MEANS FOR THE RECOVERY OF HOMOKARYOTIC SEGREGANTS FOLLOWING SEXUAL CROSSES IN *NEUROSPORA TETRASPERMA*

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MOST genetic work on *Neurospora* has been done with *N. crassa*. In this heterothallic species sexual reproduction culminates in an ascus with eight unisexual spores, each being the representative of a single meiotic product and thus giving rise to a homokaryotic mycelium. By contrast, its relative *N. tetrasperma* is a pseudohomothallic fungus whose typical ascus includes four bisexual spores the germination of which gives rise to heterokaryotic mycelia that are capable of self-mating. These bisexual spores have been organized around nuclear representatives of two different meiotic products. The morphological and functional differences of the asci in these species are based on a systematic difference in the arrangement of nuclear spindles at meiosis II and the subsequent mitosis (DODGE 1927). On the other hand, both species have a similar, if not identical, genetic basis for the determination of mating type specificities, and genetic material can be transferred, with some difficulty, from one species to the other (SRB 1965).

Because the mycelia of *N. tetrasperma* are characteristically heterokaryotic and undergo uncontrolled self-mating, genetic analysis in this species is more difficult and far less direct than in *N. crassa*, accounting in part, no doubt, for the latter's much greater popularity with geneticists. Nevertheless, extensive work by HOWE (e.g., HOWE 1963) has provided valuable basic genetic information about *N. tetrasperma*. METZENBERG and AHLGREN (1969) have shown the species to be a valuable source of enzyme variants. PINCHEIRA and SRB (1969) have utilized a mutant affecting the sexual reproductive apparatus to increase understanding of similarities and differences in ascus development in *N. tetrasperma* and *N. crassa*. Both for reasons of inherent interest and of its potential for comparative biochemical and developmental studies, *N. tetrasperma* is likely to become increasingly important as an experimental genetic organism. Current interest, then, makes appropriate the development and evaluation of techniques that will permit efficient genetic work with *N. tetrasperma*. The primary goal is the ready isolation of homokaryotic progeny following a mating. This article describes and discusses means now available, including ones recently developed by us, for the recovery of homokaryotic progeny.

MATERIALS AND METHODS

Strains: The homokaryotic wild-type *N. tetrasperma* strains of different mating type, *T-7A* and *T-7a*, were derived from *T-220*, a pseudohomothallic strain of *N. tetrasperma* isolated in Borneo by J. H. WARCUP. Strain *Pk-1-tet* is a dominant abnormal ascus mutant isolated from a

conidial plating of *T-7A* following mutagenic treatment with dimethyl sulfate. Strain *pk-2-cra* carries the mutant gene *pk-2*, originally isolated in *N. crassa* as a recessive abnormal ascus mutant allelic to biscuit in linkage group V. In the studies described here, *pk-2* is in a genome primarily that of wild-type *N. tetrasperma*, to which the gene was transferred by more than ten generations of backcrossing (PINCHEIRA and SRB 1969). The transferred *pk-2* is designated here as *pk-2-cra* to distinguish its species of origin from that of *Pk-1-tet*. In vegetative culture, both mutants, *Pk-1-tet* and *pk-2-cra*, have tight colonial morphology. The mutant genes are alleles, as shown by both complementation and recombination studies carried out in *N. tetrasperma*. The auxotroph used as a marker is an adenine purple (*ad-3*) mutant (cistron unknown), isolated from a conidial plating of *T-7a* following treatment with N-methyl-N'-nitro-N-nitrosoguanidine. The *E* strain (DODGE 1939) is that designated as 340.6aE (Fungal Genetics Stock Center #605).

Culture techniques: Crosses in culture tubes (150×16 mm) were made either on Difco cornmeal agar or on the crossing medium of WESTERGAARD and MITCHELL (1947). The latter medium was supplemented with adenine sulfate (0.1 mg/ml) whenever the *ad-3* mutant was included in a cross. The complete medium used for ascospore isolations and maintenance of laboratory cultures is essentially that of BEADLE and TATUM (1945).

In order to differentiate heterokaryotic (bisexual) from homokaryotic (unisexual) cultures on Petri plates, a special medium was required. Initially, Petri plates were poured with WESTERGAARD & MITCHELL's crossing medium containing 0.1% sucrose and 2% sorbose (Nutritional Biochem.). Later it was found that 0.5% sucrose and 2% sorbose were more satisfactory for the purpose of distinguishing bisexual from unisexual cultures grown as colonies restricted in size. The use of medium with the increased sucrose concentration permits earlier distinction between the two types of colonies. With 0.5% sucrose, the first signs of self-fertile colonies can be observed in as few as five days after plating ascospores while as many as 10 days are required when 0.1% sucrose is used.

Ascospore plating technique: Ascospore platings were carried out as follows: (1) Using an inoculating loop, ejected ascospores were scraped from the wall of a crossing tube and transferred into a culture tube (150×16 mm) containing 8 ml of a 10% solution of Clorox, a procedure used to eliminate any live vegetative material. (2) The spores were collected by centrifugation and resuspended in 8 ml of sterile water. (3) spores were heat activated at 60°C for 30 min and after dilution were plated onto a suitable crossing medium. Spore concentrations for plating ranged from 50–100 spores in 0.1 ml. After a little practice, an appropriate concentration for plating can be identified by simple visual inspection of the spore suspension.

RESULTS

Hand isolation of occasionally produced small homokaryotic spores: A wild-type cross of *N. tetrasperma* produces asci almost all of which contain four spores. Originally each spore includes two haploid nuclei (is tetranucleate following the mitosis within the spore) and is heterokaryotic for all loci that were heteroallelic in the zygote and that were not involved in a gene-centromere crossover at meiosis (DODGE 1927; COLSON 1934). Because crossing over rarely occurs between the mating-type locus and the centromere of its chromosome, almost all normal spores are heterokaryotic for mating type and give rise to mycelia that self-mate. Occasionally, asci containing other than four spores are produced (Figure 1). If a five-spored ascus is produced, only three of the spores are normal in size and originally binucleate; the remaining two spores are smaller, originally uninucleate, and one is of one mating type, one of the other (Figures 2, 3). Isolation of such small haploid spores is equivalent to a random spore isolation in *N. crassa* and provides the same information. Normally, only 1 to 2% of the asci from wild-type crosses have more than four spores. The average size of 50 normal

TABLE 1

Isolation of small presumably homokaryotic spores from the cross T-7A $\times$ ad-3 a

Crossing medium*	Age of cross	Number germinated from 100 isolates	Genotype for mating type and <i>ad-3</i>		
			<i>A, a ad-3, +</i> †	<i>A +</i>	<i>a ad-3</i>
cm	3 weeks	83	10	41	32
cm	4 "	92	11	41	40
W-M	3 "	85	5	47	33
W-M	4 "	87	14	43	30

* cm = Difco cornmeal agar. W-M = WESTERGAARD and MITCHELL crossing medium.

† Instances of "errors," that is, of small spores giving rise to heterokaryotic mycelia that are self-fertile and include both *ad-3* and its normal allele.

spores produced by our wild types is 16.4×31.0 microns, while for 50 small spores the average measurements are 12.9×22.8 microns. To look for and manually isolate such small spores from among the much more frequent large spores is tedious. Moreover, although small size is a good indication of a unisexual (homokaryotic) spore, the correlation is not perfect, as is shown genetically (Table 1) and by examination of Figures 2 and 3.

More efficient means of recovering homokaryotic segregants: Two approaches can be employed to increase the efficiency of recovering homokaryotic products following meiosis in *N. tetrasperma*: (1) increase the number of small spores produced in a given cross; (2) find methods of recovering the homokaryotic products by other than hand isolation. The first approach is possible by means of appropriate mutants.

a. *Effect of strain E on frequency of small spores:* Figure 4 shows a portion of a perithecial squash from a cross of *aE* $\times$ *T-7A*. As can be seen, the effect of *E* is almost to eliminate the pseudohomothallism of *N. tetrasperma*, in that many seven- and eight-spored asci are produced, while few four-spored asci can be found. Strain *E* is useful for obtaining high frequencies of small spores, but has disadvantages. As originally described by DODGE (1939) mutant *E* when heterozygous gave rise to a high frequency of aborted asci but if asci were produced, they were eight-spored. In addition, ascospores containing *E* died at the germling stage. Ultimately, means were found to reduce somewhat both ascus abortion and spore inviability (DODGE 1939; DODGE, SINGLETON and ROLNICK 1950).

When *a E* was crossed to *T-7A*, little ascus abortion was evident and *E* strains could be recovered; however, serious problems with spore inviability were encountered. Even after five backcrosses of *E* strains to our wild-type *N. tetrasperma* only about 30% germination was obtained from spores isolated from crosses involving *E* (Table 2).

A detailed genetic analysis involving *E* has been carried out by CALHOUN and HOWE (1968). Contrasted with the results of DODGE, they report markedly less ascus abortion and better spore viability. In contrast to the results with our strains of *N. tetrasperma*, the spore viability in crosses heterozygous for *E* is better. Of 505 eight-spored asci analyzed, 66% had more than four viable spores, and spore

FIGURES 1-7.—Production and detection of homokaryotic spores in *Neurospora tetrasperma*. FIGURES 1-3: Asci from the wild-type *N. tetrasperma* cross *T-7A* $\times$ *T-7a*. FIGURE 1: Perithecial squash showing two exceptional asci, a three-spored ascus and a five-spored ascus. FIGURES 2,3:

TABLE 2

Ascospore isolations from the original cross $aE \times T-7A$ and from two different backcross generations

Cross	Backcross generation	Number germinated from 150 isolates	Genotype for mating type and <i>E</i>			
			<i>A E</i>	<i>a E</i>	<i>A +</i>	<i>a +</i>
<i>a E</i> $\times$ <i>T-7A</i>	0	21	0	3	12	6
<i>a E</i> $\times$ <i>T-7A</i>	3	41	2	4	18	17
<i>A e</i> $\times$ <i>T-7a</i>	5	46	3	1	26	16

viability approached 68% under optimum conditions. In any case, *E* at best gives problems with spore viability, and whenever *E* is employed the fact must be taken into account that crosses homoallelic for *E* are always sterile.

b. *Effect of Pk-1-tet and pk-2-cra on small spore production*: JOHNSON and SRB (1962) observed that *pk-2-cra* increases the percentage of small spores in *N. tetrasperma*. The effect of *pk-2-cra* when crossed with wild-type *N. tetrasperma* is similar to the result observed for *E* (Figure 5). Although eight-spored asci are rarely produced, asci containing more than four spores occur at a frequency of 42% (PINCHEIRA and SRB 1969).

That other colonial mutants might have a similar effect was verified by the finding that colonial mutant *Pk-1-tet* also increases small spore production when heterozygous in a cross. *Pk-1-tet* differs from the other mutants, however, in producing asci that are balloon-shaped rather than linear (Figures 6A-6B). The exceptional asci produced by *Pk-1-tet* are almost always five- or six-spored. When heterozygous in a cross, *Pk-1-tet* normally yields 20-30% small homokaryotic spores (as determined by plating ascospores on sorbose plates and scoring the percentage of unisexual progeny produced).

Neither *pk* mutant has a pronouncedly depressing effect on spore germination (70-80% germination is routinely obtained). In addition, unlike *E*, which has essentially wild-type morphology, both *pk* mutants are easily recognized by their colonial growth habit. Thus, while the colonials can be used to increase the production of small spores, if so desired, they can readily be avoided among progeny isolates that are to be used in future experiments.

c. *Recovery of homothallic cultures by a plating technique*: The second approach for effective isolation of homokaryotic cultures is to find methods of recovery other than isolation of individual spores by hand. The basis of one such

Three asci from a single perithecium and at the same magnification. The size of the small spores in the five-spored ascus of Figure 2 approximates the size of the normal spores in the five-spored ascus of Figure 3. This kind of discrepancy in spore size accounts for some small spores proving to be heterokaryotic. FIGURE 4: Asci from the cross *aE* $\times$ *T-7A*. Visible are one four-spored ascus, three six-spored asci, one seven-spored ascus, and four eight-spored asci. FIGURE 5: Asci from the cross *pk-2-cra A* $\times$ *T-7a*. FIGURES 6A,6B: Two asci from the cross *Pk-1-tet A* $\times$ *T-7a*. FIGURE 7: Section of Petri plate showing one self-sterile colony (with no perithecia) among nine self-fertile colonies. Photo was taken eight days after spores were plated on WESTERGAARD & MITCHELL cross-
ing medium containing 0.5% sucrose and 2% sorbose.

method is illustrated in Figure 7. Ascospores from the cross $T-7A \times ad-3 a$ were plated on a specially adjusted crossing medium (see MATERIALS AND METHODS). On the basis of presence or absence of perithecia, heterokaryotic self-fertile colonies are easily distinguished from homokaryotic self-sterile colonies. The homokaryotic colonies can be used to provide data of the kind represented in Tables 1 and 2 but obtained there by manual isolation of spores. When appropriate, vegetative transfers can be made from homokaryotic colonies in order to establish progeny isolates for further testing. Or if use is made of markers whose phenotypes are visually distinguishable on plates, data can be taken directly. The technique has been used, for example, to confirm with much larger numbers the close linkage of *ad-3* and mating type shown in Table 1. Although not without some built-in error, the technique provides accurate results and is efficient. Of all colonies selected from such plates because perithecia did not form, fully 95% turned out on further testing to be truly homokaryotic.

DISCUSSION

The standard technique for recovering homokaryotic progeny from an *N. tetrasperma* cross is to search visually and then isolate small ascospores. The task is tedious, especially in crosses of wild-type *N. tetrasperma*, where small spores are scarce. At present, a highly efficient way to increase the production of small spores as such is to use strain *E*. But because of relatively poor spore viability and because *E* gives complete sterility when homoallelic in a cross, the use of *E* as a standard means of increasing small spore production has proved to be of limited practicality with our strains.

Asci initiated by zygotes homoallelic for *pk-2-cra* provide small spores in about the same frequency as do crosses involving *E* (PINCHEIRA and SRB 1969). Routine utilization of homozygous *pk-2-cra* for genetic manipulation and analysis in *N. tetrasperma*, however, has the sometimes awkward feature that both parents and all progeny will have tight colonial growth habit. On the other hand, certain colonial mutants with dominant effects can be used as heterozygotes to increase small spore production with little of the deleterious effect on viability that comes with use of *E*. And because the colonial phenotype is recognizable by simple inspection, colonials are readily discernible among progeny isolates if culling is desirable. In *N. crassa*, mutants whose effects on the ascus are similar to those of *pk-2-cra* have been found at several other loci (SRB and BASL 1969). Screening for corresponding mutants in *N. tetrasperma* may yield strains that are even more effective in the production of small spores than are the presently available mutants.

Whether or not a mutant gene is employed to increase the frequency of small spores, the finding that homokaryotic progeny can be distinguished from heterokaryotic progeny on Petri plates eliminates the need for hand isolation of small spores. But an effective and simple basis for genetic analyses in *N. tetrasperma* is to employ a mutant to increase small spore number and then use the plating technique to detect the homokaryotic progeny. The plating technique, as de-

scribed, rests on the oversimplified assumption that only truly homokaryotic spores will be unisexual. Because crossing over between the mating-type locus and its centromere does occur, although infrequently (DODGE 1931; HOWE 1963), homokaryosis for mating type does not necessarily mean homokaryosis for other loci. As noted by HOWE (1964), instances of second-division segregation for mating type can be identified by including in a cross a marker gene tightly linked to its centromere and in a linkage group other than that of mating type. Marker genes of this kind are available in *N. tetrasperma*. Since simultaneous homokaryosis (resulting from second-division segregation) for two genes closely linked to their respective centromeres is highly unlikely, the doubly marked system offers effective controls. Finally, normal-sized, presumably heterokaryotic spores of *N. tetrasperma* sometimes prove to behave unisexually for reasons not accountable by gene-centromere crossovers, e.g., nuclear death (HOWE 1964). Such exceptional occurrences, if they affect nuclei randomly, do not bias the results obtained with the plating technique.

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SUMMARY

Routine genetic analysis with *N. tetrasperma* is made difficult by the fact that most ascospores from wild-type crosses give rise to heterokaryotic self-mating mycelia. In order to obtain unambiguous genetic material, the usual procedure is to search for the rarely produced small spores that give homokaryotic mycelia. The occurrence of such small spores can be greatly increased by employing certain mutants such as the *E* strain of DODGE; however, the practicability of use of *E* is reduced by its effects on fertility. When heterozygous in crosses, certain morphological mutants also have the effect of increasing the frequency of small spores. Such mutants do not have the undesirable effects of *E* and can be used to great advantage. Self-fertile can be distinguished from self-sterile colonies by means of a simple plating technique that is described. The plating technique can be used to maximum efficiency when one of the parents in a cross includes a mutant gene whose effect is to increase the frequency of small spores.

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