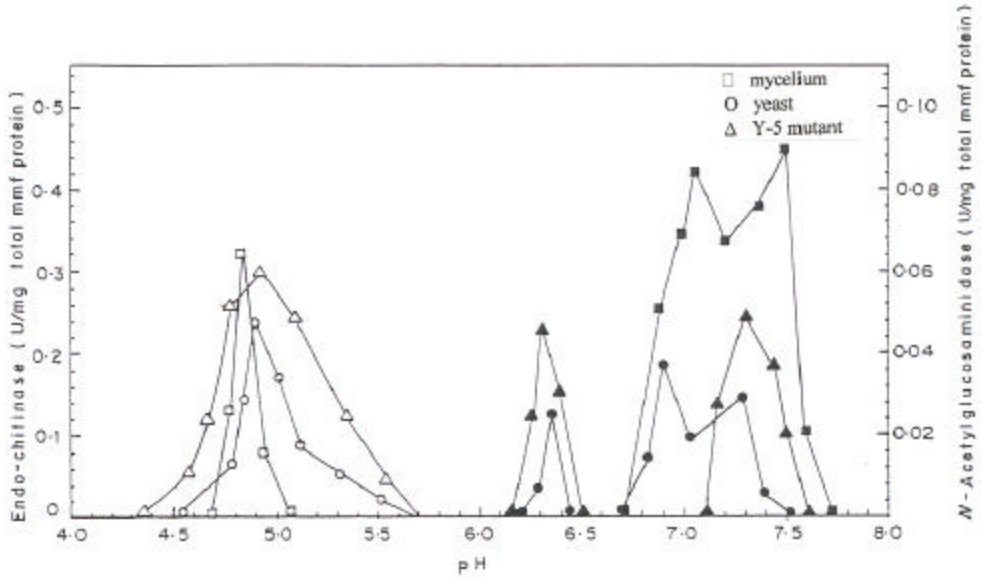


Figure 5A. 3: Isoelectricfocussing of particulate fraction- free mixed membrane fractions of *Benjaminiella poitrasii* mycelium, yeast and Y-5 mutant cells. Open symbols are for endo-chitinase activity (U/mg total mmf protein) and solid symbols are for *N*-acetylglucosaminidase activity (U x 2 x 10^{-1}/mg total mmf protein).

Effect of b-glycosidase inhibitor on whole cell chitinolytic activities and yeast-mycelium transition of *Benjaminiella poitrasii*.

Glucono-δ- lactone, a known inhibitor of β-glycosidases, was used to study its effect on the intracellular endo-chitinase and *N*-acetylglucosaminidase activities and subsequently on yeast-mycelium transition. The enzyme activities were estimated by permeabilizing whole cells with ethanol (50% v/v) as described by O'Donnell (1991). The presence of increasing concentrations of glucono-δ-lactone (2.5- 10 mg / ml) during yeast-mycelium transition increasingly inhibited enzyme activities and retarded germ tube formation. At 2.5 mg / ml concentration, the *N*-acetylglucosaminidase activity measured with 4-MU-(GlcNAc) was inhibited to the extent 65% (i.e. 0.088 ± 0.01 U / mg wet weight and 0.261 ± 0.05 U / mg wet weight in presence and absence of glucono-δ-lactone, respectively). The endo-chitinase activity measured with 4-MU-$(GlcNAc)_3$ was inhibited to 45% as compared to the control (0.211 ± 0.01 U / mg wet weight and 0.389 ± 0.08 U / mg wet weight, respectively). In presence of 2.5 mg glucono-δ-lactone / ml, 60% inhibition in germ tube formation was observed relative to the control (22 ± 4 % in presence of glucono-δ-lactone as compared to minus inhibitor i.e. 55 ± 8 % germ tube formation) during the initial 12 h of transition. When the concentration of glucono-δ-lactone was further increased to 10 mg/ml, the *N*-acetylglucosaminidase activity was found to be inhibited > 80% (0.044 ± 0.004 U / mg wet weight) while endo-chitinase was inhibited 65% (0.131 ± 0.012 U / mg wet weight) and the yeast-mycelium transition was not detected. It was interesting to note that there was no effect of glucono-δ-lactone on the growth of yeast by budding.

Discussion

The chitinolytic enzymes present as cell wall-bound, microsomal or cytosolic significantly influence the morphogenetic event in fungi (Adams *et al.* 1993). In *S. cerevisiae,* it has been observed that most of the chitinase was secreted into the growth medium. However, in *B. poitrasii,* detectable levels of extracellular chitinolytic activities were not observed. The intracellular distribution of these activities was found to be significantly different. The chitinolytic activities bound (either tightly or loosely) to the membranes isolated following identical protocols indeed exhibit morphology related difference in the levels (Table 5A. 1 and 2). Additionally, reports available in the literature suggested that membrane bound-chitinases significantly contributed in the morphogenetic event in fungi (Adams *et al*. 1993; Humphreys and Gooday 1984a, b), therefore, membrane-bound chitinase levels were measured during transition in *B.poitrasii.*

The microsomal chitinases of *M. mucedo* were reported to be solubilised most effectively by Triton X-100 (Humphreys and Gooday 1984b). In *B. poitrasii*, the treatment of mixed membrane fraction with Triton X-100 also increased the *N*-acetylglucosaminidase activity. Furthermore, after digitonin solubilization, the total *N*-acetylglucosaminidase activities of membrane fraction increased 12% and 30 % in case of yeast and mycelial cells (Table 5A. 2). However, the endo-chitinase activity was found to be decreased 45-65% for yeast- and mycelial- form fractions. This can be attributed to the possible requirement of lipophilic environment for the endo-chitinases in *B. poitrasii* as observed in *M. mucedo* by Humphreys & Gooday (1984b).

During the early stages of yeast-mycelium transition in *B. poitrasii,* a sudden increase in the endo-chitinase activity was observed (Fig. 5A. 1). This initial increase can be correlated with its hydrolytic role in weakening cell walls for germ tube formation. In *C. albicans* germ tube formation was reported to be accompanied by an increase in *N*-

acetylglucosaminidase activity (Sullivan *et al*, 1984). Rast *et al* (1991) suggested that *N*-acetylglucosaminidase activity may be participating in chitin synthesis by supplying *N*-acetylglucosamine for wall synthesis. The increase in the *N*-acetylglucosaminidase activity at a later stage may, therefore, be important in mycelial proliferation of *Benjaminiella*. During mycelium-yeast transition (Fig. 5A. 2) the trend of the specific activities was reverse to the trend observed for yeast-mycelium transition (Fig. 5A. 1). The high quantities of chitinolytic enzymes of mycelial cells appeared to be decreased. There was no detectable extracellular secretion of chitinolytic enzymes during transition. The decrease in the specific activities could be attributed to either proteolysis of the enzymes which were not required at the given point of time or the inhibition by specific inhibitors, if any (Deshpande, 1992).

Sullivan *et al*. (1984) reported that in *C. albicans*, GlcNAc and some of its related derivatives favoured germ tube formation and induced *N*-acetylglucosaminidase activity. The enzyme activity *in situ* is in a privileged state, protected especially from inhibitors and pH changes (Gooday *et al.* 1992). Therefore, the effect of chitinase inducer like GlcNAc and inhibitor, glucono-δ-lactone was studied in the whole cells. The *in situ* measurements of enzyme activities showed that in *B. poitrasii* GlcNAc enhanced the germ tube formation and increased the *N*-acetylglucosaminidase activity (data not presented). On the other hand, glucono-1,5-lactone, a powerful competitive inhibitor of glycosidases (Reese and Maguire, 1971), drastically reduced germ tube formation. This can be correlated with the inhibition of *N*-acetylglucosaminidase activity.

Isoelectricfocussing of the soluble portion of membrane fractions of mycelium, yeast and of mutant cells repeatedly exhibited distinct profiles for endo-chitinases in the pH range 4.3-5.7 and for *N*-acetylglucosaminidases in the pH ranges 6.1-6.5 and 6.7-7.7. The endo-chitinases of mycelium- and yeast-form cells (both parent and Y-5) showed relatively similar pattern of resolution. In contrast, a distinct yeast-form specific peak for the *N*-

acetylglucosaminidase activity was noted around pH 6.3-6.4 in parent as well as in the mutant (Fig. 5A. 3). It was interesting to note that the predominant *N*-acetylglucosaminidase activities observed at pHs 6.9 for yeast and 7.1 for mycelium fraction of parent strain could not be detected in the mixed membrane preparation of Y-5 mutant. Further purification and characterization of *N*-acetylglucosaminidases of the parent strain membrane fraction may be useful to understand their definite role in the morphological transition in *B. poitrasii*.

B) *Benjaminiella poitrasii* as a model for the screening for antifungal agents as chitinase/ ODC inhibitors

Introduction

Systemic fungal infections in human beings are steadily increasing due to the fall in immunity levels accredited to diseases like AIDS, diabetes and the advent of modern surgical procedures (Georgopapadakou and Walsh, 1994). Furthermore, the current antifungal strategy faces the problem of toxicity as in the case of amphotericin B as well efficacy and aquired resistance considering the azole drugs in use (Hunter, 1995). This necessitates a search for new antifungals which will prove to be effective in halting the spread of fungal infections.

As most of the pathogenic fungi change from the saprophytic to the infective form, the prevention of this transition is the main target for newer antifungal drugs. One of the potential target process is the cell wall biosynthesis of polymers such as chitin, glucan, mannan. Earlier glucan and mannan synthesis have been studied as a potential target for antifungal drugs. Antifungals like papulacandin, aculeacin A and echinocandin B, inhibit glucan synthase that is involved in the synthesis of glucan, an important wall polymer (Wood, 1998). However the pathogenic fungi like *P.brasiliensis, C.immititis* and a few zygomycetes do not possess β 1-3 glucan in their cell walls as a major component (Gooday,1995). Antifungal compounds like pradimicins and benanomycins targetting the cell wall mannoproteins and causing disruption of the cell membrane are effective against pathogenic yeasts and dermatophytes but not zygomycetes (Gooday, 1995). Chitin is the backbone in the wall architecture of fungi and inhibitors of chitin metabolism are becoming popular as novel antifungal drugs that inhibit fungi specifically with no toxic effect on mammals (Gooday, 1990). Essentially these drugs inhibit the action of enzymes involved in the synthesis and degradation of chitin. Among the chitin metabolizing enzymes, the inhibition of chitin synthase by specific inhibitors such as polyoxins and nikkomycins has been shown to be potentially useful in restricting the proliferation of fungal pathogen. The fungal cell wall

needs to be maintained in a plastic/flexible state for its dynamic growth and the chitinase activity is implicated in this function. Therefore, a chitinase inhibitor may be developed as a new type of antifungal (Gooday, 1990; Sakuda, 1996). In this regard, allosamidin, a pseudotrisaccharide from *Streptomyces* sp was reported to inhibit the chitinase activity of *Candida* and *N.crassa* (Mcnab and Glover, 1991; Milewski *et al*,1992).

A complete chitinase complex comprising of endo-chitinase (EC 3.2.1.14) and *N*-acetylglucosaminidase (EC 3.2.1.30 now EC 3.2.1.52) activities is produced by *Benjaminiella poitrasii*. The studies mentioned in the previous section (Chapter Va) suggested that the chitinolytic enzymes, particularly, *N*-acetylglucosaminidase significantly contributed in the yeast-mycelium transition in *B. poitrasii*. In the present investigations, *B. poitrasii* was employed to screen potential microbial, bacterial and fungal, cultures which produce inhibitors of chitin metabolizing enzymes. For the testing of the efficacy of the antifungal agents three stages/ phases of the life cycle *viz.* yeast –mycelium transition, sporangiolum germination, and the hyphal tip elongation by monitoring bursting of the tip, were identified. Additionally the *in vitro,* inhibition of chitinase and *N*-acetylglucosaminidase activities of *B.poitrasii* was also studied.

Results

Screening of potential natural antifungal agents

Different bacterial and fungal soil isolates were grown in YPG (1%, glucose) medium at 28° C for 96 h. The cell free supernatants were tested for their antifungal potential using *B.poitrasii* as a model.

Effect on the yeast-mycelium transition

B. poitrasii yeast cells were grown in YPG (0.5% glucose) medium at 28° C for 24 h were used as an inoculum. The transition was studied in YPG (0.1% glucose) with and without the addition of an inhibitor (100 µl/ 5 ml medium) at 28° C for 6 h. It can be seen from Table 5B. 1 that in the presence of few of the inhibitor preparations the % germ tube formation was decreased (85%) as compared to the control (100%) with no addition. The preparations from *Bacillus* sp. 102 reduced the germ tube formation to 56% while 35-45% inhibition in the germ tube formation with *S. rolfsii* and *Chaetomium* sp. was noted in 12 h. The crude culture filtrates of *Bacillus* sp. 101 and *Streptomyces* sp. retarded germ tube formation by 15% (Table 5B.1).

Effect of sporangiolum germination

A sporangiola suspension (8×10^3 spores/plate) was spread on the YPG agar plate and the filter discs (0.5 cm soaked with 100 µl inhibitor) were placed in the plates and the plates were incubated at 28° C for 48 h. Except with the preparation of *S. rolfsii* appreciable inhibition in the spore germination measured as inhibition zone was not seen for the preparations which inhibited yeast-mycelium transition (Table 5b.1). This can be correlated to either the requirement of higher concentration of the inhibitors or the diffusion barrier on the solid agar media. In most of the cases, mycelial colonies were seen, however, the yeast colonies were seen at the periphery of the inhibition zone observed for *S. rolfsii* preparation.

The inhibitors from the culture filtrates of fungal origin *Volutella, Chaetomium* and *Sclerotium* and *Bacillus* sp 101, *Bacillus* sp 102 and *Streptomyces* sp did not inhibit spore germination and the development of sporangiospore and zygospore formation was normal (Table 5B. 1). In *Sclerotium* though there was no zone of inhibition the zone of differentiation showed only yeast formation.

Table 5B .1 Effect of culture filtrate on the yeast-mycelium transition, spore germination, hyphal tip bursting, *N*-acetylglucosaminidase and endo-chitinase activity.

Culture filtrate	Y-M transition	Spore germination	Hyphal Tip bursting	*N*-Acetylgluco saminidase (% activity)	Endo-chitinase (% activity)
Volutella	84.5 %	-	-	-	-
Sclerotium	56 %	+	-	-	-
Chaetomium	65 %	-	60 ± 10	131.0 ± 6.5	272.0±10.0
Bacillus sp No.10	84.5 %	-	50 ± 10	63.5 ± 10	75.7 ± 2.0
Bacillus sp No.10	56 %	-	80 ± 5	72.6 ± 2.7	60.6 ± 2.5
Streptomyces sp	84.5 %	-	45 ± 5	65.8 ± 3.3	196.0 ± 5.5

Yeast-mycelium transition was seen in 0.1% YPG liq. medium at 28°C for 6 h.

Germ tube percentage is with respect to the control having 71% germination which was considered as 100%.

Spore germination was carried on YPG (1% glucose) at 28°C with 8×10^3 spores / plate.

Culture filtrates were concentrated to 1/ 10 their volume and 100 µl of this concentrate was used.

- not detected
ND not determined

Hyphal tip bursting

The culture filtrate from the fungal isolate *Chaetomium* applied to 18h grown mycelia showed 65% bursting (Table 5B. 1). Among the bacterial isolates the culture filterate of *Bacillus* sp no 102 showed highest 80% hyphal tip bursting. *Bacillus* no101 and *Streptomyces* sp showed 45-50% hyphal tip bursting (Table 5B. 1).

***B.poitrasii*: a model to screen cell growth inhibitors**

The cell wall synthesis as well as the cell metabolism inhibitors were tested for their effect on *B.poitrasii*

Yeast-mycelium transition

Among the inhibitors for the cell wall synthesis nikkomycin (10μg/ml) and tunicamycin (40 μg/ml) completely halted the transition into mycelium (Table 5B. 2). The yeast cells treated with nikkomycin showed enlargement of the cells. In case of tunicamycin treatment, the plasma membrane had a folded appearance in yeast cells of normal size (12-15μ).

Benanomycin A (10 μg/ml) and benanomycin B (5 μg/ml) reduced the germ tube formation by 15% considering that the control displayed 100% germ tube formation (Table 5B. 2). The cells were normal in appearance. The serine protease inhibitor PMSF, (1mg/ml) and the glycosidase inhibitor, glucono-δ-lactone (2.5 mg/ml) halted the germ tube formation completely (Table 5B. 2). Cycloheximide reduced the germ tube formation by 72%. The inhibitor of αrnithine decarboxylase 1,4 diamino –2- butanone (10 mM) showed 30% germ tube formation (Table 5B. 2). The methylation antagonist , 5 azacytidine (10 mM) increased the germ tube formation by 12% (Table 5B. 2). Phalloidin (1μg/ml) showed 15% reduction in germ tube formation with normal germ tube in length and appearance (Table 5B. 2).

Table 5B. 2 Effect of inhibitors on the yeast-mycelium transition and spore germination and hyphal tip bursting.

Inhibitor	Y- M transition	Spore germination	Hyphal tip bursting
Cell wall synthesis inhibitors			
Nikkomycin	No GT	+++	-
Tunicamycin	No GT	+++	-
Benanomycin A	84.5 %	++	-
Benanomycin B	84.5 %	++	-
Protein synthesis inhibitor			
Cycloheximide	28.16%	++	-
Protease inhibitor			
PMSF	No GT	++	ND
Glycosidase inhibitor			
Glucono-δ-lactone	No GT	-	-
Ornithine decarboxylase inhibitor			
1,4 Diamino-2-butanone	77 %	++	ND
Methylation antagonist			
5 Azacytidine	112 %	+	ND
Actin inhibitor			
Phalloidin	84.5 %	-	ND

Yeast-mycelium transition was seen in 0.1% YPG liq. medium at 28°C for 6 h.

Germ tube percentage is with respect to the control having 71% germination which was considered as 100%.

Spore germination was carried on YPG (1% glucose) at 28°C with 8×10^3 spores / plate.

- not detected
ND not determi

Sporangiolum germination

Among the cell wall synthesis inhibitors nikkomycin totally inhibited the spore germination (Table 5B. 2). There was a uniform zone of inhibition till 4cm with no spore germination. The other cell wall inhibitor tunicamycin had 0.5 cm zone of inhibition from the antibiotic loaded filter disc followed by a 0.5 cm zone where the germ tube ballooned at the tips (Table 5B. 2). Benanomycin A did not show any zone of inhibition and benanomycin B had 0./5 cm zone of inhibition followed by a 0.5 cm zone of differentiation where the germ tubes showed ballooning at the tips (Table 5B. 2).

In case of the glycosidase inhibitor glucono-δ-lactone, no zone of inhibition was observed (Table 5B. 2). The protease inhibitor PMSF had 0.5 cm zone of inhibition (Table 5B. 2). Cycloheximide (1mg/ml) inhibited spore germination in a zone of 0.5 cm and further in the zone of differentiation, the mycelium showed a beaded appearance with profuse branching (Table 5B.2). The sporangiospore and the zygospore formation was limited as compared to the normal.

The ornithine decarboxylase inhibitor 1,4 diamino-2-butanone developed 0.5 cm zone of inhibition succeeded by 1.0 cm zone of differentiation where the spores showed excessive swelling than normal and the mycelium was uneven (Table 5b. 2). The zone of normal growth showed formation of aerial hyphae without formation of sporangiola and zygospores. 5-Azacytidine had 0.5 cm zone of inhibition (Table 5B. 2).

In the presence of Phalloidin (0.1 mg/ml) there was no spore germination inhibition and growth of germ tube as well as sporangiospore and zygospore development was normal (Table 5B. 2).

Hyphal tip bursting

Hyphal tip bursting was observed only in case of nikkomycin (80%). The other inhibitors did not show bursting.

Effect of the potential cell metabolism inhibitors on chitinolytic enzymes

Among all the tested crude culture filtrate, the inhibition of chitinase activity measured on 4 MU$(GlcNAc)_{1\&3}$ was significant with *Bacillus* 102 culture filtrate(Table 5B. 2).The chitinase activity was in the presence of the crude culture filtrate from *Bacillus* sp 101 and *Streptomyces* sp NCL1 the *N*-acetylglucosaminidase was inhibited by 28-35%(Table 5B. 2). The *Bacillus* sp 101 inhibited the endochitinase activity (25%) while in the presence of *Streptomyces* preparation endo-chitinase activity was found to be increased two fold. The *Chaetomium* sp. MY3 culture filtrate inhibited showed prominent activating effect on both *N*-acetylglucosaminidase and endo-chitinase activities (Table 5B. 2). The *Bacillus* sp. 102 preparation inhibited *N*-acetylglucosaminidase and endo-chitinase activities (30-40%) (Table 5B. 1). Further fractionation of the preparation has been carried in our laboratory (communicated to Letters in Applied Microbiology).

Discussion

The enzymes involved in chitin hydrolysis have been implicated in several aspects in the life cycle of fungi, such as hyphal tip growth, spore germination, cell separation, autolysis, etc (Kuranda & Robbins 1991; Rast *et al*, 1991; Sahai & Manocha 1993; Yanai *et al*, 1992). It has been suggested that in *B.poitrasii* the *N*-acetylglucosaminidase and endochitinase play an important role in the morphogenetic process (Chapter Va ; Ghormade *et al*, 1999). Chitinases can be targetted by inhibitors to halt morphogenesis not only in case of pathogenic fungi but also insects (Cohen, 1993; Sakuda *et al*, 1987) This wide spectrum application of the chitinase inhibitors led to the testing of potential antifungals using *B.poitrasii* as a screen. As the biochemical changes are reflected by the morphological changes *Benjaminiella* could be used as a test system to evaluate the effect of inhibitors *in vitro* and *in vivo.*

Here we have used three different stages including yeast-mycelium transition, hyphal tip elongation and spore germination in the life cycle of *B.poitrasii* to screen for the potential antifungal activity of natural as well as known inhibitors *in vivo*. In this study the effect of the inhibitors was studied on the yeast mycelium transition as well as the spore germination. Most of the human pathogenic fungi change their form (mycelium to yeast or *vice versa*) during pathogenesis (Gow, 1995; Deshpande, 1998). Therefore, the effect on the germ tube formation was used to evaluate the antifungal potential of the inhibitors. The spore germination stage was identified because the spores are important as etiological agents in spread of fungal disease. It has been mentioned in chapter IIIa that targetting this stage by antifungal drugs would stall the spread of the fungus at the initial stages. The hyphal tip elongation was pin pointed as a rapid test for the inhibition of the metabolic processes involved in the formation of the cell wall in the apical portion.

The index used for the inhibitory capacity of the various inhibitors and culture filtrate was the percentage of bursting observed in the specified time period.

Among all the potential inhibitors the preparation from the *Bacillus* sp102 affected the yeast-mycelium transition (44%) and the hyphal tip elongation (80%) significantly (Table 5B. 1). The effect on the spore germination was undetected which could be due to the inadequate diffusion of the inhibitor on the agar plate. The culture filterate from *Volutella, Chaetomium, Sclerotium, Bacillus* sp 101 and *Streptomyces* sp affected the yeast-mycelium transition and the spore germination to a lesser extent (Table 5B.1).

All three phases in *B. poitrasii* can be used to screen cell metabolism inhibitors. In case of the *B.cinerea* and *M.rouxii* two stages, the spore germination and hyphal extension test were used to screen for the inhibition by nikkomycin (Smith *et al*, 1990; Zhu and Gooday, 1992). The yeast-mycelium morphological transition in *C.albicans* and the whole cell of *S.cerevisiae* were used for the detection of antifungal compounds (Frost *et al*, 1998;

Sakurai *et al*, 1999). Tariq and Devlin (1996) observed the effect of the inhibitor in *G.candidum, M.plumbeus, T.koningii* and *F.oxysporum* on the mycelial extension.

In *B.poitrasii* all three tests were useful for nikkomycin, a chitin synthase inhibitor which arrested the yeast-mycelium transition, spore germination and gave 80% hyphal tip bursting (Table 5B. 1). This implies that the enzyme participation is important during all the three morphogenetic processes. Other cell wall inhibitors like tunicamycin and the benanomycins (A and B) did not show bursting with the hyphal tip elongation test which may be due to their affect on the the cell wall matrix synthesis (Table 5B. 1). Tunicamycin (Table 5B. 2) halted the formation of the germ tubes during the yeast-mycelium change and affected the spore germination which may indicate towards the glycoprotein involvement in the cell wall building process (Elorza *et al*, 1987). The marginal effect of the benanomycins (A and B), which are known to bind to the mannan or mannoproteins in the cell wall or the cell membrane, on the other two tests may be due to the lesser involvement of the mannans in the cell wall building process. However, in case of *S.cerevisiae* the yeast cells which were metabolically active were susceptible to the action of benanomycin (Watanabe *et al*, 1997).

The glycosidase inhibitor, glucono-δ-lactone halted the yeast-mycelium transition but did not affect the spore germination or the hyphal tip elongation which may be ascribed to the reduced accessibility due to diffusion on the agar medium (Table 5B.2).

Most of the commercially available cell metabolism inhibitors like cycloheximide and PMSF affected yeast-mycelium transition and sporangiolum germination as they interfere with the vital processes involved in general metabolism (Table 5B. 2). Another target identified for the development of antifungal drugs was the cytoskeleton where the polymerization of actin or tubulin is implicated in the morphogenesis. The actin polymerization inhibitor phalloidin affected the transition marginally at the concentration and did not affect the germination (Table 5b. 2).

The effect of the 1,4 diamino-2-butanone, an inhibitor of ornithine decarboxylase which causes hypermethylation due to reduction in the polyamine levels was reflected in the reduced germ tube formation during yeast-mycelium transition and spore germination test (Table 5B. 2). These observation suggest that the polyamines may be involved in the morphogenesis of *B.poitrasii*. The DNA methylation levels are reported to be different in yeast and mycelial form of dimorphic fungi (Cano-Canchola *et al*, 1987; Russell *et al*, 1987). The methylation antagonist, 5-azacytidine showed increase in the germ tube formation. These studies were also used to evaluate the role of DNA methylation in the dimorphic transition in *B. poitrasii.* Therefore *B.poitrasii* can be used as a model for i) the screening of chitin metabolism inhibitors using all three tests ii) General cell metabolism inhibitors using two events the yeast-mycelium transition and the spore germination iii) Other cell metabolism inhibitors, as methylation inhibitors can be tested for their effect using the yeast-mycelium shift and the spore germination.

The morphogenetic events that are visually apparent are the manifestation of the biochemical changes taking place *in vivo*. As the chitinolytic enzymes were involved in the morphogenetic process, the endochitinase and *N*-acetylglucosaminidase activities of *B.poitrasii* in the presence and the absence of the crude culture filterate were estimated to assess its inhibiting potential. The maximum hyphal tip bursting of *Bacillus* sp 102 was correlated to the 30-40 % inhibition of endo-chitinase and *N*-acetylglucosaminidase activities (72.6 ± 2.7 and 60.6 ± 2.5) (Table 5B. 2). It has been suggested that chitin hydrolysis may be one of the regulating processes for chitin synthesis in fungal cell wall (Adams *et al.* 1993; Ghormade *et al*. 1999; Gooday *et al*. 1992). Therefore, hyphal tip bursting can also be correlated to the inhibition of *N*-acetylglucosaminidase and endo-chitinase activities which by supplying *N*-acetylglucosamine contributed to hyphal tip growth.

The effect of *Bacillus* sp. 102 culture filtrate, that inhibited chitinases of *B. poitrasii*, on mycolytic enzyme preparation of *M. verrucaria* having chitinase activity have been tested (communicated, Letters in Applied Microbiology). The chitinase activity measured in its presence, on acid swollen chitin and *N*-acetylglucosamindase on *p*NP- *N* acetylglucosaminide were found to be unaffected. Such type of selectivity in the inhibition of insect chitinase by allosamidin has also been reported (Koga *et al,* 1987). Further identification and structure-function relationship studies of the pure inhibitor from *Bacillus* sp.102 would be useful to identify its role explicitly.

Our studies show that *B.poitrasii* is an effective bioassay where the different phases of its life–cycle can be used to test the effect of various antifungals on the metabolic processes involved in morphogenesis *in vivo* and *in vitro.* The effect of the antifungals during the dimorphic transition and spore germination in *Benjaminiella* can also be used for the study of morphogenesis.

CHAPTER VI

Conclusion

Conclusion

The investigations on the life cycle of *B.poitrasii* as a model system for the study morphogenesis and for screening for antifungal drugs revealed the following advantages over other systems:

1. A complete and well characterized life cycle
2. A rapid, easy reversible transition with no interference of polymorphic forms unlike W*angiella* and other well studied human pathogens.
3. Temperature as well as glucose dependent dimorphism, that can be employed to study pathogenesis in human pathogens.

Exploring the presence of the dimorphic character in the asexual, sexual and vegetative phases of *Benjaminiella poitrasii,* suggested that dimorphism is an intrinsic property and the expression of this character is not restricted to the vegetative phase. Different microscopy methods of light, scanning and fluorescence were used to study the different phases of the life cycle of the dimorphic zygomycetous fungus *B.poitrasii* and its mutant. Scanning electron microscopy (SEM) revealed the details of asexual and sexual spore formation. Asexual spore formation showed the swelling of the hyphal tip followed by emergence of stub like outgrowth on the surface of the vesicle that later formed the sporangiolum on their tips. Sexual spore formation revealed the fusion of the two opposing gametangia during zygospore formation. In both the cases, formation of the reproductory structures in *B. poitrasii,* was not influenced by the yeast favouring conditions, as the mycelium was the prerequisite. On the other hand, both the spores displayed the dual mode of germination either into yeast or mycelium in response to their respective conditions. Such type of duality in germination was reported for sporangiospore in *M.rouxii* (Bartnicki-Garcia, 1968a). While the zygospore was found to germinate by germ-sporangial as well as mycelial type. In case of *B. poitrasii* it is the first time that the bud type germination of the

zygospore has been reported among dimorphic mucoraceous fungi. As dimorphic organisms are intermediate to the unicellular yeast and the filamentous forms, understanding of dimorphism as in intrinsic character will be useful to trace the evolutionary relatedness among fungi. Dimorphic fungi are important pathogens of humans and animals. The zygospore germination into budding yeast has implications in the pathogenesis of zygomycetous fungi and the antifungal strategy where the biology of the fungi is important to identify the infective propagules (Ghormade and Deshpande, 2000).

The genetic characterization of the budding yeast cells (Y_Z) produced from the germinating spores was important to understand the complete life cycle of *B. poitrasii*. The traditional methods such as DNA measurement using spectrophotometry, effect of UV radiation along with the recent method of flow cytometry of the compliant parent yeast (Y) cells, Y_Z , the asexual spores revealed the similar DNA contents in all three. The data on monomorphic yeast-form (Y-5) mutant confirmed the findings on ploidy of the yeast cells. As the DNA contents remain unchanged in the major portion of the life cycle the fungus prevails in a haploid state, as is confirmed by the UV kill method. The zygospore represents the only stage where the diploid character is maintained and meiosis occurred before the development of yeast during its germination.

Another interesting facet of the dimorphism is the response of the organism to the different environmental conditions for yeast-mycelium or *vice versa* transition. For instance, *C. albicans* exhibits dimorphism in response to pH, GlcNAc, serum, temperature, etc (Gow, 1995), while *M. rouxii* shows glucose and CO_2 dependent dimorphism (Sypherd *et al*, 1978). Therefore, it is possible that the fungus may respond preferentially to certain conditions to achieve the desirable morphology for its survival and proliferation. Although it has been suggested that dimorphism in *B. poitrasii* is an intrinsic property and the expression of this character is not restricted to the vegetative phase, mainly the yeast-mycelium transition was

studied in order to understand relative significance of the environmental triggers such as glucose, pH and temperature in the morphogenesis. It was indeed difficult to design the biochemical and molecular experiments, therefore, microbiological approach, *viz.* monitoring of yeast-mycelium transition after subjecting the yeast inoculum to the sudden exposure to 22° C and /or glucose (10%) in different orders, was followed. Various combinations of environmental changes suggested that *B. poitrasii* significantly responded temperature change than the glucose change. However, pH (either 4.0 or 8.0) showed an overriding effect on the yeast-mycelium transition. Of course, the further elucidation of the signal transduction would reveal information about the pathway traversed by the individual signals and the possible crosstalk between them.

The regulation of chitin synthesis is one of the important phenomena in deciding the morphological outcome (Adams *et al*, 1993). The proteolytic activation of chitin synthase (Cabib *et al*, 1990; Fevre *et al*, 1990), membrane stress (Deshpande *et al*, 1997), presence of competitive inhibitor for chitin synthase (Wenke *et al*, 1993) and the presence of chitinolytic enzymes (Adams *et al*, 1993) are the different mechanisms suggested to be involved in the regulation of chitin synthesis. The work on the biochemical characterization of intracellular chitinolytic enzymes of *B. poitrasii* showed the significant relationship with the yeast-mycelium and reverse transition. The specific activities of chitinases of cell wall-free, particularly in membrane fraction were significantly different in yeast-, mycelial-form cells. The predominant *N*-acetylglucosaminidase activity observed at pHs 6.9 and 7.1 during isoelectricfocussing of the parent strain membrane fraction was undetected in the mutant's preparation. The studies suggested that the membrane-bound (either tightly or loosely) chitinolytic enzymes, particularly, *N*-acetylglucosaminidase significantly contributed in the morphological changes in *B. poitrasii.* (Ghormade *et al,* 1999).

The regulatory processes for cell wall, particularly chitin synthesis were also explored for their use as a target for the antifungal agents. The three stages of the life cycle, namely sporangiolum germination, yeast-mycelium transition and hyphal tip growth measured by hyphal tip bursting were used to screen different potential antifungal producers. Among the tested isolates, *Bacillus* sp. 102 culture filtrate effectively affected all the three stages in the life cycle of *B. poitrasii*. Interestingly *in vitro* testing showed that it had a strong chitinase inhibitory activity. Further identification and structure-function relationship studies of the isolated inhibitor from *Bacillus* sp.102 will be useful to identify its role explicitly. Additionally, these studies suggested that chitinolytic enzymes played a significant role in the life cycle of *B. poitrasii*. Other than cell wall metabolism inhibitors, polyamine, cytoskeleton, protease and protein synthesis and DNA methylation inhibitors were also tested successfully using *B. poitrasii*. However, in the present scenario, the chitin metabolism is one of the promising targets to develop antifungal agents against.

Finally, a number of medically important fungi, especially those species causing systemic infections like *Candida, Histoplasma, Blastomyces* and *Paracoccidioides* are dimorphic in nature. To beat the physiological and cellular defenses of the host, most of them change to a convenient morphological form such as unicellular yeast or filamentous mycelium. This change is reversible and is the suggested target for development of antifungal drugs. In case of human pathogenic fungi the spores are the etiological agent which spread the disease. Therefore, the possibility of halting the germination of the spores as an antifungal strategy, to stem the spread of pathogenic fungi in the human body is important. Hence these studies validate the use of *Benjaminiella poitrasii* as a model for the study of morphogenesis and for the screening of antifungals agents.

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3. Patil R.S., **Ghormade V.** and Deshpande M.V. (2000) ChitinolyticEnzymes: An exploration. *Enzyme Microb. Technol* **26**: 473-483**.**

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4. **Ghormade V.,** Sainkar S.R. and Deshpande M.V. Light scanning electron microscopy studies of dimorphism by the vegetative and reproductive forms in a zygomycete *Benjaminiella poitrasii* [Manuscript revised and communicated to *Mycologia*].