Perithecial Development and Nuclear Behavior in Pyricularia

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ABSTRACT

In fertile matings of isolates of *Pyricularia grisea*, perithecia originate as small masses of thicker-than-normal hyphae wound together. Cells of these hyphae are uninucleate in early stages. Binucleate ascogenous hyphae appear later and give rise to asci by crozier formation. Nuclear fusion in the young ascus is followed by three nuclear divisions, resulting in eight nuclei around which ascospores are delimited. Two additional nuclear divisions occur in the ascospores, and septa are formed between daughter nuclei following each division. Mature ascospores have four uninucleate cells. Most cells of the mycelium, conidia, and conidiophores are uninucleate, although more than one nucleus was occasionally seen in cells of old hyphae. The single nucleus of the young conidium divides, and a two-celled conidium is formed. The nucleus of the apical cell divides again, which completes the formation of the three-celled mature conidium. Chromosomes were most easily counted in the first nuclear division after ascospore delimitation. The haploid number of chromosomes was usually found to be six, although occasionally only five could be distinguished. No differences in nuclear behavior were observed between *P. oryzae* and *P. grisea* in the asexual state.

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Pyricularia grisea (Cke.) Sacc. was described from crabgrass (*Digitaria sanguinalis* L.). Morphologically similar pathogens cause diseases of rice, banana, pearl millet, maize, and a number of grass species (1, 2, 10, 12, 15, 17). According to Sprague (15) these pathogens should probably all be known as *P. grisea*. Asuyama (1) suggested that the morphologically similar pathogens reasonably may be included in one species, and the species subdivided into specialized forms on the basis of pathogenicity. At present, most authors use the name *P. oryzae* for the pathogen on rice and *P. grisea* for the pathogens on the other hosts. Recently, however, Hashioka described the pathogen on banana as a new species, and used the previously published specific names for the pathogens on pearl millet and *Leersia* (4, 5). In this paper, *P. grisea* is used to designate isolates of the pathogen from crabgrass and *P. oryzae* to designate isolates from rice.

Rice blast, caused by *P. oryzae*, is one of the most serious diseases of rice. An important means of control of blast is the development and use of resistant cultivars (8). One problem with this means of control is the occurrence of new races of the pathogen capable of attacking previously resistant cultivars (18). The nature of this pathogenic variation has not been elucidated. Ou and Ayad (14) reported that monoconidial subcultures from a monoconidial culture could be differentiated into numerous pathogenic races. Giatgong and Frederiksen (7) found that even after three generations of monoconidial subcultures, as many as one-fourth of the subcultures were different pathogenically from the parent culture. In contrast, Latterell (9) found little pathogenic variation among monoconidial subcultures from isolates of the fungus. Suzuki (16) reported that cells of the mycelium and conidia are multinucleate, but others (7, 19) have found that most of these cells are uninucleate. There is also disagreement among these authors as to the number of chromosomes found in this pathogen. A better understanding of the nuclear behavior of this fungus is needed to aid in explaining variation, and for future genetic studies. No sexual state is known for *P. oryzae*, but the perfect state of *P. grisea* has been obtained in culture and described as *Ceratosphaeria grisea* (6). Since these two pathogens are so closely related morphologically, it seems likely that they would have similar nuclear behavior. This paper describes the nuclear behavior of *P. grisea* during ascus and ascospore development. Observations were also made on early stages of perithecial development, and on the nuclear behavior in the asexual state of both *P. grisea* and *P. oryzae*.

MATERIALS AND METHODS.—Ascospore isolates 32 and 40 of *P. grisea* and isolates TH67-22, TH68-41, and TH68-141 of *P. oryzae* were principally used in this study. Perithecia were induced to form in culture by mating fertile isolates of *P. grisea*, as previously described (6). To study the development of asci and ascospores, matings were incubated at 20 C and harvested after 3-4 weeks. Perithecia were placed on clean microscope slides in water and prepared for staining by the squash technique. Conidia and conidiophores for observation were produced abundantly by the following method (3): Mycelium was placed on oatmeal agar in petri dishes and incubated for six to eight days at 25 C. The aerial mycelium was then removed by brush in water. The dishes were covered with thin plastic food wrap, and

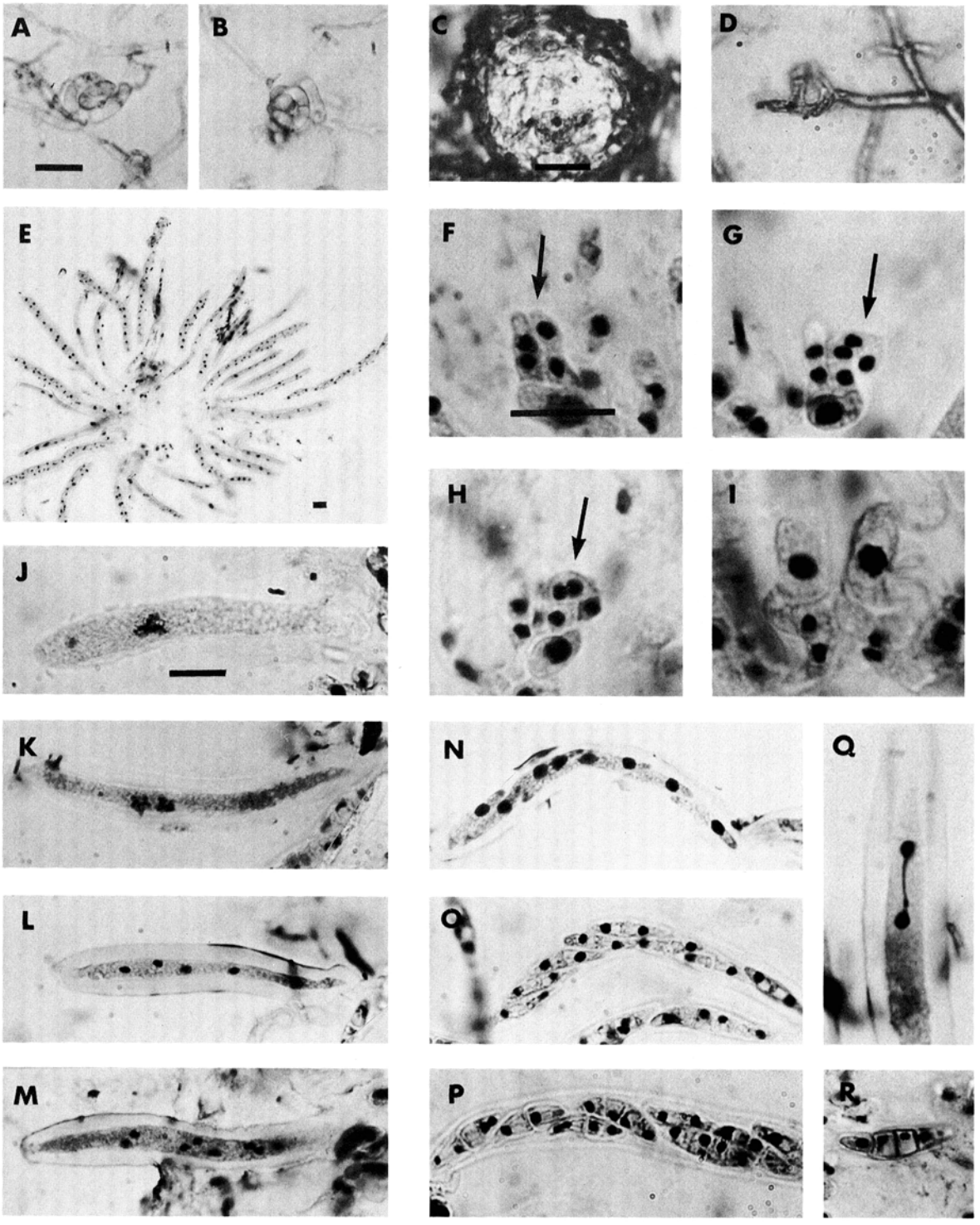

Fig. 1-(A to R). Perithecium development and nuclear behavior in the sexual state of *Pyricularia grisea*. **A,B)** Development of perithecial initial. **C)** Cross section of young perithecium. **D)** Lateral branches of hyphal strand. **E)** Asci in various stages of development. **F)** Early binucleate crozier stage. **G)** Four-nucleate crozier. **H)** Three-celled crozier. **I)** Basal cell of crozier with two nuclei. **J)** Diploid stage of ascus. **K)** Nuclear meiotic division (Division I). **L)** Four-nucleate stage of ascus. **M)** Eight-nucleate stage of ascus. **N)** Ascospore delimitation. **O)** Binucleate ascospores following Division IV. **P)** Division V in some ascospores, but not in others. **Q)** Stained thread joining two daughter nuclei. **R)** Ascospore with four uninucleate cells. Black scale bar in the first photograph of each of the following five groups represents 10 μm for all photographs of that group: A-B, C-D, E, F-G-H-I-Q, J-K-L-M-N-O-P-R.

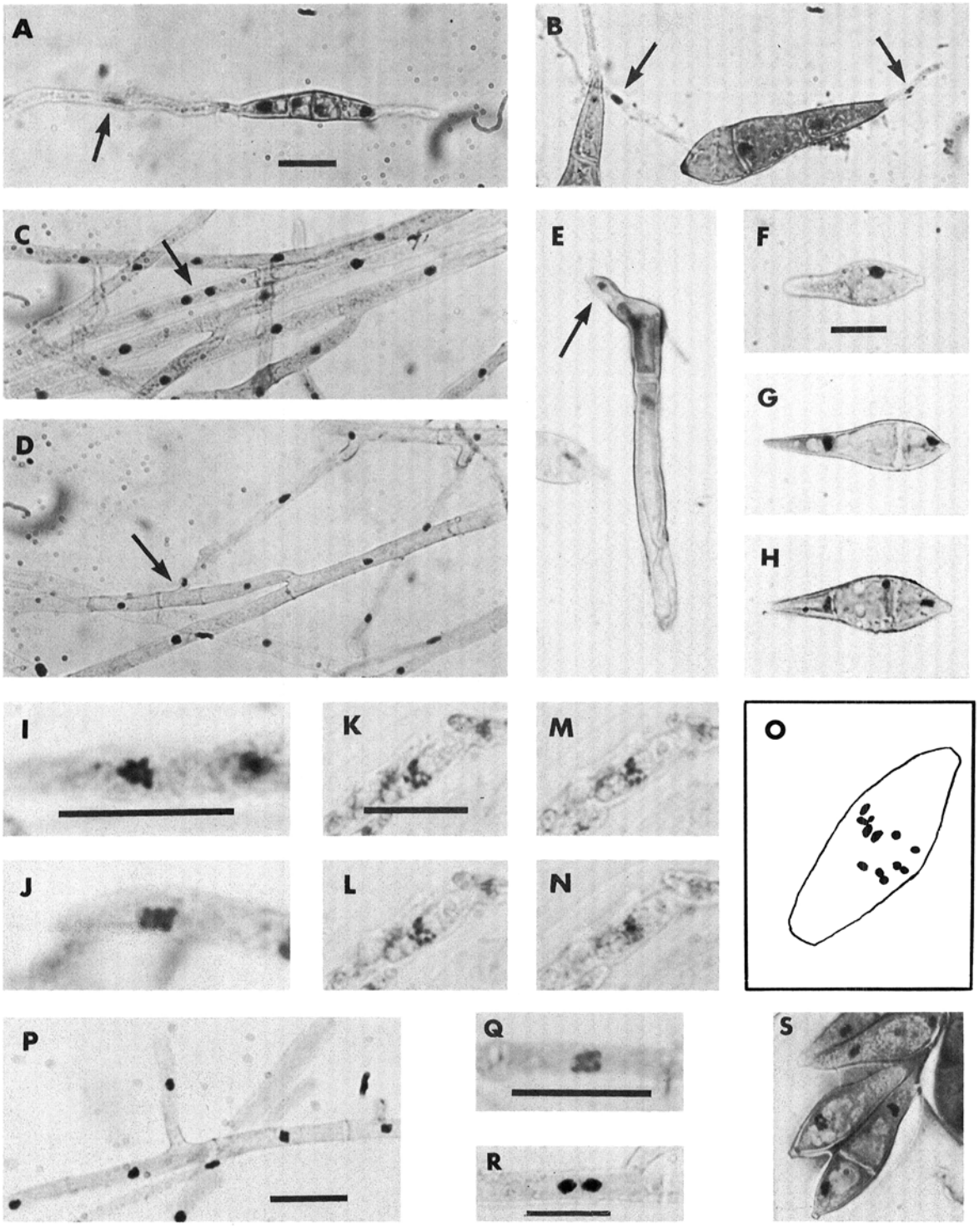

Fig. 2-(A to S). Nuclear behavior and chromosome number in *Pyricularia grisea* (A to O) and *P. oryzae* (P to S). **A,B)** Nuclear migration into germ tube of ascospore and conidium, respectively. Arrows indicate nuclei migrated into germ tube. **C,D)** Nuclei in mycelium. Arrow in C indicates two nuclei in hyphal cell. Arrow in D indicates hyphal anastomosis. **E)** Nucleus in terminal cell of conidiophore. **F,G,H)** One, two and three-celled conidia showing one nucleus per cell. **I,J)** Six chromosomes at metaphase in ascospore. **K,L,M,N)** Four focal planes of same dividing nucleus at early anaphase IV. **O)** Diagram of chromosomes in K-N. **P)** Uninucleate hyphal cells. **Q)** Six chromosomes in hyphal cell. **R)** Daughter nuclei after division. **S)** Conidia with one and two uninucleate cells. Black scale bar in the first photograph of each of the following groups represents 10 μm for all photographs of that group: A-B-C-D-E, F-G-H-S, I-J, K-L-M-N, P, Q, R.

incubated again under fluorescent light. Cover slips coated with albumin were pressed briefly against the surface of sporulating cultures. Conidia and conidiophores adhering to the surface of the cover slips were stained. The same technique was used for germinated ascospores and conidia in distilled water. Somatic nuclei were observed in hyphae growing out of small colonies in yeast extract on slides. The HCl-Giemsa method of Yamasaki and Niizeki (19) was used for staining nuclei. To observe the early stages of perithecial development, small blocks of agar bearing perithecia were embedded in paraffin and sections 10-μm thick were prepared for staining.

RESULTS AND DISCUSSION.—*Perithecial development.*—An early stage in the development of perithecia was small masses of thicker-than-normal hyphae wound together (Fig. 1-A,B). At this stage it was not possible to determine whether both parents contributed to the production of these small masses of hyphae, or whether they were formed by only one of the parents. Hyphae continued to grow around these masses to form larger white globular bodies (Fig. 1-C). As the perithecia approached maturity, the outer layer of hyphae turned brown to form the thick-walled cells of the peridium, the neck began to form, and the first asci appeared inside the perithecium. An indication that perithecial initials are formed on one of the parental strains, and later fertilized by the other parental strain, is that the inner coil of thick hyphae in the early stage of perithecial development appears to have uninucleate cells (Fig. 1-C). No binucleate cells were observed at this stage. Also, occasionally lateral branches were observed on a hyphal strand (Fig. 1-D) similar to the perithecial initials reported for a self-fertile strain of *Glomerella* (11). However, the strains used in this study failed to produce perithecia when selfed. Further work is needed to determine when and how fertilization takes place.

Nuclear behavior.—Asci are produced by crozier formation (Fig. 1-F,G,H). The crozier arises from a binucleate cell (Fig. 1-F) as reported for other ascomycetes (13). The binucleate cell becomes hook-like in shape, and the two nuclei divide simultaneously (Fig. 1-G). Two cross walls form, resulting in three cells (Fig. 1-H), a terminal uninucleate cell, a penultimate cell containing two nuclei, and a basal uninucleate cell. The two nuclei in the penultimate cell fuse before much elongation of the ascus takes place. The nucleus of the terminal cell seems to migrate into the basal cell after fusion of the terminal cell with the basal cell, to form a binucleate cell (Fig. 1-I). From the basal cell containing two nuclei, a new crozier is again produced, and the process repeated.

Various stages of ascus and ascospore development may be found in the same young perithecium (Fig. 1-E). The only diploid stage in the life cycle is shown in Fig. 1-J, but it was not possible to count the chromosomes at this stage. Elongation of the ascus appears to be rapid, and it is followed by meiotic division (Fig. 1-K). Occasionally, a thread of stained material was observed between the two daughter nuclei after nuclear division (Fig. 1-Q). Four nuclei (Fig. 1-L) are formed after the second division, and the third division soon follows, resulting in eight nuclei (Fig. 1-M). Spore delimitation takes place at the eight-nucleate stage (Fig. 1-N). Originally, only one haploid nucleus is present in each of the eight ascospores. Two more nuclear divisions occur in the delimited ascospores (Fig. 1-O,P). The last division does not always begin simultaneously in all nuclei (Fig. 1-P). Septa are formed between daughter nuclei after each division. The mature ascospore has four cells, each with a single nucleus (Fig. 1-R). The four nuclei in each ascospore are daughter nuclei of the original haploid nucleus, and are thus genetically homogeneous.

Various aspects of nuclear behavior in germinated ascospores, mycelia, conidiophores, conidia, and germinated conidia are shown in Fig. 2. Germ tubes frequently come out of the end cells of the spores. The nucleus may migrate directly into the germ tube, or one of the daughter nuclei may migrate into the germ tube after the nucleus divides. This was true with both ascospores (Fig. 2-A) and conidia (Fig. 2-B). Similar observations have been reported by Giatgong and Frederiksen (7) and Yamasaki and Niizeki (19) with conidia. These results show that this also occurs with ascospores. Although most cells of the vegetative hyphae are uninucleate (Fig. 2-C,D), multinucleate cells are occasionally observed in older hyphae (Fig. 2-C). Possible explanations for the presence of multinucleate cells in older hyphae, are that nuclear division in the older cells may not be accompanied by septal formation or branching, and that nuclear migration might occur by hyphal anastomosis. Hyphal anastomosis (Fig. 2-D) was observed often in monoconidial cultures, and sometimes nuclear migration was observed. The apical cells of conidiophores are uninucleate (Fig. 2-E). It seems likely that the nucleus in the terminal cell of the conidiophore divides to provide the single nucleus that migrates into the young conidium (Fig. 2-F). Division of the nucleus of the young conidium is followed by septal formation between daughter nuclei (Fig. 2-G). The nucleus of the apical cell of the conidium divides again, forming the typical three-celled conidium (Fig. 2-H). Thus, it appears that the three nuclei found in the mature conidium originate from a single nucleus in the young conidium, and that they are genetically homogeneous.

No differences in asexual nuclear behavior were observed between *P. oryzae* and *P. grisea*. Most mycelial cells of *P. oryzae* are uninucleate (Fig. 2-P). They are binucleate soon after nuclear division (Fig. 2-R). Conidia are uninucleate at first, and form two uninucleate cells after the nucleus divides (Fig. 2-S). Formation of mature conidia with three uninucleate cells and germination of conidia are as described for *P. grisea*. Our results agree with those of Giatgong and Frederiksen (7) and Yamasaki and Niizeki (19) who found that most cells of *P. oryzae* are uninucleate.

Chromosome number.—Chromosomes were most easily counted in the perfect state of *P. grisea* at Division IV, the first nuclear division following delimitation of the ascospore. The haploid number of chromosomes found at metaphase was six (Fig. 2-I,J). At early anaphase 12 chromosomes could be counted (Fig. 2-K, L, M, N, O) as each of the six chromosomes from metaphase divided and separated. Occasionally only five chromosomes could be distinguished at metaphase. This may have been due to overlapping of chromosomes. However, the possibility that only five chromosomes are present in some nuclei cannot be excluded. Six chromosomes were also found in

most mycelial cells of *P. oryzae* (Fig. 2-Q).

Studies of the number of chromosomes in the asexual state of *P. oryzae* have produced contradicting reports. Suzuki (16) found that the number of chromosomes was inconsistent, and varied from two to five. Yamasaki and Niizeki (19) reported that five or six chromosomes were present in some nuclei and only three chromosomes in others. They postulated that nuclei with three chromosomes may have been haploid and those with five or six chromosomes diploid. Giatgong and Frederiksen (7) found that six chromosomes predominated in most cells of *P. oryzae*, although two, three, four, or five chromosomes were occasionally observed. In agreement with the latter report, we found six chromosomes in most cells of *P. oryzae*. The presence of six chromosomes also in the perfect state of *P. grisea* indicates that the basic haploid number of chromosomes in these pathogens is six.

A possible explanation for the conflicting reports in the literature regarding the number of nuclei in vegetative hyphae of *P. oryzae* may be the age of the mycelium, since young hyphae are generally uninucleate, but old hyphae may sometimes be multinucleate. Also the staining procedure is critical. In some preparations, particularly those not adequately destained, several stained bodies could be observed in most cells. These varied greatly in size and shape, and were not considered to be nuclei.

Ou and Ayad (14) considered heterocaryosis to be a plausible explanation for the great variation in pathogenicity they obtained in monoconidial subcultures from monoconidial cultures. They cited reports by Suzuki (16) and others that cells of mycelium and conidia of *P. oryzae* are multinucleate. Giatgong and Frederiksen (7), on the other hand, found it difficult to accept heterocaryosis as an explanation for the variation they observed. According to our results, pathogenic variation among monoconidial subcultures of single spore cultures cannot be explained on the basis of heterocaryosis because the spores are homocaryotic.

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