THE EFFECTS OF SUPPLEMENTATION AND PLATING DENSITIES ON APPARENTLY ABERRANT MEIOTIC AND MITOTIC SEGREGATION IN *ASPERGILLUS RUGULOSUS*¹

D. O. COY² AND R. W. TUVESON

Department of Botany, University of Chicago, Chicago, Illinois

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IT was recently observed that when random ascospores from hybrid cleistothecia were analyzed, markers conferring nutritional competence were recovered in a significantly higher proportion than would be expected on a random basis (COY and TUVESON 1964a). In this same investigation, non-nutritional markers such as those controlling conidiation and color of conidia were recovered with the expected frequencies. The present paper presents evidence which suggests that the distorted ratios for nutritional markers are not the result of aberrant mechanism(s) of segregation or the formation of "partial hybrid" cleistothecia (PONTECORVO, ROPER, HEMMONS, MACDONALD and BUFTON 1953; COY and TUVESON 1964b), but rather may be explained on the basis of a reduced "fitness" of specific mutants. Further, evidence is presented which suggests that mutant alleles which are not recovered at random among random ascospores will not be recovered among somatic segregants.

MATERIALS AND METHODS

The source of the isolate of *Aspergillus rugulosus* and the techniques for the selection and identification of mutants have been given (COY and TUVESON 1964a).

The minimal medium was Czapek's solution agar (Difco). Supplements were added in concentrations given by TUVESON and GARBER (1959). The complex medium used was potato dextrose agar supplemented with 5 percent yeast extract (Difco). For the dry weight experiments, the agar was filtered out of the complex medium, and the liquor was then supplemented as required and sterilized. Thirty-ml volumes of this liquid were pipetted into Petri dishes and inoculated with approximately 100 conidia per plate. After 4 days incubation, the resulting mycelium was collected on dried, weighed filter paper. The paper plus mycelium was then dried for 24 hr at 60°C and reweighed.

The following mutants were used: γ , yellow conidia; w , white conidia; *lys-1*, lysineless; *arg-1*, arginineless; *iso-1*, isoleucineless; *paba-1*, para-aminobenzoic acidless; *prol-1*, prolineless; *ade-1*, adenineless; NP-1, inability to synthesize an unidentified antimicrobial agent.

All cultures were grown at 37°C.

Presumptive heterocaryons were established between those strains indicated in Tables 1, 2, and 4 as described by COY and TUVESON (1964a). After 9 to 14 days incubation, it was possible to remove mature cleistothecia from the mycelial mat. Methods for the detection of hybrid cleistothecia were essentially the same as those employed for random ascospore analyses in A.

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² Present address: Department of Biology, Millikan University, Decatur, Illinois.

nidulans (PONTECORVO *et al.*, 1953). The total contents of a cleistothecium scored as hybrid on the basis of multicolored colonies or varied colonial morphology along the test streak were plated onto complex medium employing 0.1 ml of inoculum per plate. The number of spores per plate varied with the dilution used. Germination of spores was almost complete as judged from hemocytometer counts and known dilutions. Isolations were made from individual colonies and these were formed into grids of 26 colonies for the determination of nutritional requirements.

To detect the somatic diploid, mycelial fragments of a mat formed between the strains indicated in Table 4 were plated onto minimal medium containing 0.02 mg adenine per ml. The fragments gave rise to a slow-growing presumptive heterocaryon from which a rapidly growing fan of mycelium producing green conidia emerged. The fan was transferred to minimal medium supplemented with adenine and to complex medium. After 7 days on complex medium it was possible to obtain a suspension of conidia from the presumed somatic diploid. The presumed diploid conidia were counted and plated onto complex medium and to complex medium supplemented as indicated in Table 4. The resulting green colonies exhibited a number of single heads, or spots with a few heads or sectors producing white or yellow conidia. The recovery of yellow and white conidial heads, together with conidial size, suggests that the green strain was indeed a somatic diploid. Conidia from the yellow or white spots or from the sectors were streaked onto complex medium supplemented with the requirements of both parental strains. Supplementation of the complex medium insured abundant conidiation of the colonies produced by the conidia from the various sources. Purified colonies were then picked and formed into grids of 26 colonies on supplemented complex medium. Not more than one white and one yellow segregant were selected from each diploid colony. The requirements of conidia from each colony were determined by replicating the colonies to minimal medium plus all the nutrilites required by the parentals, to complex medium, to minimal medium, to minimal medium plus adenine, and to a series of minimal medium plates lacking in turn one of the nutrilites required by the parental strains. Slides were made of the conidia from each segregant colony to determine by diameter measurements if the colonies were haploid or diploid.

RESULTS

Table 1 typifies observations showing apparently aberrant segregation of markers. It was observed that non-nutritional conidial color markers segregate in the expected manner; white being epistatic to yellow, the results indicate an extremely close approximation to the expected 1:2:1 ratio of yellow : white : green. It is the nutritional markers, here lysine and arginine, which apparently segregate abnormally, and show a highly significant deviation from the expected 1:1.

Random ascospores from a single cleistothecium were plated on supplemented and unsupplemented complex medium at three different plating densities (Table 2). In this experiment lysine-competent strains were recovered in excess on unsupplemented complex medium irrespective of the plating density employed.

TABLE 1

Apparently aberrant segregation among random colonies from the cross:
 $y^+ w^+ lys^-1 arg^-1 \times y^+ w^+ lys^-1 arg^-1$

	yellow	white	green	lysine ⁻	lysine ⁺	arginine ⁻	arginine ⁺
Observed	48	105	53	73	123	13	183
Expected	51.5	103.5	51.5	98	98	98	98
	(not significant)			(highly significant)		(highly significant)	

Spores were plated on potato dextrose agar plus 0.5 percent yeast extract.

TABLE 2

*Number of non-mutant alleles recovered at several loci among random products from a single hybrid cleistothecium**

Ascospore plating medium	Mean no. of ascospores plated	Total no. of colonies analysed	Wild-type alleles recovered at each locus					
			γ^+	<i>iso</i> ⁺	<i>pab</i> ⁺	<i>prol</i> ⁺	<i>lys</i> ⁺	NP-1 ⁺
Potato dextrose agar								
+ 0.5% yeast extract	76.5	204	97	114	101	103	140**	89
	35.8	205	104	109	104	112	136**	96
	20.1	205	114	103	95	97	128**	89
Potato dextrose agar								
+ 0.5% yeast extract								
+ 1.0 mg/ml lysine	88.5	206	111	105	105	106	105	92
	36.1	203	107	102	89	130**	119	97
	18.3	204	102	116	82	132**	111	105
* Cross:								
	Strain 1	γ^+ $\frac{5}{isol-1}$	$\frac{pab-1}{+}$	$\frac{30}{+}$	$\frac{lys-1}{+}$	$\frac{NP-1}{+}$		
	Strain 2	$\frac{+}{+}$	$\frac{+}{prol-1}$	$\frac{+}{+}$	$\frac{+}{+}$	$\frac{+}{+}$		

** Deviation from 1:1 is highly significant ($P < .01$).

In addition, the recovery of the lysine-dependent strains increased on supplemented complex medium irrespective of plating density. In a single instance on supplemented complex medium (36.1 plating density), an excess of lysine-competent strains was observed. However, this deviation from the expected 101.5 lysine-competent strains is of borderline significance ($0.05 > P > 0.01$) and probably represents a random event.

The results of the experiment presented in Table 2 suggest an unexpected interaction between the lysine in the supplemented complex medium and the growth of the proline-deficient strains. The recovery of an excess of proline-competent strains is highly significant at plating densities of 36.1 and 18.3. It should be noted that no such excess was observed at a plating density of 88.5. It should also be observed that *paba-1* strains are recovered in greater numbers than one would predict on a random basis at plating densities of 36.1 and 18.3 on supplemented complex medium. This result would, however, be anticipated since *prol-1* and *paba-1* are linked and came into the cross in repulsion.

The effects of supplementing the complex medium on growth of the same nutritional mutants were investigated. Dry weights of mycelia were determined (Table 3). The green, lysine-deficient mutant (γ^+ , *lys-1*) was stimulated by the addition of lysine to the medium. The difference in dry weight of mycelium obtained when the lysine-deficient mutant was grown in supplemented and unsupplemented complex medium is statistically significant, implying that the complex medium is deficient in lysine. Table 3 further suggests that the isoleucine-deficient strain is stimulated by the addition of isoleucine to the medium. The *paba-1* mutant is not affected by supplementation of complex medium with either isoleucine or lysine. The total dry weight decreased significantly as a result of supplementation only when the green, *prol-1* strain was grown on complex medium supplemented with lysine.

TABLE 3
Dry weight determinations

	Medium								
	PDA + YE	PDA + YE + 0.4 mg/ml iso				PDA + YE + 0.5 mg/ml lys			
	Dry weight*	Dry weight*	d.f.	t value†	P	Dry weight*	d.f.	t value†	P
green prototroph	0.295	0.304	15	3.87	<.01	0.299	14	1.30	>.10
green, <i>lys-1</i>	0.079	0.085	7	2.71	<.05	0.113	8	11.49	<.001
yellow, <i>iso-1</i>	0.264	0.276	8	0.14	>.001	0.261	8	0.49	>.10
green, <i>paba-1</i>	0.263	0.260	8	0.14	>.10	0.265	8	0.76	>.10
green, <i>prol-1</i>	0.252	0.250	7	0.02	>.10	0.240	8	7.10	<.001

* Mean dry weight in grams, based on five to nine replicates.

† As compared with mean values on standard medium, PDA + YE.

Table 4 presents results of an analysis of segregants obtained from a somatic diploid carrying markers which had been employed in meiotic analyses. All the diploid segregants selected on the basis of color from supplemented complex medium were isoleucine and lysine independent. Four diploid segregants producing white conidia and requiring isoleucine and adenine were isolated from unsupplemented complex medium. One diploid yellow, isoleucineless, lysineless, adenineless segregant was obtained from a diploid colony growing on complex medium. Segregation of the color markers was as expected, white being recovered twice as frequently as yellow on supplemented and unsupplemented medium. None of the haploids were both yellow and isoleucine-deficient. Among the haploid segregants the isoleucine mutant was recovered at random. It is obvious that

TABLE 4
*Analysis of haploid segregants obtained from a somatic diploid**

	Medium on which spores were plated†			
	Potato dextrose agar + 0.5% yeast extract		Potato dextrose agar + 0.5% yeast extract + 1 mg/ml lys, iso	
	Observed	Expected	Observed	Expected
<i>w</i>	29	28.4	26	30.0
<i>γ</i>	15	14.7	19	15.0
<i>iso</i> ⁻	26	22.0	20	22.5
<i>iso</i> ⁺	18	22.0	25	22.5
<i>lys</i> ⁻	9	22.0	17	22.5
<i>lys</i> ⁺	35***	22.0	28	22.5
Total segregants analyzed				
haploid	44		45	
diploid	40		42	

* Genetic constitution of somatic diploid.

Strain 1	<i>y</i>	+	<i>lys-1</i>		<i>ade-1</i>	+
Strain 2						
	+	<i>iso-1</i>	+		<i>ade-1</i>	<i>w</i>

† Plating density approximately five spores per plate.

*** Deviation from 1:1 highly significant ($P < 0.0005$), all other ratios not significantly different from 1:1.

the lysine marker was not recovered with a similar 1:1 ratio on unsupplemented complex medium.

DISCUSSION

COY and TUVESON (1964a) determined that markers for conidial versus non-conidial and white versus green conidial color segregated in the expected manner, giving an acceptable 1:1 ratio in each case. However, the 1:1 hypothesis had to be rejected for *lys-1* which consistently gave an excess of lysine-competent strains on complex medium. In all crosses investigated to date, the lysine-dependent strains (*lys-1*) were recovered at significantly reduced frequencies when plating was done on unsupplemented complex medium, although other markers yielded the expected 1:1 ratio. We suggest that the lysine-deficient strains were at a competitive disadvantage based on either slow-germination or an inadequate supply of lysine.

Presumably the aneuploid strains from certain somatic diploid strains in *A. nidulans* are at a competitive disadvantage. However, KÄFER (1961) has shown that such strains are able to grow and be recovered if diploid spores are plated at low densities on complex medium (ten or fewer spores/plate). It is assumed that this recovery of aneuploid strains is due to the removal of competition from diploid strains through dilution. By analogy, it was hypothesized that the plating of ascospores at low densities might permit growth and recovery of lysine-deficient strains of *A. rugulosus*. However, as shown in Table 2, it is the level of lysine supplementation and not the plating density that limits their recovery. The difference in dry weight of mycelium obtained when the lysine mutant was grown in supplemented and unsupplemented complex medium is statistically significant, also implying that the complex medium is deficient in lysine.

The significant excess of proline-competent strains isolated at plating densities 36.1 and 18.3 (Table 2) suggests an inhibitory effect of lysine on *prol-1*. Table 3 indicates that supplementation of complex medium with lysine has an inhibitory effect on proline-dependent strains. The failure of proline-competent strains to occur in excess at plating density 88.5 (Table 2) is probably due to rapid depletion of lysine by the growth of many lysine-requiring strains. GRIGGS (1958) has demonstrated that plating high concentrations of auxotrophic *Neurospora* conidia suppresses certain phenotypes. PONTECORVO (1953) reported that arginine intake or utilization by an *A. nidulans* arginine-requiring strain is competitively inhibited by exogenous lysine.

Table 2 results suggest the prediction that the recovery of the proline mutant from a somatic diploid might be determined by the concentration of lysine in the medium, and that lysine-deficient strains should be recovered from a somatic diploid at a significantly reduced frequency when plated on complex medium without lysine supplementation.

Although no somatic diploid containing the proline marker has been synthesized to date, a somatic diploid carrying the lysine marker was analyzed (Table 4). The sectors from the diploid yielded approximately equal numbers of diploid and haploid color segregants. Analysis of the haploid and diploid segregants

obtained from the somatic diploid indicates that the model explaining somatic segregation in *A. nidulans* applies in *A. rugulosus*. From the *A. nidulans* model, one would not expect to find any recombinant γ *iso-1* strains among the haploids, and none were observed. One would predict that white segregants would occur among the haploids at twice the frequency of yellow segregants, and that the isoleucine mutant should be recovered at random among the haploid segregants. Clearly, these predictions are met in the results presented in Table 4. The excess of lysine-competent strains on unsupplemented complex medium does not meet the prediction, however, but the acceptable 1:1 ratio of lysine-competent to lysine-deficient strains on lysine supplemented complex medium does so. The recovery of excess lysine-competent strains from somatic diploid colonies growing on unsupplemented complex medium conforms with the results in Table 2, where lysine-competent strains were recovered in excess when random ascospores were plated on unsupplemented complex medium. Apparently, the recovery of mutants from a diploid can be predicted using information obtained from random ascospores.

Four *w*, *iso-1*, *ade-1* segregants and a single γ , *iso-1*, *lys-1*, *ade-1* segregant were isolated from somatic diploid colonies growing on unsupplemented complex medium and were classified as diploid on the basis of spore size. Such diploid segregants would not be expected from the model proposed to explain somatic segregation in *A. nidulans* (PONTECORVO 1958; KÄFER 1961). However, haploid segregants of these types would be expected. We propose that the five unexpected diploid segregants represent misclassified haploid segregants. If this were true, the conclusions drawn from the haploid data in Table 4 would remain unaltered.

The results suggest that the composition of the medium and the nature of the mutants employed may profoundly influence linkage results based on random ascospores and somatic segregants. Nonrandom recovery of somatic segregants, which has been reported in *A. fumigatus* (STRØMNAES and GARBER 1963), *A. amstelodami* (LEWIS and BARRON 1964), and which appears to occur in *Verticillium albo-atrum* (HASTIE 1962), may be based on the nature of the medium and properties of the mutants rather than on any mechanism of somatic segregation fundamentally different from that which occurs in *A. nidulans*.

SUMMARY

Analysis of random ascospores indicate that specific auxotrophic markers are at a competitive disadvantage irrespective of plating densities on complex medium. Prototrophic recombinants may be recovered more frequently than the parental classes. This competition was investigated using supplementation and dilution techniques. Lysine-competent strains were recovered in excess on unsupplemented complex medium irrespective of the density of plating. Proline competent strains were recovered in excess at low plating densities on supplemented complex medium. For other markers in the same cross, an acceptable 1:1 ratio of mutant to wild-type alleles was obtained at all plating densities. Both plating density and level of supplementation of the medium, even "complete"

medium, are important in the recovery of auxotrophic alleles, and certain nutrients may inhibit the growth, and therefore the recovery, of specific mutant alleles among random ascospores. Selection appears to play a role also in the isolation of segregants from somatic diploid colonies, and the recovery of mitotic recombinants from a somatic diploid can be predicted using information from random ascospores.

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