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Physiological and Morphological Correlation of *Rhizopus stolonifer* Spore Germination[1]

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Sporangiospores of *Rhizopus stolonifer* were examined at various stages of germination by scanning electron and phase-contrast microscopy. These observations were correlated with changes in spore dry weight, spore volume, respiration, and syntheses of ribonucleic acid, deoxyribonucleic acid, and protein during germination.

One of the impressive morphological developments in the life cycle of a mycelial fungus is the conversion of a dormant spore into an actively growing mycelium. The initiation of spore germination leads to rapid increases in the rates of respiration and in protein and nucleic acid syntheses. Our previous studies have focused on the isolation and characterization of the components involved in protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) syntheses from ungerminated and germinated spores of the fungi *Rhizopus stolonifer* and *Botryodiplodia theobromae* (5, 7, 14). Ultimately, we want to relate biochemical events with morphological changes. In this report, the sequential morphological changes of *R. stolonifer* sporangiospores during germination are depicted by phase-contrast and scanning electron microscopy; correlation is made with the syntheses of protein, RNA, and DNA as well as with alterations in the rate of respiration.

MATERIALS AND METHODS

Organism and cultural conditions. *R. stolonifer* (mating strain +) was obtained from W. Gauger, Botany Department, Univ. of Nebraska, Lincoln, Neb. The techniques for growth, harvesting, and germination of sporangiospores were identical to those previously described (18) except that the spores were grown in a medium containing glucose, 20 g; asparagine, 2 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.26 g; agar, 20 g; and water to 1 liter. The spores were germinated at a concentration of 1.0 mg/ml in the same medium without agar.

Analytical measurements. Total RNA was determined by the orcinol procedure (17), and total protein was measured by the method of Lowry et al. (13) after chemical fractionation of the cells (8). Total DNA was determined by the absorbance of the isolated DNA at 260 nm (4).

Polarographic measurements of oxygen consumption by germinating spores were made with Clark-type oxygen electrodes as previously described (5) except that the temperature was maintained at 29 C.

Labeled precursor incorporation assays. At various times during the germination period, 5-ml samples of the spore suspension were removed and incubated an additional 15 min at 29 C in the presence of [^{14}C]uracil or [^{14}C]leucine (0.05 μCi of spore suspension per ml). At the end of this period, 2 ml of 30% (wt/vol) trichloroacetic acid was added, and the assay mixtures were stored on ice for at least 1 h. The spores were transferred to glass fiber disks and processed as described previously (1). The time at which DNA synthesis was initiated was determined by adding 7.5 μCi of [^{14}C]guanine to 25 ml of the spore suspension and incubating for 1 h. The labeled spores were diluted with 1 liter of unlabeled spores, the DNA was isolated from them and centrifuged to equilibrium on CsCl gradients, and the radioactivity was determined as described previously (4).

Microscopy. The techniques for observing specimens by phase-contrast and scanning electron microscopy were modified from those described earlier (3, 19). For phase-contrast microscopy, mounting slides were prepared by spreading a thin film of 1% Noble agar evenly over the surface of glass microscope slides. A small amount (about 0.05 ml) of a cell suspension was placed on the solidified agar surface and covered with a cover slip. Cells were photographed on Panatomic-X film through Neofluar phase optics of a WL Zeiss microscope.

Squares (10 by 10 mm) cut from glass microscope slides were placed on aluminum specimen stubs for scanning electron microscopy. About 0.05 ml of a diluted cell suspension was spread over the mounting surface, dried, and coated with aluminum to a thickness of 15 nm. Specimens were examined in a Cambridge Stereoscan Mark II scanning electron micro-

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scope at an accelerating voltage of 20 kV; the final aperture was 200 μm, and the beam specimen angle was 45°.

RESULTS

Germ tube formation, oxygen uptake, and dry weight at various times during germination are shown in Fig. 1. Sporangiospores began to swell between 1 and 2 h (T_1 and T_2), and germ tube emergence was first observed at $T_{3.5}$ to T_4. By T_6, 90% of the spores had formed germ tubes and the dry weight of the cultures had increased about three- to fourfold. During the swelling process the spore diameter increased from 8.8 to about 15 μm. Assuming that the spores are spherical, their volume increased about fivefold during swelling. The rate of O_2 uptake increased approximately 50% from T_0 to $T_{0.5}$ and, thereafter, continued to increase linearly until it had increased 10- to 12-fold at T_4; the initial $Q(O_2)$ was 13 and increased to 86 at $T_{4.5}$. The rate of O_2 uptake usually remained constant during the remainder of the germination process.

The incorporation of leucine into protein and uracil into RNA at various stages of spore germination is demonstrated in Fig. 2. The kinetics of incorporation revealed that protein and RNA syntheses began within the first 30 min and rapidly increased throughout the germination process. All three types of RNA, ribosomal, transfer, and messenger, initiate synthesis within this time period (J. R. Roheim, R. H. Knight, and J. L. Van Etten, unpublished data). The rates of leucine and uracil incorporation decreased at the time of germ tube emergence (T_{3-4}). This reduction was probably the result of increases in the endogenous pools of protein and RNA precursors rather than decreases in their synthetic rates because total protein and total RNA continued to increase throughout the germination process. Similar kinetics were observed for the incorporation of either [^{14}C]guanine or [^{14}C]adenosine. However, the reduced incorporation rate observed after T_{3-4} was not as pronounced with adenosine as it was with uracil or guanine. Separate experiments indicated that a high percentage of the labeled precursors was actually incorporated into protein or RNA. Spores were incubated for 2 h in the presence of either [^{14}C]leucine or [^{14}C]uracil; after chemical fractionation of the spores (8), 90% of [^{14}C]leucine appeared in the fraction that contained hydrolyzed protein and 91% of [^{14}C]uracil appeared in the fraction that contained hydrolyzed nucleic acid. In addition, if the spores labeled with [^{14}C]uracil were incubated in 1 M KOH for 18 h at 37 C, all of the radioactivity was completely solubilized. Therefore, the [^{14}C]uracil entered the RNA and not the DNA of the spores.

The changes in DNA content and the incorporation of [^{14}C]guanine into DNA during spore germination are given in Table 1. Guanine was used to monitor DNA synthesis because *R.*

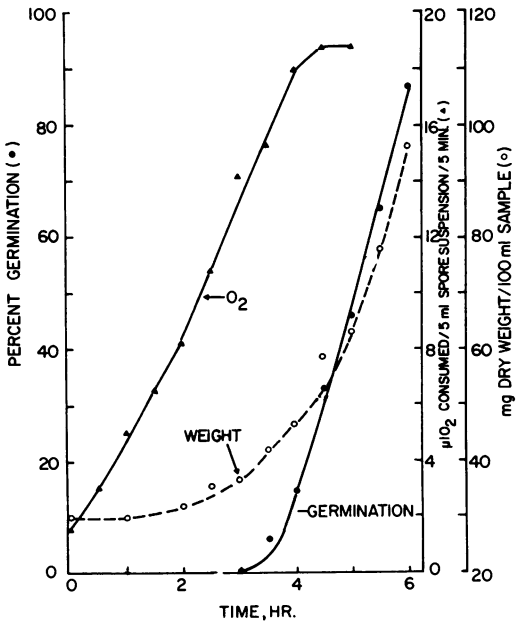


FIG. 1. *Percentage of germination* (●), *rate of oxygen uptake* (▲), *and dry weight of spores* (○), *at various time periods during germination of R. stolonifer.*

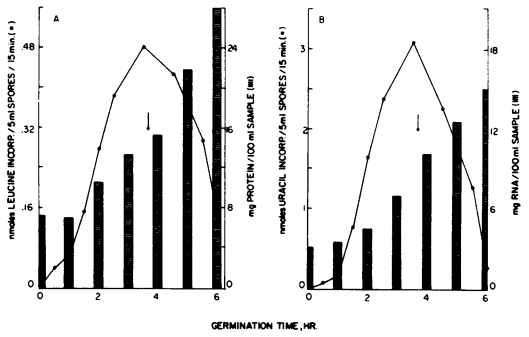


FIG. 2. *A, Incorporation of leucine into protein during 15-min pulse periods* (●) *and total protein content (bar); B, incorporation of uracil into RNA during 15-min pulse periods* (●) *and total RNA content (bar) of the spores at various stages of germination. The total RNA or protein was determined from 100-ml samples of the spore suspension at the time periods indicated. The time at which germ tubes were initially observed is indicated by the arrows.*

TABLE 1. *Changes in DNA content and the incorporation of [^{14}C]guanine into DNA during spore germination of R. stolonifer*

Germination time (h)	Spore suspension[a] (mg of DNA/100 ml)	Counts per min per A_{260}[b] of DNA per h[c]
0	0.05	
1	0.05	0
2	0.07	351
3	0.11	2,546
4	0.16	4,000
5	0.23	
6	0.28	

[a] Values are not absolute because inherent with the extraction process is an approximate 50% loss of DNA.

[b] Absorbancy at 260 nm.

[c] Germination time represents the conclusion of the 1-h pulse period.

stolonifer spores do not readily incorporate thymidine into DNA, presumably due to the absence of thymidine kinase (9). DNA synthesis began between T_1 and T_2 and increased during the remainder of the germination process.

The scanning electron and phase-contrast (inserts) micrographs in Fig. 4 disclose the sequence of morphological change from a dormant spore through germ tube formation. The surface of an ungerminated (T_0) sporangiospore of *R. stolonifer* appeared striated in light optics (Fig. 4A, insert) and in the scanning microscope exhibited prominent cylindrical ridges. Previous observations by scanning microscopy (6) have indicated that such surface structures are reliable characteristics for grouping species. Figure 4B shows a swollen spore at $T_{2\text{-}3}$. Characteristic of 90 to 95% of all spores examined at this period is their triangular shape. By T_3 (Fig. 4C), the spore appeared elongated with no apparent change in its surface configuration. Slight collapse of the spore suggests than an internal modification within the spore decreased its turgidity during treatment for electron microscopic examination. Germ tube emergence ($T_{3.5}$) and extension (T_6) are depicted in Fig. 4D and E, respectively. The tip of the germ tube appeared distinctly different from the wall behind the apical dome (see Fig. 4D). A similar appearance was noted during germination of pycnidiospores of *B. theobromae* (19).

DISCUSSION

As was true for *Saccharomyces cerevisiae* (15, 16) and *B. theobromae* (4, 18, 19), correlation of differential modification in surface topography of *R. stolonifer* sporangiospores with various physiological activities during germ tube development presents an interesting aspect of cellular differentiation. The sequence of certain biochemical events and alterations in overall spore shape during germination and germ tube development of *R. stolonifer* are outlined in Fig. 3. The events from spore through germ tube formation are time framed and are delineated as follows: (i) onset of RNA and protein synthesis and increased O_2 uptake by $T_{0.5}$; (ii) onset of DNA synthesis with enhanced RNA and protein syntheses, increases in rate of O_2 uptake, spore volume, and initial increase in spore dry weight by T_2; (iii) further increases in O_2 uptake, dry weight, and syntheses of DNA, RNA, and protein at $T_{2\text{-}6}$; (iv) visual appearance of germ tubes and germ tube elongation at $T_{3.5}$ to T_6. Whereas protein synthesis precedes RNA synthesis during germination of *S. cerevisiae* ascospores (15) and *B. theobromae* pycnidiospores (1), RNA synthesis and protein synthesis are concomitant in *R. stolonifer* and begin immediately upon initiation of sporangiospore germination.

An intriguing morphological feature of *R. stolonifer* germination is the contrast between the extended germ tube and its parent sporangiospore. In no instance during our observations did we see any change in spore surface striation of the sporangiospores except at the point of germ tube emergence. Apparently, initial cellular differentiation is distinctly internal and perhaps unrelated to the physiology of the outer spore wall. Previous investigations have demonstrated that the germ tube wall of certain *Rhizopus* species is formed by a continuation of an inner wall layer of the spore (2, 10–12). During the initial stages of germ tube development, specific physiological activities may occur at the site of emergence and may be displayed by the formation of a smooth tip. The subtle difference between the germ tube tip surface and the remaining portion of the extended germ tube also may be a reflection of relative rates of metabolic activity. Further studies are being done on the biochemical and

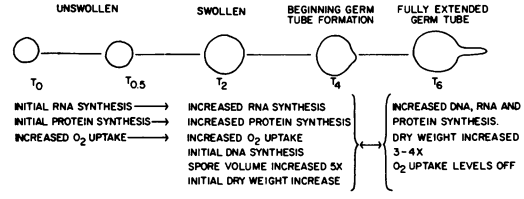
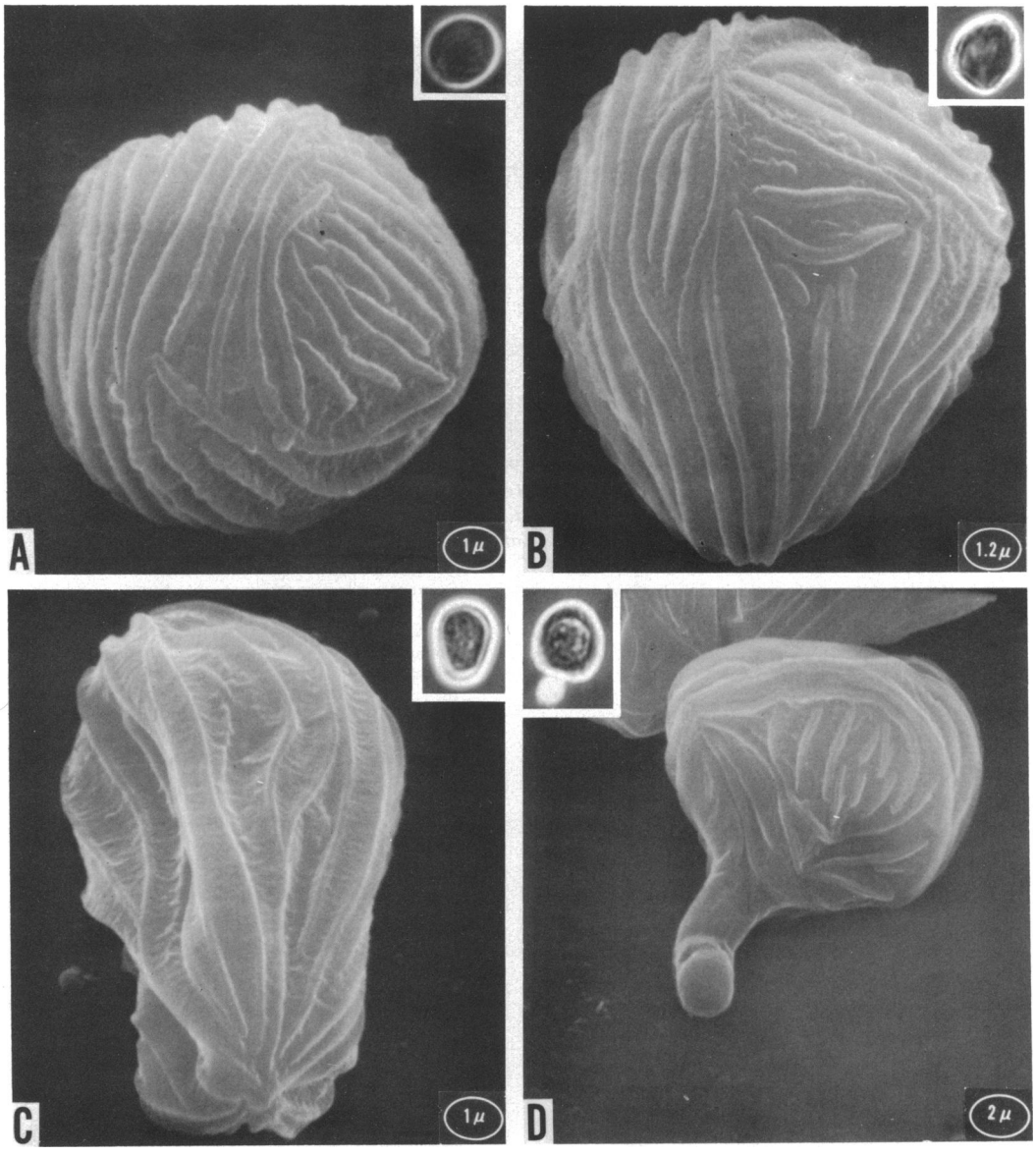


FIG. 3. *Schematic diagram of the time sequence of certain events during the germination of R. stolonifer sporangiospores.*


A
1μ
B
1.2μ
C
1μ
D
2μ

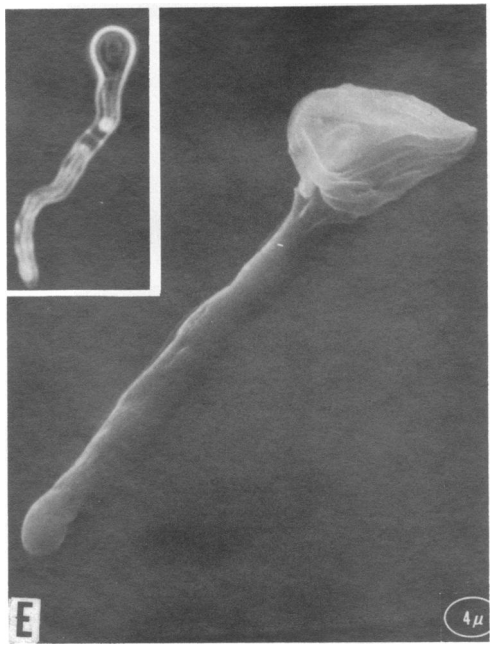


FIG. 4. *Scanning electron micrographs of R. stolonifer spores at various stages of germination with corresponding phase contrast micrographs (×2,375) inserted. A, Ungerminated spore, T_0; B, swollen spore, $T_{2.5}$; C, elongated spore, T_3; D, germ tube emergence, $T_{3.5}$; and E, germ tube elongation, T_6.*

physiological events of germination and germ tube formation in this organism.

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