NUCLEAR ORIENTATION IN THE ASCOSPORES OF *NEUROSPORA TETRASPERMA*¹

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FOUR successive nuclear divisions occur during ascus development in *Neurospora tetrasperma*. The first two divisions are meiotic; the last two, mitotic. Previous cytological studies of ascus development have emphasized primarily the importance of the second and third divisions, during which rather elaborate mechanisms of nuclear movement provide that descendants of each of the two nuclei separated at the first division will be present in each of the four ascospores of the ascus (DODGE 1927; COLSON 1934; DODGE, SINGLETON and ROLNICK 1950; PINCHEIRA 1963). These mechanisms are important in providing the basis for the secondarily homothallic ascospores (i.e., heterokaryotic for mating type) which are characteristic of this species (SANSOME 1946).

The present investigation concerns the fourth division and its consequences. It will be shown that this division, the only one of the four occurring after ascospore delimitation, may accomplish more than merely doubling the number of ascospore nuclei from two to four. Our studies of the nuclear composition of hyphal tips excised from both ends of germinating ascospores indicate that nuclei of opposite mating type consistently emerge into each tip. Our cytological observations suggest that a mechanism of nuclear movement occurs at the fourth division which may account for this high frequency of heterokaryotic hyphal tips, although our data cannot exclude certain alternative mechanisms. Nevertheless, it is clear that heterokaryosis for mating type in *N. tetrasperma* is present in ascospore germlings, rather than being dependent upon initiation by post-germinational hyphal fusions.

MATERIALS AND METHODS

The five *N. tetrasperma* stocks used were albino, *al*(102); pantothenic, *pan*(124); tryptophan, *tryp*(126); and wild types 85A and 85a. The *al* gene has shown no recombination with mating type or with the centromere in linkage group I; *pan*(124) is 4.8 units from centromere in IV; and *tryp*(126) is 10 units from centromere in II (HOWE and HAYSMAN 1966).

Ascospores were dispersed at wide intervals over the surface of petri dishes containing 4% water-agar, and heat-shocked in an oven at 60°C for 30 minutes. Ascospores of normal size which had produced a germ tube at each end were selected under a stereomicroscope for experimentation. The germ tubes were excised close to each end of the ascospores just before branching occurred, at which time the tubes were approximately 60 to 150 microns long. Wild-type ascospores required 4 to 5 hours at room temperature to reach such lengths; multiply marked asco-

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spores, 12 to 15 hours, a period which could not be appreciably shortened by substituting standard growth media for the 4% water-agar. Excising such short tips probably reduced the viability but was done to avoid confusing a given tip with tips from other ascospores or from opposite ends of the same ascospore. Excisions were made by means of tiny stainless steel razor blade fragments mounted in a special holder. The excised germ tubes were transferred to appropriately supplemented slants of Westergaard-Mitchell medium (WESTERGAARD and MITCHELL 1947). Conidia of the cultures grown from the germ tube isolates were plated on supplemented Westergaard-Mitchell sorbose agar (NEWMAYER 1954). Twenty or more colonies were usually isolated from each culture plated, and a random sample of several of these isolates subsequently found to be homokaryotic, as judged by self-sterility, was scored for all four segregating markers in order to determine the component nuclei.

For cytological examinations, three to four-day-old perithecia were used from crosses in which the *A* strain had served as protoperithecial parent. Fixation of material in acetic acid-alcohol-lactic acid fixative and staining in aceto-orcein were carried out essentially as described by BARRY (1966). Dissection of perithecia in preparation for staining followed the technique of PINCHEIRA (1963). Microscopy was by means of a Zeiss Photomicroscope equipped with color-corrected 1.4 na condenser, 0.4 na 16 $\times$ objective, 3.2 $\times$ intermediate lens, 1.25 $\times$ Optovar magnification changer, and 6 volt tungsten light source. This system gives a total magnification of 64 $\times$. A Wratten B58 filter was also used. Photographs were taken with Kodak High Contrast Copy film.

Figure 1 summarizes the procedure used to infer nuclear orientation in the ascospores on the basis of the nuclear types which might emerge into the hyphal tips. With respect to a pair of segregating alleles, eight different nuclear arrangements in the tetranucleate ascospores are theoretically possible, six of them heterokaryotic and two homokaryotic. These eight arrangements are placed into groups A, B, and C to facilitate reference to them; the members of group A and the members of group B are not phenotypically distinguishable from others within the same group by means of hyphal-tip analysis. The relative frequency of the heterokaryotic versus homokaryotic arrangements depends upon the distance of a marker from the centromere. Mating type is particularly suitable for these studies because of such close proximity to centromere that

FIGURE 1.—The eight possible nuclear arrangements in the tetranucleate ascospore, with respect to mating type (upper row) and a recessive marker (lower row). Phenotypes of hyphal-tip cultures derived from both ends of each ascospore are given below the diagrams, assuming that at germination the nuclei pass pairwise into the nearest hyphal tip.

only ascospores heterokaryotic for mating type occur, which eliminates the need for considering group C arrangements for mating type. Therefore, if the six heterokaryotic arrangements for mating type occur randomly, and if at germination the nuclei pass pairwise into the nearest hyphal tip, two thirds of ascospores tested would be expected to produce self-fertile hyphal-tip cultures at each end; one third, self-sterile hyphal-tip cultures at each end (Figure 1, upper row). A significant deviation from this 2:1 ratio would indicate nonrandomness. The use of the other three markers, recessives chosen for increasing centromere distances, is illustrated in Figure 1, lower row. One of these markers, albino, has never been found to recombine with mating type and hence, on a random basis, would be expected to show the 2:1 ratio of heterokaryotic arrangements. The remaining two markers, *pan* and *tryp*, are farther from their centromeres and hence should give some homokaryotic arrangements, which are readily distinguishable; however, heterokaryotic arrangements for these two markers, if random, should also show the 2:1 ratio.

Figure 2 summarizes how the rather large proportion of ascospores, from which the hyphal-tip cultures apparently had one or more inviable component nuclei, were included in the data. There are six main phenotypically distinguishable ascospore types, based upon viability and self-fertility or self-sterility of derived hyphal-tip cultures. (There are 13 additional permutations of these six main types, but none alters inferences made from the six types and hence they are not considered). Nuclear arrangements were first determined for type I ascospores exclusively, because the recovery of all four nuclei provided the greatest reliability. Secondly, after analysis of a large number of type I ascospores had revealed that only certain ones of the theoretically possible nuclear arrangements seemed to occur, the genotypes of the inviable nuclei in types II-V were readily deduced. All five of these ascospore types are included in the tables but are listed separately so that inferences about nuclear arrangements may be made from any or all of the five types.

RESULTS

Hyphal tip analysis of multiply-marked ascospores: Pairs of hyphal tips were excised from 177 ascospores from the cross *tryp*⁺; *pan*⁺; *al* *A* × *tryp*; *pan*; *al*⁺ *a*. Hyphal-tip pairs from 27 of these ascospores failed to grow after transfer to slants (type VI, Figure 2) and are not considered further. The nuclear arrangements inferred from hyphal-tip analysis of the remaining 150 ascospores are listed in Table 1. The most striking finding is that none of these 150 ascospores showed the group A nuclear arrangements (Figure 1) with respect to any of the four markers employed. A total of 145 of the 150 ascospores were heterokaryotic for all four markers, and all 145 showed the group B nuclear arrangements (Figure

FIGURE 2.—The six main phenotypically distinguishable ascospore types with respect to viability and resultant self-fertility or self-sterility of hyphal-tip cultures derived from each end. Phenotypes of the hyphal-tip cultures are given below the diagrams, assuming pairwise emergence of the nuclei into the nearest hyphal tip. Solid nuclei, inviable; open nuclei, viable and, unless designated, containing whichever mating-type allele survives by chance. First division segregation of mating type is assumed.

TABLE 1
Nuclear arrangements of 150 ascospores from the cross $\text{tryp}^+; \text{pan}^+; \text{al} A \times \text{tryp}; \text{pan}; \text{al}^+ a$, inferred from hyphal-tip analysis

Ascospore type	Genotypes of the four nuclei of each ascospore*										Total number of ascospores
	Heterokaryotic arrangements				Partially homokaryotic arrangements						
	$t;p;al A \mid +; +; + a$ $t;p;al A \mid +; +; + a$	$+p;al A \mid t; +; + a$ $+p;al A \mid t; +; + a$	$t; +; al A \mid +p; + a$ $t; +; al A \mid +p; + a$	$+; +; al A \mid t;p; + a$ $+; +; al A \mid t;p; + a$	$+p;al A \mid +; +; + a$ $+p;al A \mid +; +; + a$	$+; +; al A \mid +p; + a$ $+; +; al A \mid +p; + a$	$t; +; al A \mid t;p; + a$ $t; +; al A \mid t;p; + a$	$+p; + a \mid t; +; al A$ $+p; + a \mid t; +; al A$	$t;p; + a \mid +; +; al A$ $t;p; + a \mid +; +; al A$	$+; +; al A \mid t; +; + a$ $+; +; al A \mid t; +; + a$	
I	22	21	14	19	2	1	0	0	1	1	80
II	1	3	3	2	0	0	0	0	0	0	9
III	2	1	0	5†	0	0	0	0	0	0	8
IV	11	4	19	10	0	0	0	1	0	0	45
V	3	2	1	2	0	0	0	0	0	0	8
Total number of ascospores	39	31	37	38	2	1	1	1	1	1	150

Genotypes of the inviable nuclei in ascospore types II-V were deduced from the surviving nuclei in these same ascospores, based upon nuclear arrangements found in type I.

* Each upper pair of genotypes represents the two component nuclei of the hyphal tip from one end of each ascospore; each lower pair, from the other end.

† One ascospore had unlike mating-type alleles at each end; the other four, like mating-type alleles.

1) exclusively. Independent assortment of the unlinked *al*, *pan*, and *tryp* markers, as well as the lack of any recombination whatsoever between *al* and mating type, is evident in Table 1.

The remaining five ascospores in Table 1 were homokaryotic for either the *pan* or *tryp* loci (but not both), which can be accounted for as second division segregations for these two loci; in support of this view is the fact that the *al* and mating type markers in these five ascospores were heterokaryotic and showed the usual group B nuclear arrangements. Therefore, the nuclei apparently did not move randomly into the germ tubes, for the only nuclear orientations found were those ascribed to group B. The five instances of homokaryosis for single markers in the same germ tube may be attributed to second division segregation.

A complete listing of genotypes of all nuclei, both viable and inviable, for each of the 150 ascospores would be too unwieldy for presentation here but may be extracted by the interested reader from the information given in Tables 1 and 2.

Hyphal tip analysis of wild-type ascospores: Before doing the analysis with the multiply marked strains just described, hyphal tips were isolated from 242 ascospores from a cross of the two wild-type strains, 85A and 85a. Only the mating-type locus was available as a marker in this material. The pairs of hyphal tips from 162 of these ascospores failed to grow. This poor viability was probably caused partly by our inexperience in these early experiments and partly by the fact that hyphal tips from the wild-type crosses tended to branch earlier and hence were excised at shorter lengths.

Among the remaining 80 ascospores there were 17 type I, 3 type II, 0 type III, 52 type IV, and 8 type V. Nonrandomness was clearly evident despite the poor viability, however, because all 17 of the type I ascospores showed only group B nuclear arrangements, whereas on a random basis one third would have been expected to show group A arrangements.

Cytological observations and interpretations: Following the third division in the developing ascus, each ascospore contains two nuclei, which are usually located opposite each other at the sides of the spore (Figure 3). The genetic data

TABLE 2

Deduced numbers and genotypes of inviable nuclei in Ascospore Types II-V, from the cross $tryp^+; pan^+; al A \times tryp; pan; al^+ a$

Ascospore type and observed numbers	Deduced genotypes of inviable nuclei								Total number of inviable nuclei
	<i>t;p;al A</i> +;+;+ <i>a</i> +;+; <i>al A</i> <i>t</i> ;+;+ <i>a</i> <i>t</i> ;+; <i>al A</i> +; <i>p</i> ;+ <i>a</i> +;+; <i>al A</i> <i>t;p</i> ;+ <i>a</i>								
II-9	0	1	1	2	2	1	1	1	9
III-8	4	0	0	2	0	0	5	5	16
IV-45	11	11	4	4	20*	19	10	11*	90
V-8	5	4	4	2	1	2	2	4	24
Total number of inviable nuclei	20	16	9	10	23	22	18	21	139

* One nucleus of this genotype was from the type IV ascospore which showed second division segregation.

FIGURES 3, 3A, -8, 8A.—Arrows are used in the photographs to indicate pertinent ascospores, because cytological events within an ascus are frequently not synchronized. These same spores are interpreted in the diagrams by use of solid and open circles to distinguish genetically differ-

indicate that these two nuclei are nonsisters (Figure 3A). Division IV ensues, and nuclear separation continues laterally until two nuclei, usually very difficult to resolve, are located at each side of the spore (Figure 4). Internuclear strands are usually visible at this stage. Similar strands are occasionally found at subsequent stages. These strands may be spindles but if so are more erratic in occurrence and staining quality than are the spindles of earlier divisions in the ascus. Whether spindles or not, these strands are nevertheless helpful in tracing relationships between the four nuclei.

There are two points following division IV at which critical interpretations of nuclear movements may be made as bases for explaining why the hyphal tips are consistently heterokaryotic for certain markers. The first point concerns the identity of the four nuclei in Figure 4. Our interpretation is that the two nuclei at each side of the spore are nonsisters (Figure 4A). Next, two nuclei on one side move toward opposite ends of the spore so that the four nuclei now form a V-configuration, which is more apparent if the internuclear strands are still visible at this time (Figure 5). The second point for critical interpretation of nuclear movement concerns the significance of the V-configuration. Our interpretation is that the two nuclei at the apex of the V pass around each other at about this time (Figure 5A), orienting nonsister nuclei toward each germ pore. Internuclear strands still visible may show an X-configuration (Figure 6), as nonsister nuclei now move further toward the spore ends (Figure 6A). Movement may continue endward to some extent, after which rib formation (Figures 7, 7A) and elaboration of melanin obscure further observations. At germination, nonsister nuclei pass into the hyphae emerging from each end of the ascospore (Figures 8, 8A).

Inviolate nuclear types: Assuming that the four ascospore nuclei pass pairwise into the germ tubes, the failure to find a particular component nucleus during subsequent analysis by conidial plating was interpreted as owing to nuclear lethality. Alternatively, nuclear or hyphal loss following excision, as well as other causative factors, could have been operative. The frequencies of these ascospore types (Figure 2) showed a perplexing distribution. For example, type III ascospores are assumed to represent two inviolate nuclei; type II ascospores, only

ent nuclei. FIGURE 3.—The binucleate stage resulting from division III. 3A. Diagram indicates genetic nonidentity of nuclei in each spore. FIGURE 4.—An early tetranucleate stage resulting from division IV and subsequent, lateral nuclear separation. Internuclear strands arranged laterally. 4A. Diagram indicates that the nuclei at each side of the spore are genetically non-identical. FIGURE 5.—Two nuclei on one side have moved toward opposite ends of the spore. Internuclear strands arranged obliquely, forming a V-configuration. Nuclear passing occurs at the apex of the V. 5A. Diagram interpreting nuclear movements. FIGURE 6.—All four nuclei have moved toward the spore ends. Internuclear strands arranged in an X-configuration. 6A. Diagram interpreting nuclear types. FIGURE 7.—A late tetranucleate stage with the constricted nuclei near the spore ends difficult to see because of rib formation. 7A. Diagram interpreting nuclear types. FIGURE 8.—Hyphae emerging from each end of the ascospore. Nuclei are visible but accurate counts are unobtainable because of hyphal shrinkage incurred during slide preparation. 8A. Diagram indicating heterokaryosis in the hyphal tips but not implying the number of nuclei actually present.

one. Yet both types occurred with similar frequencies. Types III and IV are both assumed to represent two inviable nuclei, yet type IV was found with much greater frequency than was type III. In fact there was a markedly high frequency of type IV ascospores from both the mutant and wild type crosses.

Whatever the cause of the distribution of ascospore types shown in Figure 2, nuclear loss did not seem to be associated with a disproportionate loss of reciprocal genotypes. Table 2 shows the four pairs of reciprocal genotypes with respect to the *al*, *pan*, and *tryp* markers, as deduced for the inviable nuclei from the viable nuclei. The ratios of 20:16, 9:10, 23:22, and 18:21 found in these deduced ascospore nuclei do not differ significantly from the 1:1 chance expectation. From these ratios, allele ratios were derived and were also found to be random: *al*:*al*⁺, 70:69; *pan*: *pan*⁺, 72:67; and *tryp*: *tryp*⁺, 74:65. The mating-type alleles did not recombine with *al* and have the same allele ratios as *al*.

Nuclear ratios in the heterokaryons: Hyphal-tip analysis of the multiply marked ascospores provided an opportunity for examining nuclear ratios in a large number of heterokaryons, since 247 viable cultures were obtained from the 177 hyphal-tip pairs excised. A total of 214 of these 247 cultures was self-fertile and hence obviously heterokaryotic, which necessitated conidial plating to determine nuclear content. (Two of the 214 cultures proved to be exceptional and are described subsequently.) The isolates from these platings, when grown to maturity, were classified as either self-fertile or self-sterile, after which the self-sterile (presumably homokaryotic) isolates were classified as either *al* or *al*⁺. The 3,997 self-sterile isolates obtained from the 212 platings gave the following results:

Allele ratios	Number of heterokaryons	
	<i>al</i> : <i>al</i> ⁺	<i>al</i> ⁺ : <i>al</i>
1:1	35	
2- 5:1	51	49
6-10:1	25	14
11-20:1	9	14
21-30:1	6	4
31-60:1	2	1
65:1	1	1

Although considerable variation was found, it is evident that most of the heterokaryons were sufficiently well balanced so that nuclear determinations could be made from the 20 colonies per culture routinely sampled. It is particularly noteworthy that, since all 212 of these heterokaryons were self-fertile and at least partially pigmented (i.e., not albino), the mating-type alleles and the *al*⁺ allele are expressed even under unbalanced nuclear conditions as extreme as 65:1.

The two exceptional heterokaryons mentioned above were both derived from the same ascospore and were completely refractory to the usual plating analysis. Each was pigmented and self-fertile, yet a total of 268 self-sterile homokaryons obtained from conidial platings of these two heterokaryons all proved to be *al pan*⁺ *tryp* A. These two heterokaryons evidently carried a spontaneous, irreparable mutation in the presumptive *al*⁺ *pan tryp*⁺ *a* nuclear component, which prevented growth as a homokaryon on complete medium (yeast and malt extract supplements). To test for the presence of such a component, each of the two

heterokaryons was grown with an *al⁺ pan tryp a* strain, and conidia from the two presumptive trikaryons were plated. If the mutation hypothesis is correct, at least some *al⁺ pan tryp⁺ a*, *al⁺ pan tryp a* isolates from the platings would be expected, which should be viable because of the dikaryotic condition and recognizable because of the pantothenate requirement. Such isolates were indeed found.

DISCUSSION

Previous cytological and genetic studies have established that each of the four ascospores in the ascus of *Neurospora tetrasperma* is tetranucleate and heterokaryotic for markers, such as mating type, which segregate at the first meiotic division. The present investigation shows that both of the hyphal tips of germinating ascospores are also heterokaryotic for these same markers. This species, therefore, has a mechanism which assures heterokaryosis for certain genes in the hyphae from the onset of ascospore germination. The constancy of heterokaryosis in the ascospore germings seems firmly established on the basis of a study of the hyphal-tip pairs of 80 multiply marked ascospores and 17 wild-type ascospores (type I), from each of which all four nuclear components were recovered and genetically analyzed. The agreement between the findings with both multiply marked and wild-type ascospores indicates that the results are not due to mere selection for heterokaryosis for nutritional deficiencies, since the wild-type ascospores carried no nutritional markers. The technique of isolating hyphal tips from opposite ends of germinating ascospores has been previously employed by PITTINGER (1958), who investigated the mitotic breakdown of disomic nuclei of pseudo-wild types in *N. crassa*.

In the present investigation of the basis for the heterokaryotic hyphal tips, it was assumed that during germination there is random pairwise movement of the four nuclei of the ascospores into both hyphal tips. Although on this assumption one third of hyphal-tip pairs would be expected to be homokaryotic for markers which segregated at the first meiotic division, significantly fewer homokaryotic tips were found. The cytological mechanism proposed for this apparent non-randomness is nuclear passing at the fourth division in the ascospore. It is of interest that this nuclear shifting seems to be necessitated by the lateral arrangement of the two fourth-division axes, which orient sister nuclei toward each end of the ascospore (Figure 4A). If these axes were oriented longitudinally with respect to the ascospore, nonsister nuclei would be delivered toward each germ pore, in accordance with the observed genetic results. The postdivisional V-configurations of the nuclei and the occasionally observable internuclear strands are interpreted as manifestations of nuclear passing. These V-configurations are also shown by PINCHEIRA (1963) in his Figure 41. The internuclear strands, which were helpful in interpreting nuclear movements, are unfortunately difficult to see in our strains. The ascospore, in fact, is not particularly satisfactory material for cytological observations, because vacuolation of the cytoplasm soon becomes pronounced, and rib formation and elaboration of melanin commence shortly there-

after. It is not unlikely that other cytological patterns occur, just as they do at the second meiotic division (DODGE 1927).

Additional ascospores analyzed included 70 multiply marked and 63 wild-type ascospores (types II-V), from which one to three of the four nuclear components were not recoverable and hence presumably inviable. Actually, nuclear lethality was probably not the sole factor involved, for these types showed complex numerical distributions. The high frequency of type IV ascospores, for example, could have owed partly to nuclear or hyphal loss following excision rather than to nuclear lethality, or to a tendency for all four nuclei to pass out of the same germ pore. Previous estimates of the frequency of nuclear lethality (HOWE 1964) are probably not applicable here, because hyphal-tip analysis was not used, and hence hyphal loss did not contribute to the frequencies obtained.

The most logical way to infer the genotypes of the inviable nuclei in types II-V is on the basis of the nuclear orientation patterns already established in type I, which showed no lethality. Because this was done, it is not surprising that no differences in nuclear orientation patterns in the various types were found. The sample size of type I alone, however, seems sufficient to establish the nuclear orientation patterns that occurred. The only group A nuclear arrangements that could have been missed by the procedure used to infer genotypes of inviable nuclei would be in certain ascospores of types III and V. Type III ascospores would be expected, on a random basis, to occur in two subtypes in a 1:1 ratio, namely those with hyphal tips of like mating type, and those with hyphal tips of unlike mating type. The observed ratio of these two subtypes among the eight type III ascospores, however, was 7:1. The finding that in seven of these eight spores the two nuclei not recovered were sister nuclei supports nuclear lethality as the causative factor. The seven type III ascospores with hyphal tips of like mating type could not have been in group A, and could have been in group C only if postreduction of mating type had occurred, which is unlikely; these seven ascospores, therefore, probably belonged to group B. The remaining type III spore could have been in either group A or group B, but not in group C. Even if we assume that this spore and the eight type V spores all had group A arrangements, a maximum of only nine group A patterns among the 150 ascospores could have occurred.

It is important to consider alternatives to the apparent nonrandom nuclear orientation observed, because the results obtained may be accounted for in other ways. Random instead of nonrandom movement of nuclei into the germ tubes could also give high frequencies of heterokaryotic hyphal tips, if one or more rounds of nuclear divisions occurred after melanin formation in the spore walls so that more than the four nuclei per spore actually observed were present. If, during the germination process, the nuclei undergo a certain degree of mixing by movements through or back into the ascospore, such mixing could override nuclear orientations previously established at the fourth division. Because of the development of pigment in the ascospore wall, we were not able to observe nuclear movements between the time of heat shocking and actual emergence; hence pairwise nuclear emergence has not been cytologically verified. Counts of the number

of nuclei in the germ tubes at successive intervals in subsequent investigations should prove helpful in validating alternative hypotheses.

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SUMMARY

Analysis of a large sample of hyphal-tip pairs excised from both multiply-marked and wild-type ascospores has shown a pronounced tendency for both tips of each pair to be heterokaryotic for mating type and other markers which segregated at the first meiotic division. Cytological observations suggest that fourth-divisional nuclear passing may account for this apparent nonrandomness. The data, however, do not exclude alternative interpretations.

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