A SYSTEM OF CYTOPLASMIC VARIATION IN *ASPERGILLUS NIDULANS*

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1. INTRODUCTION

ERRATIC variation in the production of perithecia by the homothallic Ascomycete *Aspergillus nidulans* (Eidam), Winter, has been observed in both mycological (Smith, 1957) and genetical studies (Mahoney and Wilkie, 1958). These general observations have shown that the production of perithecia is uncertain and spasmodic. Some strains are regularly sexual while others produce only conidia. Still others give the imperfect form alone through a series of cultures and then suddenly produce abundant perithecia.

Irregular variation in perithecium production might well be of selective advantage in such a homothallic fungus provided the variation is under genotypic control. In this way the organism may combine the ability to exploit asexuality with the versatility of sexual reproduction. This situation could, of course, depend upon a system of forward and back mutation. Indeed, in fungi, where the distinction between somatic and germinal tissue does not exist, a large number of nuclei are potentially capable of providing inocula differing from the parent material. Mitotic recombination and segregation (Pontecorvo, 1956) might also be important in determining irregular variation. An alternative, and perhaps more plausible explanation depends upon the fact that the cytoplasm of the Ascomycetes plays an important part in determining phenotype (Jinks, 1956, 1958). This is all the more plausible since Jinks has found that it is possible to eliminate the sexual stage in *Aspergillus glaucus* by adjustment of the cytoplasm.

Experiments were, therefore, undertaken to determine the nature and control of variation in the sexual phenotype of *A. nidulans*. Three homokaryotic lines of the fungus were used in these investigations. These lines were originally isolated from the wild but had been maintained in the laboratory for at least four years by mass hyphal transfer at 25° C. The methods of propagation and media used are standard for this organism (Jinks, 1952; Pontecorvo *et al.*, 1953). A line marked by the genes *paba1* *y*; *co* (Käffer, 1958), was also employed in the course of the investigations; this was very kindly supplied by Professor G. Pontecorvo.

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2. RESULTS

(i) *Origin of variation*

Mahoney and Wilkie (1958) observed a segregation into perithecial and non-perithecial offspring from wild-type colonies, and the frequency of non-perithecial types was higher at 37° C. than at 25° C.

TABLE 1

Mean number of perithecia in 3 wild lines, scored as total number of perithecia in 5 random fields per colony

Line	Mean at 37° C.	Mean at 25° C.
1	3.96 ± 1.71	1.56 ± 1.10
2	2.20 ± 0.84	4.00 ± 1.70
3	4.52 ± 1.33	0.84 ± 0.54

In no instance, during a number of studies at 25° C. in this laboratory, was any phenotypic variation of such magnitude observed. This applied in the three stock lines which were probably "run down" cytoplasmically by virtue of the continued hyphal propagation. A

TABLE 2

Mean and standard deviation of the number of perithecia in replicate selections for high and low density of perithecia at 37° C. in line 2. Each mean is based on 5 random fields for 5 colonies from each of duplicate plates

Selection	Generations											
	Parent		1		2		3		4		5	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Low 1	2.2	0.8	1.2	0	6.3	1.8	3.4	2.3	7.7	1.2	9.4	1.3
2			1.2	0.9	6.6	1.7	7.1	2.7	9.1	2.0	10.2	1.9
High 3			3.2	3.5	9.2	2.6	5.7	1.9	16.8	0.3	18.1	1.9
4			3.2	4.0	7.3	2.6	10.4	1.0	17.8	1.9	18.2	2.8

comparison was made, therefore, between the conidial offspring of the apparently stable lines at both 37° C. and 25° C. The variation observed in the density of perithecia on each colony was quite large but in none of these colonies were perithecia absent (table 1).

An attempt was then made to establish wholly asexual lines by selection of the least sexual colonies derived from a sample of conidia. Line 2 proved most satisfactory for this purpose at 37° C. Conidia were taken from the two colonies with the lowest density of perithecia and also from the two colonies with the highest density of perithecia.

The response to applying such selection at a pressure of 10 per cent. over five generations is shown in table 2 and fig. 1. From these it can be seen that while the response of the high lines was marked, that for the low lines was not even in the direction of selection. Evidently the cytoplasm varies as it was possible to increase the variation in perithecial density. But, it was not possible to establish asexuality in the way used by Jinks (1956) in *A. glaucus*.

A heterokaryon test to establish the nuclear or cytoplasmic nature of the change in perithecial density was not undertaken but, by

FIG. 1.—The progress of selection; mean number of perithecia in replicate high (3 and 4) and low (1 and 2) selections for density of perithecia. Scored as mean number of perithecia in 5 random fields from 10 colonies.

analogy with Jink's work, the latter is regarded as more probable. This assumption was supported by the behaviour of ascospores from the high and low lines. Determinations of the perithecial density of colonies grown from these ascospores were uniform, as might be expected if the difference between high and low lines was cytoplasmic. This behaviour may be contrasted with the result of the ascospore propagation of a nuclear determined difference (see Section iii). The results of this experiment indicate that, in respect of perithecial density, conidia cannot always maintain the cytoplasm in the state to which it has been reduced by hyphal propagation.

The significance of this result will be discussed later.

(ii) *Morphological variants*

Each stage of selection represented a test of the conidial progeny of wild-type colonies which exhibited differences in the density of perithecia. When the second generation of selection was reached some of the colonies grown from samples of conidia gave morphological variants amongst the apparently wild-type colonies, these variants will be referred to as A to D respectively. A description of the variants and their frequency is given in table 3. Similar variants appeared in all the later stages of the selection experiment (table 4) and were always excluded from the selection itself.

TABLE 3

The morphological variants amongst the conidial offspring, grown at 37° C. and 25° C., from colonies with the highest and lowest mean number of perithecia at 37° C.

Parent	Conidial offspring			
	At 37° C.		At 25° C.	
	Total colonies recovered	Non wild-type	Total colonies recovered	Non wild-type
Low 1	15	2A	6	0
2	87	1A, 6B	55	0
High 3	63	1A, 2C	65	0
4	38	1A, 1D	28	0

A-type colonies—a mosaic in appearance including areas of growth covered almost entirely with perithecia or, alternatively, with conidial heads. These colonies also exhibited a high density of sterile hyphae giving an overall woolly-white appearance. B-type colonies—few perithecia and many sterile hyphae. C-type colonies—very small and with a very high density of perithecia. D-type colonies—wild type in appearance with the additional feature of a brown ring of pigment in the medium around the margin of the colony.

In generation four of the selection experiment several representatives of all four variants were produced at 37° C. Samples of conidia were taken from one A, three B, one C and one D colony at this temperature and grown on minimal medium at both 37° C. and 25° C. The phenotypes of the resulting colonies is given in table 5. With the exception of that from A, the progenies from all the variants showed segregation. The A variant which produced only wild-type offspring was itself seen as one of the products of the B variant.

These variants initially arose only at 37° C. and would easily have been observed had they arisen at 25° C., indeed, when subsequently they did arise at this temperature no difficulty was experienced in distinguishing them. The absence of the variants in the first place at 25° C. can only indicate that their origin was dependent upon temperature. Segregation amongst the progenies even after removal to 25° C.

shows that the change persists long enough to have an effect during conidium production at 25° C.

The segregation of the B and C variants was investigated in more detail:

(a) *Segregation of the B variant.* In two instances out of the three examined, the conidial progeny from the B variants was composed of

TABLE 4

The distribution of variants amongst the conidial offspring from 3 generations of sampling wild-type colonies with the highest and lowest mean number of perithecia. Parent wild-type colonies selected at 37° C. Scorings of offspring at 37° C. and 25° C.

Parent	Conidial offspring						
	At 37° C.			At 25° C.			
Sample no.	Total colonies recovered	Wild-type	Variants	Total colonies recovered	Wild-type	Variants	
3	Low 1 2	83 18	83 18	0 0	80 24	79 22	1C 2C
	High 3 4	270 83	264 79	2B, 2C 3A, 1C	276 92	276 89	0 1A, 1C, 1D
4	Low 1 2	158 42	156 40	2A 2A	115 67	112 67	1A, 2D 0
	High 3 4	30 252	28 240	2A 2A, 3B, 1C, 1D	24 101	24 100	0 1D
5	Low 1 2	172 101	171 101	1B 0	153 79	152 79	1B 0
	High 3 4	117 71	117 71	0 0	118 93	117 93	1B 0

three distinct classes at both 37° C. and 25° C. The first class consisted of phenotypically wild-type colonies, the second class contained colonies of parental type and the third class was phenotypically indistinguishable from the A variant. Conidia were taken from the wild-type and A type classes at both temperatures and tested at 37° C. and 25° C. The phenotypes of the resulting colonies are given in table 6. The progenies from the A type variants fell into the three classes unlike the initial A progeny tested (table 5), the difference probably depending upon the small sample taken in the latter case. No samples of conidia were taken from B type colonies on the assumption, which subsequently proved to be valid, that they would continue to segregate into three classes. In contrast to the conidial progenies, mass hyphal transfers gave

wild-type growths from all classes. Similarly sexual progenies from the A and B variants in line 2 gave only wild-type progeny.

(b) *Segregation of the C variant.* The conidial progeny from the C variant was composed of two classes, at both 37° and 25° C. In one

TABLE 5
The phenotypes of colonies obtained from conidial samples of A, B, C, and D type variants and grown at 37° C. and 25° C.

Parent		Phenotype of conidial offspring				
		Total colonies recovered	Wild-type	A-type	Parental	Very few or no Perithecia
A	37°	16	16	—	—	—
	25°	11	11	—	—	—
B ₁	37°	24	9	8	7	—
	25°	35	16	7	12	—
B ₂	37°	79	24	17	38	—
	25°	108	52	8	48	—
B ₃	37°	72	69	3	—	—
	25°	70	70	—	—	—
D ₁	37°	78	43	—	35	—
	25°	206	186	—	20	—
C	37°	75	41	—	—	34
	25°	117	13	1	—	103

TABLE 6
The phenotypes of colonies derived from samples of conidia of apparently wild-type and A-type colonies from the segregation of a B-type variant.

Parent Colony	Phenotype of conidial offspring								
	At 37° C.				At 25° C.				
	Phenotype	Total colonies recovered	Wild-type	B-type	A-type	Total colonies recovered	Wild-type	B-type	A-type
From 37° C.									
Wild-type	158	158	0	0	119	117	2	0	
A-type 1	61	43	4	14	73	52	2 + 1D	18	
A-type 2	61	59	1	1	55	49	2	4	
From 25°C.									
Wild-type	255	255	0	0	208	207	0	1	
A-type 1	138	34	17	87	71	18	33	20	
A-type 2	62	13	11	38	61	7	44	10	

class the colonies were indistinguishable from wild-type, while in the other there were few or no perithecia. The proportions in the two classes were different at the two temperatures. The C variant was itself absent from all but one of these progenies. Conidial samples were taken from both classes and proved to be pure breeding in this and subsequent generations.

All the variants obtained during the progress of the selection experiment gave wild-type progeny after mass hyphal transfer. This prevented the application of a heterokaryon test to determine whether the segregating differences were of a nuclear or a cytoplasmic origin. However, even in the absence of such proof, it seems difficult to ascribe the continued segregation of differences through uninucleate conidia to anything except a non-nuclear mechanism. Support for this interpretation follows from the fact that, in a number of cases, segregations of this kind must be attributed to the sorting out of cytoplasmic elements (Arlett, 1957; Jinks, 1959*b*). Clearly, it is often not easy to disentangle the effects of nucleus and cytoplasm (Roper, 1958), in the present case, although proof of the cytoplasmic origin of these variants is not irrefutable, it is regarded as most probable and is presented as such.

TABLE 7

Conidial progeny from 2 heterokaryons between a sterile arginine requiring line and a line requiring p. amino benzoic acid

Heterokaryon	Conidial offspring		
	Total colonies recovered	<i>paba1 y; co</i> fertile	arginineless sterile
1	444	429	15
2	190	188	2

(iii) Nuclear control of sexuality

That the A-D variants were cytoplasmic in origin is put beyond any doubt by the sharp contrast when their behaviour, described above, is compared with that of a fifth variant which depends on a stable nuclear change. This variant appeared as a white woolly sector completely devoid of both perithecia and conidia in a stock line requiring arginine. Its rate of growth on medium supplemented with hydrolysed casein was 7.59 mm. per day at 25° C., as compared with 4.35 mm. per day for the strain which gave rise to it. The nuclear nature of the change was established unambiguously by a heterokaryon test. Two heterokaryons were made between the sterile *arginine* line and the line *paba1 y; co* which required p. amino-benzoic acid for growth. These heterokaryons are, of course, capable of growth on a minimal medium. The classification of their conidial progeny is shown in table 7. It will be seen that the relationship of fertility and sterility to the marker genes was unchanged by passage through the heterokaryon, thus establishing the difference as nuclear.

These progenies also show an excess of the *paba1 y; co* nuclei in the heterokaryon. A rate of growth comparison showed no differences between *paba1 y; co* colonies from the heterokaryon and control

colonies which had not been in heterokaryotic association. Hence it was concluded that there was no influence of the sterile *arginine* nuclei on the *paba1 y; co* nuclei.

There was, however, some effect of association with *paba1 y; co* on sterile *arginine* progeny. A close examination of the seventeen sterile *arginine* colonies showed that while fourteen were completely sterile three (two from heterokaryon 1 and one from heterokaryon 2) produced a few conidia and perithecia; these numbered less than ten on each colony. Six conidial heads were separately picked off one of these colonies and plated on casein supplemented medium at 25° C. Amongst a total of three hundred and forty-two colonies recovered, two had a few conidial heads but no perithecia, the remaining sister colonies being entirely sterile. Four heads from another of the three colonies gave a total of five hundred and two daughters most of which developed a few conidial heads and perithecia, the number ranging up to 10.

Four perithecia were also crushed from this second colony and the ascospores plated on casein supplemented medium at 25° C. The progeny from each perithecium fell into two classes. Four-fifths were similar to the conidial progeny, both in general appearance and range of variation, while the remainder were completely sterile and, therefore, similar to the original isolate.

Evidently, therefore, the expression of the mutant gene responsible for the sterility in the *arginine* isolate is capable of modification by the cytoplasm for, following association with the cytoplasm of the *paba1 y; co* strain the degree of sterility varies.

3. DISCUSSION

Two points of significance emerge from these observations. The first is the relation of cytoplasmic variation to temperature. The second is the evidence for cytoplasmic restandardisation in the conidia. The results show that variation is more frequent at 37° C. than at 25° C. That this variation is cytoplasmic in origin is suggested by its failure to pass through ascospores, in contrast to the sterility determined by the nucleus in the *arginine* line. The variation might, of course, be interpreted as resulting from a higher mutation rate at 37° C. than at 25° C. though it seems hardly likely that an increase of such magnitude could be brought about solely by mutation. The more probable explanation is that the increase in variation is due to the behaviour of the cytoplasm being less regular at 37° C. This implies that the cytoplasm is not subject to the precise mechanical control which ensures that nuclear genes reproduce in step with the cell. The regular behaviour at 25° C. must mean, however, that the reproduction of cytoplasmic elements is more or less in step with cell division. Raising the temperature produces the irregularity because the rate of reproduction of the cytoplasm changes. The mechanical and numerical basis of this variation is emphasised in the threshold of the effect which is partly reversible in conidia and totally reversible in the ascospore.

Similar behaviour has also been observed in yeast (Ycas, 1956; James and Spencer, 1958), *Paramecium aurelia* (Preer, 1948) and in tumours induced by *Agrobacterium tumefaciens* (Braun, 1954). In yeast the phenotypes which depend for their origin on an unequal distribution of cytoplasmic particles are more frequent at higher temperatures. Moreover, the multiplication rate of these particles is not wholly dependent upon the rate of division of the cells. The discrepancy is particularly well demonstrated in *P. aurelia* where the frequency of *kappa* cytoplasmic particles varies according to the relative rates of cell division and particle reduplication and may be increased or reduced, at will, by changes in the feeding regime. It is also possible to eliminate the causative principle from tumours induced initially by *A. tumefaciens* by the induction of a high rate of cellular proliferation. Thus disturbances in the equilibrium of cytoplasmic systems would appear to be of widespread occurrence.

The second significant observation follows from the attempt to change the density of perithecia on the fungus by a cytoplasmic selection. Variation does, apparently, occur in the cytoplasm for this character since it was possible to raise the density of perithecia. It was not possible, however, to maintain the density of perithecia at a level to which it had been reduced by hyphal propagation. Hence it would seem that the conidia are incapable of maintaining a cytoplasm of a constitution which regularly exists in hyphæ. Jinks (1956) has shown in *A. glaucus* that a cytoplasm which can pass through conidia cannot pass through ascospores. This is also true of the present stock of *A. nidulans* but, whereas in *A. glaucus* the cytoplasm giving complete sexual sterility will pass through conidia, this is not always true of *A. nidulans*.

Evidently, therefore, conidial propagation in *A. nidulans* has something of the property of the ascospore propagation shown by both species (Mather and Jinks, 1958): it imposes a limit to the range of cytoplasmic variation that can be transmitted. This provides evidence of some, albeit weak, restandardisation of the cytoplasm by the conidia in *A. nidulans*.

The process of restandardisation is seen in a special relation to the nuclear constitution in the sterile *arginine* strain. Initially the mutant gene gave complete sterility but this was modified by association with a mixed cytoplasm, showing how the nucleus and cytoplasm interact in producing the fungal phenotype. Passage through neither the conidium nor ascospore reduces the cytoplasm to complete uniformity in respect of the mutant character. It would seem, therefore, that the process of restandardisation, whether in respect of conidia or ascospores, is related to the wild-type phenotype of the fungus.

The contrast between *A. nidulans* and *A. glaucus* is perhaps of some evolutionary interest. The variation of cytoplasm in hyphæ, conidia and ascospores may be represented as in fig. 2. A wide curve represents the range of cytoplasmic variation in hyphæ, only cytoplasm in the

narrow central portion being transmitted through ascospores. In *A. glaucus* the range of cytoplasm passing the conidia would seem to be similar to that which can exist in the hyphæ but in *A. nidulans* the range appears to be intermediate between hyphæ and ascospores (fig. 2). This is not to say that differences do not exist in *A. glaucus* for it has been shown (Briault, 1956; Jinks, 1959a) that, in relation to such systems as adaptation to sugars and poisons, conidia have a narrower range than hyphæ.

The significance can be seen in another way, for *A. glaucus* cannot be propagated indefinitely by conidia or hyphæ alone. The same

FIG. 2.—The relationship between the mode of propagation and the limits of cytoplasmic variation in *Aspergillus nidulans*.

ageing phenomenon in *A. nidulans*, however, is certainly much slower; and is probably related to a narrower range of variation in the hyphæ and to the effect of the conidia in restoring cytoplasmic balance at least partially. Clearly a fungus like *Aspergillus niger* which is effectively imperfect could not maintain itself if its cytoplasmic balance changed in the way shown by *A. glaucus*. Here only the intervention of sexual reproduction or heterokaryosis will restore the necessary level of balance after continued asexual propagation. Thus, in so far as there is a tendency towards restoration of the cytoplasmic balance in the conidia of *A. nidulans* it may well be that it shows a step towards a feature which is an essential characteristic of an imperfect fungus. This suggests, moreover, that the series *A. glaucus*, *A. nidulans*, *A. niger* is illustrative of the cytoplasmic requirement in the progress to the imperfect fungus.

4. SUMMARY

1. Three lines of *A. nidulans* which had been propagated by hyphae for four or more years at 25° C. produced morphologically stable conidial offspring at this temperature.

2. Selection amongst the least and most sexual of these wild-type colonies in line 2 at 37° C. showed:

(a) The lower limit in sexual phenotype could not be maintained on recourse to conidial propagation, indicating a conidial limit to cytoplasmic variation, and

(b) That a range of morphological variants of cytoplasmic origin were induced at a rate of approximately 2 per cent. of the colonies recovered.

3. An instance of nucleo-cytoplasmic interaction in the control of fertility was demonstrated in the behaviour of the conidial progeny from a heterokaryon between a sterile and a morphologically wild-type line.

4. The mechanism for the induction of the cytoplasmic variants is suggested as having arisen out of a possible inequality between the rate of cell division and the rate of division and distribution of cytoplasmic particles.

5. On the evidence of cytoplasmic restandardisation by the conidia of *A. nidulans* it is suggested that the series *A. glaucus*, *A. nidulans*, *A. niger* is illustrative of a cytoplasmic requirement for restandardisation in the progress to the imperfect fungi.

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