Nuclear Behavior and Division in Germinating Conidia of *Botrytis cinerea*

N. Shirane, M. Masuko, and Y. Hayashi

Aburahi Laboratories, Shionogi Research Laboratories, Shionogi & Co., Ltd., Koka-cho, Shiga 520-34, Japan.

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ABSTRACT

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Light-microscopic observations were carried out on resting and dividing nuclei in germlings of *Botrytis cinerea* incubated in potato-dextrose broth at 22 C in darkness. The conidia contained various numbers of nuclei, commonly up to 10. Nuclei in each cell were divided synchronously by mitosis. The time elapse between the initiation of nuclear division and septum formation was approximately 2 hr. The cell cycle of the fungus was about 2.7 hr. Nuclei appeared to migrate through the septal pore and aggregated in the area of the appressorium. The growing tips of many germlings collapsed after treatment with methanol-acetic acid solution, releasing chromosomes at metaphase. The number of chromosomes counted in many burst germlings was 16. The length of each chromosome at metaphase ranged from 0.6 to 1.3 μm and the width was 0.2–0.3 μm; total length of the component was 13.1 μm. One of the chromosomes had an additional threadlike structure.

Although the morphological and biochemical characteristics of *Botrytis cinerea*, an economically important phytopathogenic fungus, have been studied extensively, there are few detailed studies concerning chromosomes and mitosis itself (24,26,28). The slow progress of cytogenetics in this fungus may be partly due to the difficulty of genetic analysis and the small size of the chromosomes. Moreover, the mitotic chromosomes in mycelial nuclei are notoriously difficult to analyze because the cell cytoplasm is surrounded by rigid cell wall, the nuclear membrane remains throughout division, and the spindle is intranuclear (3,20).

Recently, we succeeded in overcoming these physical barriers using germlings of conidia by bursting the hyphal tips and releasing protoplasms from the cell with methanol-acetic acid treatment. This paper describes the cell division in germinating conidia and mitotic chromosomes of *B. cinerea*.

MATERIALS AND METHODS

Preparation of conidia. A single conidium isolate, S-v-5, of *Botrytis cinerea* Persoon, obtained from diseased cucumber fruit (gray mold rot), was grown on potato-sucrose agar medium. The fungus was incubated for 3 days at 22 C initially in darkness, exposed 2 days to near-ultraviolet light (FL 20S, BL-B, Matsushita, Osaka), and sporulated under dark conditions for 2 more days. Conidia thus formed were collected, washed three times with distilled water, concentrated to 1×10^8 spores/ml, and stored at −80 C as described previously (22).

Germination of conidia. The stored conidia were diluted and suspended in potato-dextrose broth (PDB) at a concentration of 1×10^4 spores/ml, and the suspension was poured into wells (100 μl/well) in a microtest plate (Nunc, Roskilde, Denmark). After the plate was incubated at 22 C in darkness for 1–5 hr, the conidia were stained with 0.2% cotton blue solution (lactic acid:phenol:glycerin: distilled water = 1:1:1:1, v/v) and observed under an optical microscope (Diaphot-TMD, Nikon, Tokyo).

Observation of nuclei. For observation of nuclei in the conidia, the conidia were scattered with a needle on a glass slide precoated with a thin layer of egg white. For the observation of nuclei in the germlings, droplets of PDB containing 1×10^5 spores/ml were placed on glass slides and incubated at 22 C under humid conditions in the dark for various periods. The specimens were fixed in Carnoy's fluid (ethanol:chloroform:acetic acid = 6:3:1, v/v) for 30 min, transferred to 95% ethanol, and kept in 70% ethanol at room temperature for 3 hr. After hydrolyzation in 1 N HCl for 5 min at room temperature and for 10 min at 60 C, the specimens were washed with distilled water and stained with Giemsa (4% Giemsa's Losung, Merck, Darmstadt, FRG, in 1/15 M phosphate buffer, pH 7.0) for 3 hr. After being rinsed in tap water, they were air dried and observed at 100 × (objective lens) with a light microscope (Microphot-FX, Nikon, Tokyo) under oil immersion.

Observation of chromosomes. Conidia on glass slides were incubated for 6.5 hr in PDB, and the germlings were treated with methanol-acetic acid solution (methanol:acetic acid = 17:3, v/v) for 30 min at room temperature and flame dried. They were transferred to 95 and 70% ethanol, hydrolyzed in 1 N HCl, stained with Giemsa, and rinsed in tap water as described above.

RESULTS

Unincubated conidia were stained, examined under a microscope, and found to contain one to 10 nuclei, which were 1.5–2 μm in diameter. Larger conidia contained more nuclei than smaller conidia (Fig. 1). Seventy-six percent of conidia contained 4–6 nuclei (Fig. 2). Figure 3 shows the relationship between incubation time and the number of nuclei or cells per germling, along with the germination and mitosis percentages. The conidia germinated almost synchronously within 2 to 3 hr after incubation. Mitosis was observed for the first time at 3 hr after incubation. The first peak of mitotic percentage in apical cell was found at 3.8 hr, and the second peak appeared at 6.5 hr after incubation. The number of nuclei per germling began to increase 4 hr after incubation, doubled by 6 hr, and increased fourfold by 9 hr. The number of cells per germling started to increase after 6 hr (Fig. 3 and Fig. 4B, C, and D). These data indicate that the cell cycle of the fungus is about 2.7 hr and that the period between nuclear division and septum formation is approximately 2 hr under the incubation conditions we set.

Nuclei in the conidia migrated into germ tubes as they elongated (Fig. 4A). At the stage of appressorium formation, many nuclei aggregated in the area of the appressorium, as previously observed by Akutsu et al (2), and often nuclei migrating through septal pores were observed (Fig. 4E).

With 6.5-hr-old germlings, 93% of the growing tips were burst by treatment with absolute methanol and the contents in the apical cells were released. However, the released components were not

spread sufficiently for the observation of chromosomes. On the other hand, when germlings were treated with the methanol-acetic acid solution (85% methanol, 15% acetic acid), only 68% of the growing tips of the germlings were burst, but cell components were well spread to determine the number of chromosomes per cluster (Fig. 5). Chromosomes at metaphase are shown in Fig. 6, and the number of chromosomes was 16. The length of each chromosome at metaphase ranged from 0.6 to 1.3 μm and the width was from 0.2 to 0.3 μm. The total length of each chromosome was 13.1 μm. One of the chromosomes exhibited a threadlike structure. In Figure 6, the chromosomes were arranged in descending order of length but could not be distinguished from each other except for the chromosome having the threadlike structure.

DISCUSSION

Perkins and Barry (16) stated in their review concerning cytogenetics of *Neurospora* that the meiotic chromosomes are immense compared with somatic chromosomes and within the range of study with a light microscope. Lengths of the seven chromosomes at pachynema of *Neurospora crassa* range from 7 to 9 μm (25). At metaphase I, the *Neurospora* bivalents contract to lengths of 1.7 to less than 1 μm (16). This is probably a reason why meiotic chromosomes have been studied for the karyotype analysis of this fungus. However, some economically important phytopathogenic fungi lack the perfect stage (Fungi Imperfecti); thus it is difficult to study their chromosomes. The technique described in this study shows that it is possible to observe the chromosomes during mitosis for such fungi and introduces an approach to the morphological study of mitotic chromosomes. As we described in the present study on *B. cinerea*, the mitotic chromosomes were observed clearly and the number is constant in metaphase. But there are some indefinite characteristics, such as position of the centromere or arm ratios of the mitotic chromosome complement. The threadlike structure that we observed is rather unusual. Further investigation is desirable before a more definite description is possible. We think it is not an artifact and is associated with a specific chromosome because the structure is inevitably associated with one of the chromosomes in every component as shown in Figure 5. Similar association also was detected in many other micrographs with few exceptions. We interpret the exceptions to be an overlapping faint threadlike structure with chromosomes. Then one of the chromosomes that attached to a threadlike structure is able to be identified from the others. The other chromosomes, as shown in Figure 6, cannot be identified. If the chromosome banding techniques (G-, C-, Q-, or N-banding) developed in higher plants and animals (6–8,14) can be used in the filamentous fungi, rapid progress can be expected in the karyotype analysis of this microorganism.

The release of cellular contents by bursting the growing tip of the hypha with methanol-acetic acid solution may be broadly applicable for the observation of chromosomes of other filamentous fungi. With this technique, we have succeeded in observing mitotic chromosomes of *B. allii*, *B. tulipae*, *B. fabae*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Venturia nashicola*. The detailed data will be reported elsewhere. Chromosome analysis of a broad range of fungi in future work will provide a basis of fungal classification and an aid to understanding the phylogeny.

Olive (15) suggested that the mitotic divisions occuring in the vegetative phase of fungi are essentially the same as in higher organisms. McClintock (13) and Singleton (25) showed normal chromosome morphology and behavior in *Neurospora crassa*, identified and numbered the seven chromosomes, and noted that

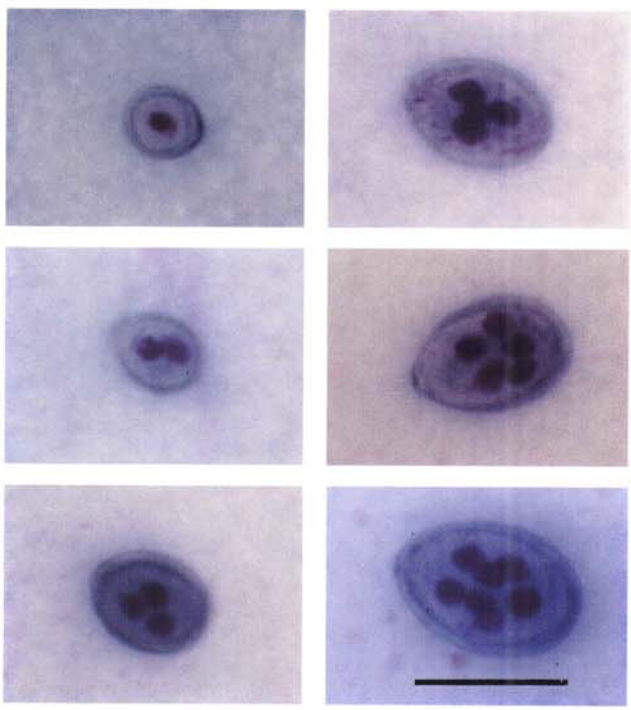

Fig. 1. Nuclei in conidia of *Botrytis cinerea*. All photographs are at the same magnification. Bar = 10 μm.

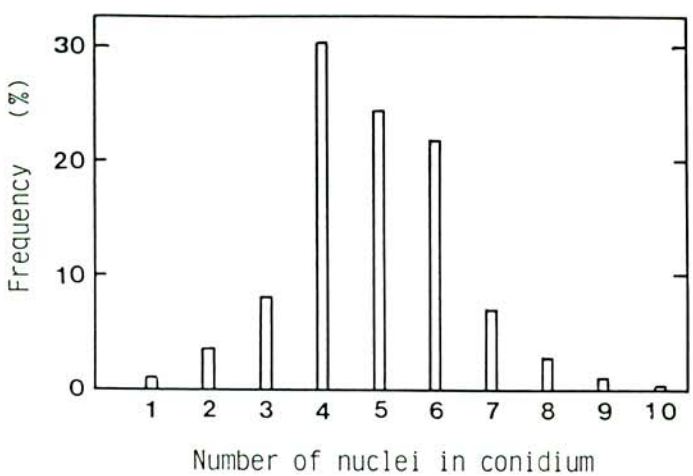

Fig. 2. Number of nuclei in the conidia of *Botrytis cinerea* (isolate S-v-5). Five hundred unincubated conidia were observed.

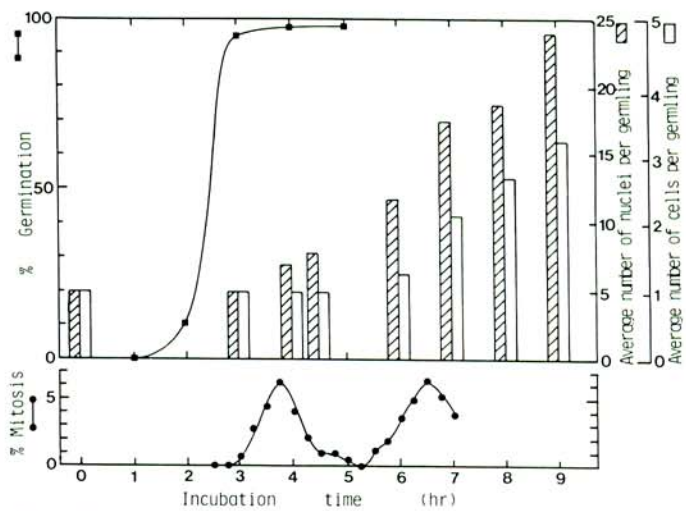

Fig. 3. Timing of spore germination and nuclear division and number of nuclei and cells per germling of *Botrytis cinerea* incubated for 1–9 hr in potato-dextrose broth at 22 C in darkness. Percentage of nuclei in mitosis was estimated for the apical cell of each germling. Note the time between the initiation of nuclear division and septum formation, and between the peaks of mitosis percentage.

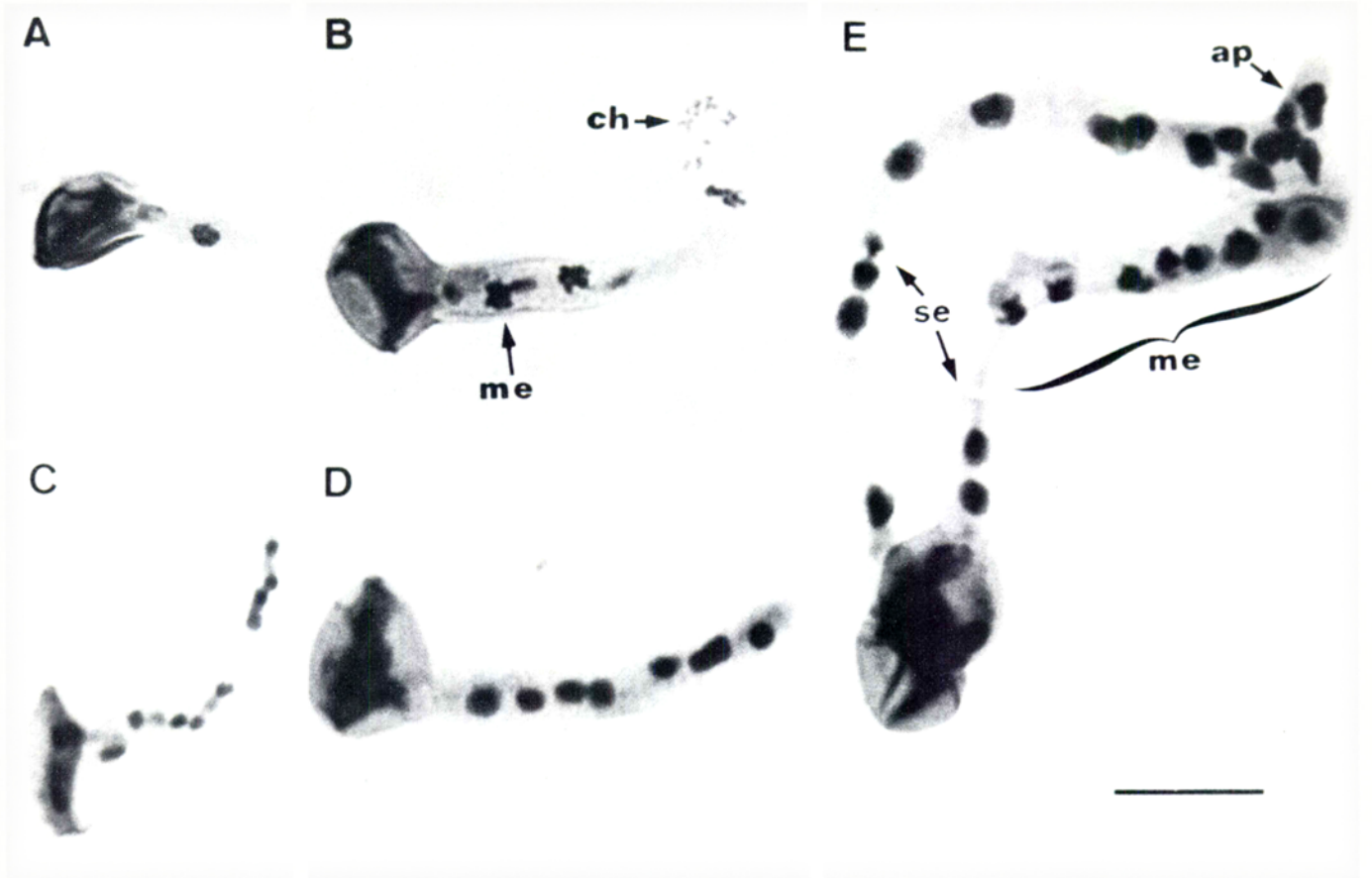

Fig. 4. Nuclei in germling of *Botrytis cinerea*. **A,** 3-hr-old germling. **B,** 4-hr-old germling at metaphase (me); the germling was treated with methanol-acetic acid fluid (17:3) and chromosomes (ch) were released from the growing tip. **C,** 4-hr-old germling at telophase. **D,** 6-hr-old germling; the number of nuclei increased twofold. **E,** 7.5-hr-old germlings; note the nuclei aggregated in the appressorium (ap) and the nucleus migrating through a septal pore. The nuclei in each cell were dividing almost synchronously (B,C,E). se = septum. All photographs are at the same magnification, and the bar represents 10 μm.

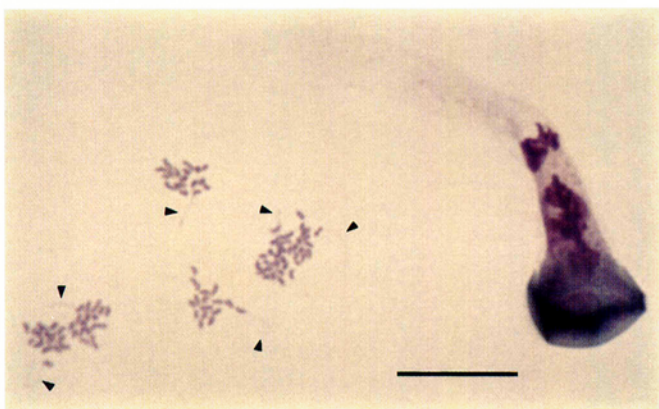

Fig. 5. Chromosomes released from the growing tip of a 6.5-hr-old germling treated with methanol-acetic acid solution. Some of the chromosomes have faintly stained threadlike structures (arrows). Bar = 10 μm.

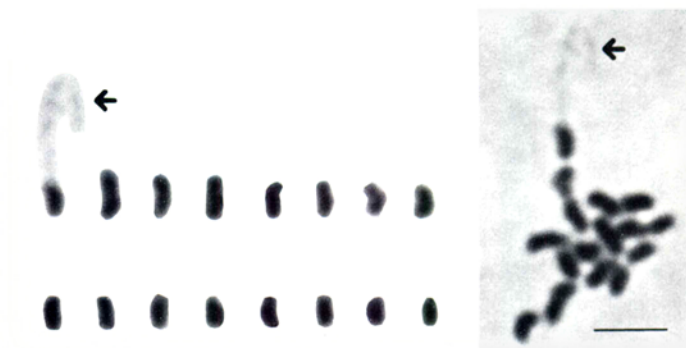

Fig. 6. Enlarged photographs of the chromosomes of *Botrytis cinerea* at metaphase. Each chromosome was arranged in descending order of length. Arrows indicate the threadlike structure. Bar = 2 μm.

meiosis was similar in its main features to that in higher eukaryotes though it differed in some details of chromosome contraction, pairling, and coiling. More recently, Heath (12) stated that details of the mitotic process in *Saprolegnia ferax* differ to varying extents from those found in higher plants and animals. Examples of interesting differences found in some fungi include persistence of nuclear membrane throughout mitotic division (5,19), segregation of kinetochores into two groups during prophase (11,12), absence of a metaphase plate (12), unusual two-track type of division (3,18), and absence of detectable chromatin condensation during mitosis of *Saprolegnia ferax* and *Saccharomyces cerevisiae* (9,10,17). The present series of observations with light microscopy indicated that mitosis of *B. cinerea* is essentially the same as that in higher organisms and the chromatin is condensed at metaphase.

Figure 4B, C, and E and Figure 5 show that somatic nuclear division is synchronous within a single cell. Other investigators (18,20,21) arrived at the same conclusion for *Aspergillus nidulans* and *B. cinerea*. These findings suggest the existence of soluble agents that stimulate the mitotic division.

Botrytis cinerea invades host cells via the appressoria (1,2,23,27). The aggregation of nuclei in the appressorium reported here may have an important role in the synthesis and supply of various enzymes and mucilaginous substances, thus facilitating mycelial penetration into host cells. The septal pores of *B. cinerea* (4) may exist to enable nuclei to migrate through the cell wall.

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