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Rhizopus stolonifer exhibits dimorphism

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This study showed that multiple morphologies could be induced from sporangiospores of *Rhizopus stolonifer* in minimal medium. These included moniliform hyphae, septate hyphae and terminal budding yeast cells. When considered along the normal coenocytic filamentous growth form, the study showed that *R. stolonifer* could be polymorphic.

Key words: *Rhizopus stolonifer*, polymorphic, multiple morphologies.

INTRODUCTION

Rhizopus stolonifer, a Zygomycete has a filamentous growth habit. Its filaments are coenocytic, that is, they are non-septate. It is the only fungus yet known to produce rhizoids which penetrate the substratum in order to obtain nutrients. The rhizoids also serve as support. Opposite the rhizoids, a sporangiophore juts into the atmosphere and this terminates in a club-shaped collumelum enclosed within the sporangial wall. Between the collumelum and wall are numerous asexual reproductive structures known as sporangiospores. This organism is also characterized by the presence of stolons, which connect rhizoid joints.

Other members of the Zygomycetes, especially species of the genus *Mucor*, have been shown to undergo fungal dimorphism (Bartnicki-Garcia and Nickerson, 1962a, b, c, d; Friedenthal et al., 1974; Schulz et al., 1974; Ruiz-Hereira, 1985; McIntyre et al., 2002; Lubberhusen et al., 2003). In such phase transitions, sporangiospores convert to yeast like cells, with a central globose mother cell which multilaterally produces daughter buds by blastic action. This occurs under CO_2 tension or high hexose concentration.

However, terminal budding yeast cells have been induced from sporangiospores of *Mucor circinelloides* Tieghem in minimal medium (Omoifo, 2005). When the external medium was subjected to K^+ variation, the 1.0 g/l K^+ supplementation was found to be critical for protoplast formation, a transient morphology, but Na^+ was required for yeast induction. Thus, a treatment of 1.0 g/l K^+, 0.10 g/l Na^+ gave optimum yeast induction; even though thallic growth forms, including holoblastic-, holothallic- and enterothallic conidia were also induced, uracil supplementation drastically diminished the presence of these thallic forms (Omoifo, 2006a). This suggested that these

ions impacted on the structural modification led to the induction of yeast cells from sporangiospores. A follow-up study showed that the incorporation of myoinositol and zinc into the minimal medium of growth of the microorganism enhanced yeast induction by more than 63%. These studies further demonstrated that environmental factors influence mould-yeast interconversion.

In the study of Bartnicki-Garcia and Nickerson (1962b), several members of the Mucurales were tested for their ability to undergo mould-yeast interconversion, but *R. stolonifer* was found to be incapable of it. Their study was conducted in solid cultures of complex nature (yeast extract-peptone-glucose, YPG) and incubated under 30% CO_2 pressure. This claim in minimal medial composition that is known to induce terminal budding yeast cells from *M. circinelloides* Tieghem was validated or otherwise disproved (Omoifo, 2005, 2006a, b).

It was also found that *Mucor* species could only convert to multipolar budding yeastlike cells under CO_2 atmosphere when there is fermentable carbon substrate like galactose, fructose, mannose, glucose and disaccharide like sucrose; when it was grown with xylose as substrate, intact sporangiospores remained in the broth after 72 h (Bartnicki-Garcia and Nickerson, 1962b).

On the other hand, McIntyre et al. (2002) cultivated *M. circinelloides* in xylose substrate-Vogel's minimal medium which was incubated in CO_2 atmosphere and the morphological expression was multipolar budding yeast like cells. Lubberhussen et al. (2003) used similar medium (Vogel's) with xylose or glucose as substrate for the cultivation of *M. circinelloides* and obtained filament-tous growth in cultures sparge under the atmosphere. Since preliminary study showed that *R. stolonifer* could utilize glucose for growth, this study examined the effect

of the pentose sugar, xylose, on its habit.

Reported here is the induction of several morphological forms of *R. stolonifer* in minimal medium.

MATERIALS AND METHODS

Fungal strain

The strain of *R. stolonifer* used in this study was a gift from Miss Elsa Thomas, the Project Assistant to Dr. G. S. Prasad of Microbial Type Culture Collection 1, Institute of Microbial Technology, Sector 39A, Chandigarh, India. It was purified through three different inoculations and subculturing and maintained as malt extract (MEA, 31.2 g/L) solid cultures which exhibited filamentous growth habit. A fresh culture was prepared after seven days.

Inoculum preparation for growth

Inoculum was obtained by pouring sterile deionized distilled water over aerobic growth and a sterile glass rod was gently passed over the surface so as to dislodge the spores. The suspension was not subjected to extreme preparation as was done in the method of Omoifo (1996, 2003, 2006a).

This was to ensure that some mycelia fragments were retained in the inoculum. This showed to be so on examination under the microscope (x400 magnification). But generally, spore count which was taken with Neubauer Haemocytometer. (BSS No. 784 Hawksley, London Vol. 1/4000) was adjusted to 10×10^5 spores per ml in sterile deionized distilled water, with the aid of tally counter.

Reagent and culture media

This has been described (Omoifo, 1996, 2006a) and subsequently modified. Briefly, media were prepared per litre: Xylose (Hymedia), 10 g; $(NH_4)_2SO_4$ (Merck), 5 g; K_2HPO_4 (Qualigens), 5 g; KH_2PO_4 (Merck), 5 g; $MgSO_4$ (Qualigens), 2 g; $FeSO_4$ (Hymedia), 0.1 g; NaCl (Qualigens), 0.1 g; $CuSO_4$ (Analytical Rasayan), 0.06 g; $MnSO_4$ (Qualigens), 0.065 g; $ZnSO_4$ (BDH), 0.06 g; $CaCl_2$ (Sigma) uracil (Hymedia), 100 mg; myoinositol (Sigma), 0.25 g.

The media where prepared in 2000 ml beaker. Weights of buffer components 0.2 M Na_2HPO_4: 0.1 M citrate were obtained using H54Ar meter balance and added to the beaker. The pH was adjusted to 4.5 with 2 N NaOH or 1 N HCl using a control dynamics pH meter model APX 175 E/C in the 2000 ml beaker before dispensing 80 ml of broth in each duplicate of 250 ml Erlenmeyer flasks for the test. The solution in each flask was made up to 100 ml with glass distilled deionized water and sterilized at 121°C per 15 min.

Inoculation, growth conditions and sample collection

A 1 ml spore suspension was drawn and inoculated into each broth flask using Eppendorf micropipette. Each culture flask was then shaken for 30 s and thereafter incubated at 28 ± 1°C (laboratory temperature). Culture flasks were examined after 72 and 168 h of growth.

The culture flasks were returned for further incubation. Slides were prepared for viewing by putting one or two drops of lactophenol on a glass slide with the aid of wire loop, one drop from the culture flask properly shaken was then added and covered with a glass cover slip and viewed under a binocular microscope at x 1000 magnification using the microscope Olympus BX51 attached with camera model No. C-3250. The observed morphologies were recorded.

RESULTS

Visual examination showed the emergence of mycelia on the surface of growth medium. This appeared white to grey on broth surface but aerial mycelia were grey.

The culture was cloudy and sediments were formed by day 3. After one week, aerial mycelia became profuse and thick sediment was formed at the bottom of the culture. Microscopic examination revealed that, aerial mycelia originated from broth level mycelia made up of rhizoids and stolons. Figure 1a showed a section of a long sporangiophore, which is vertically opposite the rhizoids that penetrate into the broth as well as a side (horizontal) branch, which is a section of the stolon interconnecting two rhizoid joints. Shown in Figure 1b is globose sporangium enclosing numerous sporangio-spores; within it also is the collumelum which is the terminal end of the characteristically long sporangiophore. Figure 1c showed collapsed sporangia that had released their content of spores. Sporangiospores are grey to dark brown in colour, have striated walls and vary in shape (Figure 1d).

Three different types of hyphae were formed within the broth. These were tan to light brown coenocytic hyphae (Figure 2c), dark brown septate hyphae (Figure 2s) and moniliform hyphae which has narrow diameter and simple septa-like partitions which have no cell wall material deposition (Figure 2m).

On the other hand, yeast cells which were profusely produced, were terminal budding and assumed varying shapes. They could be globose, subglobose, variations of cylindrical and allantoidal cells (Figure 2a and y). Yeast formation also occurred by moniliform budding whereby the growing tip enlarges; a constriction occurs behind the expanded apex which assumes the yeast shape. It could be globose, subglobose or ellipsoidal. This eventually detaches from the hyphae into the medium where it continues to proliferate by terminal budding (Figure 3a and b).

DISCUSSION

Several growth forms were observed in submerged cultures in this study. These included coenocytic hyphae, septate hyphae, moniliform hyphae and terminal budding yeast cells, which was preponderant. Therefore, *R. stolonifer* is polymorphic in minimal medium.

The study of Bartnicki-Garcia and Nickerson (1962b) showed that *R. stolonifer* could not convert to yeast-like morphology under carbon dioxide pressure, unlike the *Mucor rouxii* used in their study. In the aforementioned study, it was shown that *M. rouxii* proliferate by multipolar budding, whereby a yeast mother cell gave rise to numerous multipolar daughter buds by blastic action.
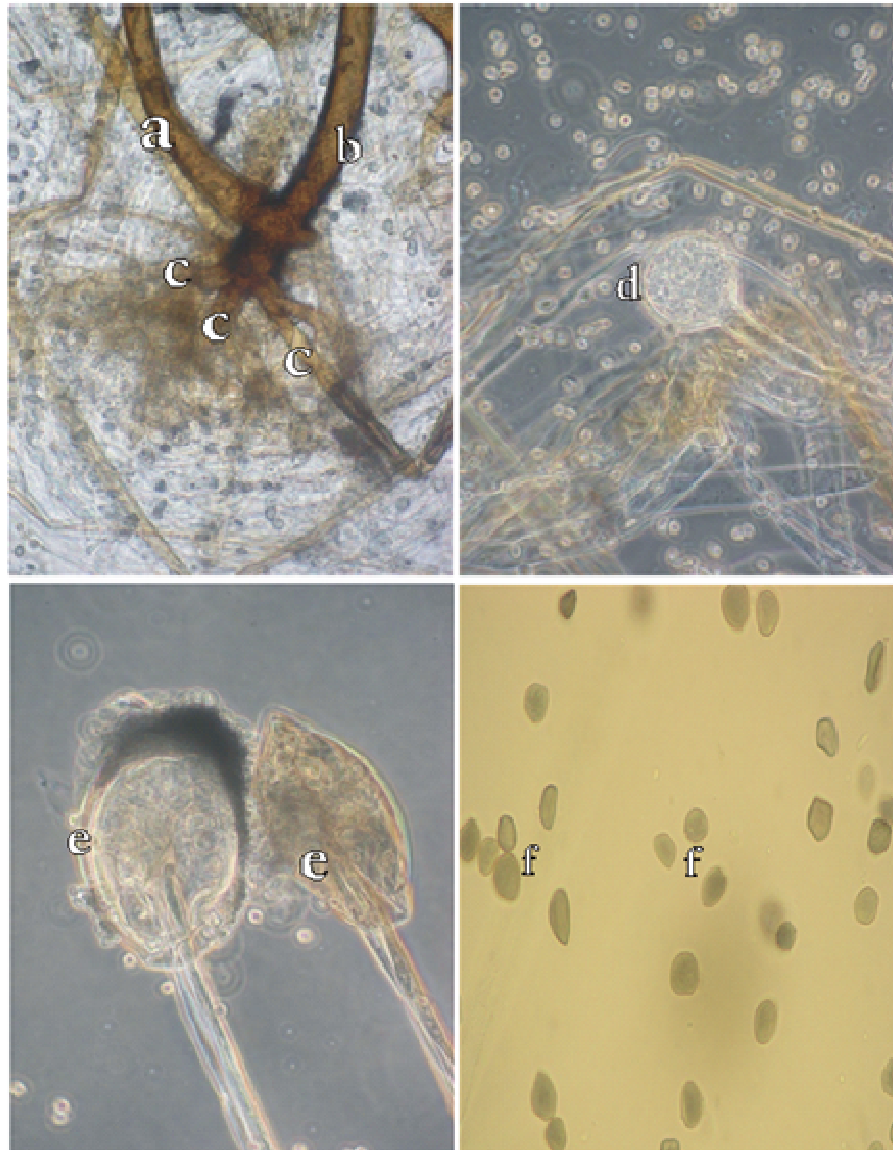
Figure 1. Substrate-level and aerial mycelia of Rhizopus stolonifer showing (a) part of stolon; (b) part of sporangiophore; (c) rhizoids; (d) sporangium; (e) collapsed sporangia and (f) striated sporangiospores.

This is in contrast with the results obtained in the present study whereby *R. stolonifer* exhibited different growth forms in minimal medium. A weight proportion for each morphological type in submerged culture was not determined. Subjective assessment however showed that the terminal budding yeast morphology was the dominant form.

Although, nuclei were not visualized in this study, it should be pointed out that dimorphic *M. rouxii* produces yeast like cells which are multipolar budding and the mother cell and daughter buds, each contain numerous nuclei, as revealed by Giemsa staining (Bartnicki-Garcia and Nickerson, 1962b) or 6-diamidino 2-phenylidole (DAPI) staining (McIntyre et al., 2002; Lubberhussen et al., 2003), whereas terminal budding yeast cell like that of *Saccharomyces cerevisiae*, harbours one nucleus per cell. Thus, after conversion to the yeast cell from sporangiospore or after intermediary stage as in moniliform hypha, proliferation of *R. stolonifer* was in the manner similar to the budding pattern of yeast cells of *S. cerevisiae*.

It does not appear that formation of septa by *Zygomycetous* species in minimal medium has been previously reported. In the study of McIntyre et al. (2002), the use of calcofluor white revealed the presence of septa in filaments formed by *M. circinelloides*. But in that case, it was due to the formation of arthrospores that occurred after the growth and ceased (Lubberhussen et
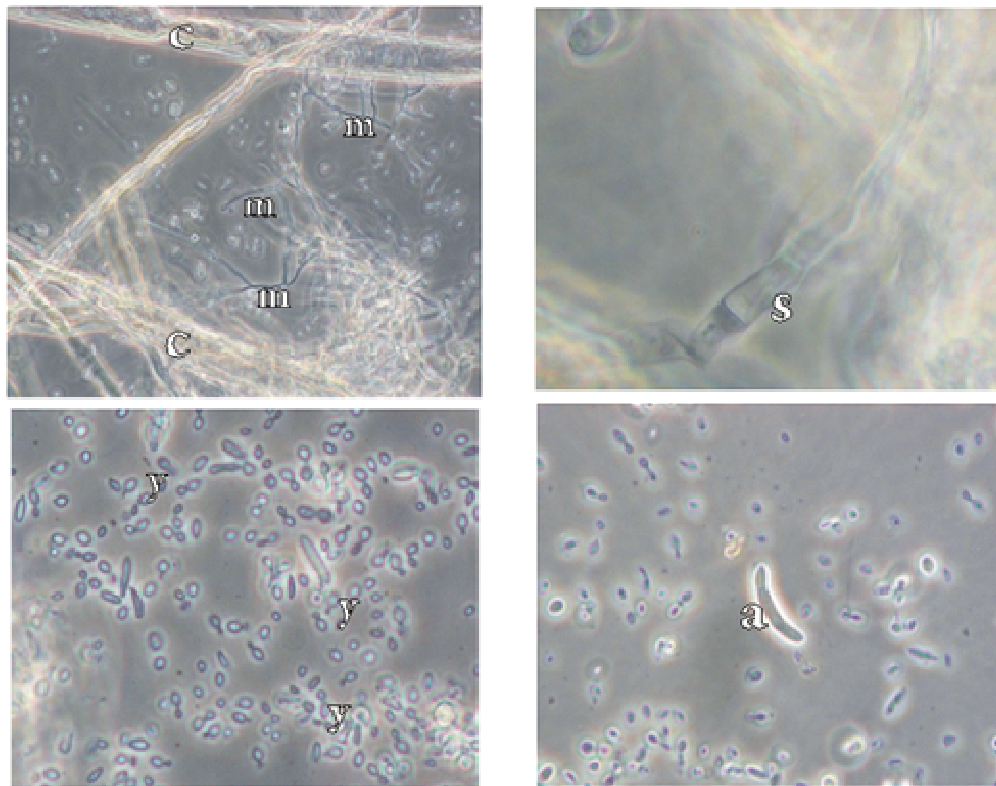
Figure 2. Submerged mycelia of R. stolonifer showing (c) coenocytic hyphae; (m) moniliform hyphae; (s) septate hypha; (c) terminal budding yeast cells and (a) subglobose, ellipsoidal and allantoid yeast cells, which may be 1 to 3 celled.

al., 2003). In the present study, arthrospores were not observed. Septation is a characteristic of the higher fungi where it compartmentalizes cellular components. Since septate hyphae were observed in submerged culture in this study, it thus means that *R. stolonifer* could exhibit characteristics of the higher fungi. As this study showed, it also applies to the formation of terminal budding yeast cells.

Formation of terminal budding yeast cell took two different lines. These include induction from (a) sporangiospore and (b) moniliform hypha. The process of induction of terminal budding yeast cells from sporangiospores has been previously observed (Omoifo, 2003, 2005).

Swollen and then lysed sporangiospore, released nucleates into medium of growth. These gave rise to protoplasts which subsequently became yeast cells, thereafter, proliferation by terminal bud formation occurred. Omoifo (2006a, b) demonstrated the effect of myoinositol and zinc on the induction and subsequent proliferation of terminal budding yeast cells from sporangiospores of *M. circinelloides* Tieghem in a system thought to be of high ionic strength that possibly led to the establishment of a pH-transmembrane gradient; such would stimulate second messengers generation and signals transduction within the living cell (Dawes, 1986;

Mitchell, 1978).

It was hypothesized that, activity at the molecular level activated mitogenic factors that led to yeast form induction (Omoifo, 2005). Thereafter, it could assume that terminal yeast budding observed here is in the manner of proliferation of *S. cerevisiae*, that is, daughter bud production by blastic action whereby the inner wall of the mother cell extends into the new bud, and this entails the extension of the existing cell wall (Marchant and Smith, 1968; Sentandreu and Northcote, 1969).

As shown in this study, formation of an intermediary structure, similar to that elaborated for the initial stages of formation of sporogenous hyphae was involved in moniliform yeast induction (Omoifo, 2005). It was shown that sporogenous hyphae emerges from sporangiospore as coenocytic thin walled vegetative filament which possesses thin lumen that becomes compartmentalized; with the centripetal growth of the plasma membrane thus forming septa-like partition (without the involvement of cell wall). A morphologically distinct conidium is produced at the distal end of this filament, and this holoblastically produced *catenate conidia* (Omoifo, 2005). In the present study, the growing tip of moniliform filament formed yeast mother cell instead, and this on detachment becomes terminal budding. This method of primary bud formation by *R. stolonifer* contrasts with that described for
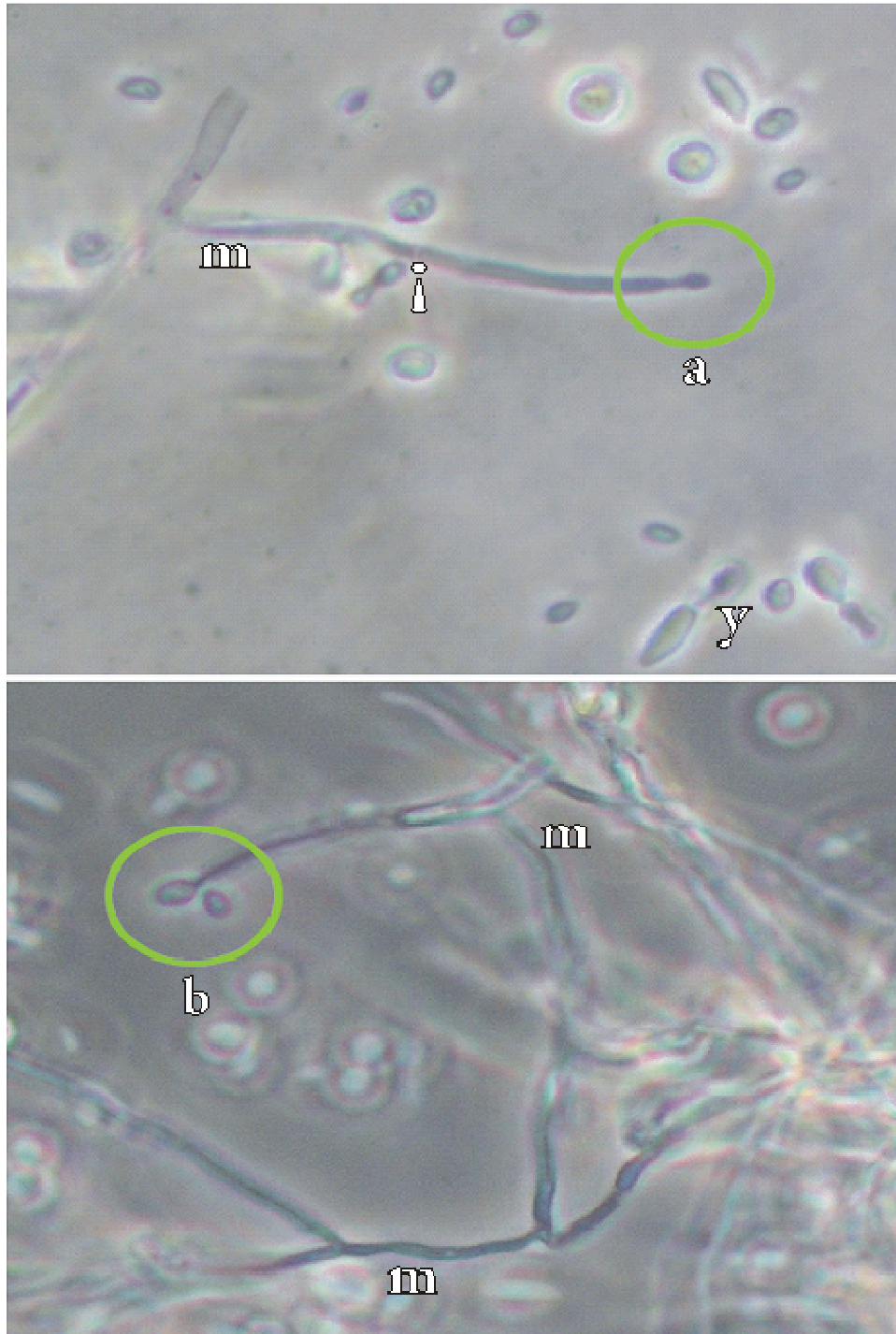
Figure 3. Yeast cells (y) and moniliform hyphae (m) of *R. stolonifer* budding off yeast cells; (a) young bud; (b) mature bud near detachment. The moniliform hypha may have simple septa-like partition, (i).

Phialophora dermatitidis whereby there is bud eruption from hyphal wall; bud wall is continuous with inner hyphal wall, whereas the outer wall forms a collar (Grove et al., 1973). In contrast too, is the study of Hardcastle and Szaniszlo (1974) that showed that moniliform hyphae emerged from induced yeast cells of *Cladosporium*

werneckii but these produced lateral conidia, which on release did not exhibit any form of budding.

In the present study, terminal budding yeast cells were induced from sporangiospores in two different ways, viz (a) sporangiospore to nucleates to protoplasts to yeast cells; (b) sporangiospore to moniliform hypha to yeast

cell. This study confirmed earlier reports which showed that terminal budding yeast cells can be induced from sporangiospores (Omoifo, 1996, 1997, 2003, 2006a, b; Omoifo et al., 2006). For biochemical and molecular studies to be conducted successfully, pure cultures of the morphotypes or anamorphs of any particular fungus is required. The achievement of this objective has been demonstrated in several studies (Omoifo, 2003, 2005, 2006a, b). This is further confirmed in the foregoing report.

ACKNOWLEDGEMENTS

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