HETEROKARYON COMPATIBILITY OF UNRELATED STRAINS IN THE *ASPERGILLUS NIDULANS* GROUP

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1. INTRODUCTION

HETEROKARYOSIS, the occurrence of genetically different nuclei within a common cytoplasm, is not only a system of somatic flexibility in wild fungi (Jinks, 1952*b*) but it is also an essential first step in a number of processes which lead to the recombination of nuclear genes and reassortment of cytoplasmic differences in fungi. For example, it is a necessary preliminary to the parasexual cycle in both sexual and imperfect fungi and to outcrossing in homothallic species (see Pontecorvo, 1956 and Roper, 1962 for reviews). The formation of a heterokaryon between different strains involves anastomosis of their hyphæ so that both the nuclei and the cytoplasms of the strains are brought together in the same cell lineage. Thus, in those species having no other mechanism for the transfer of genetic material from one strain to another, the ability of different strains to form heterokaryons, that is, their heterokaryon compatibility determines whether heritable differences, transmitted by the nucleus or the cytoplasm, can be exchanged. Consequently, it may indicate whether variation within such species arises by recombination and reassortment rather than by direct mutation alone.

The present paper is the first of a series in which the heterokaryon compatibility of a number of wild strains of *Aspergillus nidulans* and allied species will be described and related to the morphology and geographical derivation of the isolates involved. *A. nidulans* has been chosen as the test organism because it is known to form heterokaryons and undergo both sexual and parasexual recombination in the laboratory (Pontecorvo, 1953).

2. MATERIALS AND METHODS

Twenty-five isolates, most of them *A. nidulans*, were obtained from a variety of sources. Relevant information on their origins is included in the list of strains given in table 1. Strains acquired from culture collections are named and described in accordance with the information given in the appropriate culture lists; those from British universities are given the designations supplied by the donors; Birmingham isolates were identified from IMI type culture 16040 and both strains of *A. nidulans* var. *echinulatus* were identified by Mr J. J. Elphick of the CMI staff.

All isolates were maintained in the laboratory at 25° C. on Czapek minimal medium (MM) containing New Zealand agar supplied by G. T. Gurr Ltd. of London.

Auxotrophs were maintained on the complete medium (CM) described by Pontecorvo (1953).

To obtain measurements of growth-rate, sporulation and sexuality, all cultures were grown on 20 ml. MM in 9 cm. Petri dishes at 25° C. Each growth-rate

TABLE 1

List of strains

(CMI = IMI = Commonwealth Mycological Instit., Kew; CBS = Centraalbureau voor Schimmelcultures, Baarn ; NRRL = Northern Utilisation Research Branch, U.S.D.A., U.S.A.)

Strain no.	Isolation source	Species	Immediate source
1	Airborne, B'ham, 1954	*A. nidulans*	B'ham, 1954
2	Airborne, B'ham, 1954	*A. nidulans*	B'ham, 1954
3	Airborne, B'ham, 1954	*A. nidulans*	B'ham, 1954
4	? ?	*A. nidulans.* CBS-CMI 16040	CMI 1960
5	Cotton lint, Bombay, 1947	*A. nidulans.* IMI 16643	CMI 1960
6	Tobacco, S. Rhodesia	*A. nidulans.* IMI 31386	CMI 1960
7bi⁻	Induced mutant	*A. nidulans.* NRRL 194 derivative	Glasgow, 1958
7y pro⁻	Induced mutant	*A. nidulans.* NRRL 194 derivative	Glasgow, 1958
8	Soil, B'ham, 1954	*A nidulans var. echinulatus*	B'ham, 1954
10	? ?	*A. nidulans* mut. *fertilior* Henrard	Baarn, 1961
11	? ?	*A. nidulans* var. *Nicollei* Nicollet et Pinoy	Baarn, 1961
12	? ?	*A. quadrilineatus* IMI (number unknown) *	Aberystwyth, 1961
13	? ?	*A. nidulans*	Aberystwyth, 1961
14	? ?	*A. nidulans*	Manchester, 1961
15	Soil, Suffolk, 1947	*A. nidulans.* IMI 28644	CMI 1961
16	Gum arabic, York, 1951	*A. nidulans.* IMI 49026	CMI 1961
17	Fabric, New Mexico, 1952	*A. nidulans* var. *acristatus.* IMI 61453	CMI 1961
18w	Culture mutant	*A. nidulans* var. *albus.* IMI 16037	CMI 1961
19	Soil, Baghdad 1958	*A. quadrilineatus.* IMI 72731 †	CMI 1961
20	? ?	*A. nidulans* var. *latus.* IMI 74181	CMI 1961
21	Gum arabic, York, 1951	*A. nidulans* var. *latus.* IMI 45625	CMI 1961
22	Soil, Baghdad, 1958	*A. quadrilineatus.* IMI 72733 †	CMI 1961
23	? Buenos Aires, 1950	*A. nidulans* var. *echinulatus.* IMI 61454	CMI 1961
24	? Banaras, India, 1958	*A. nidulans.* IMI 75006	CMI 1961
25	*Collybia velutipes*, B'ham, 1961	*A. nidulans* var. *echinulatus*	B'ham, 1961
26	*Collybia velutipes*, B'ham, 1961	*A. nidulans*	B'ham, 1961

* Received from CMI as strain of *A. nidulans.*
† Originally catalogued as *A. nidulans.*

measurement indicates the average daily increase in colony diameter of 4 replicate colonies as measured from the 3-8 days after inoculation. Estimates of perithecial density were obtained by marking circles of 7 mm. diam. on each of 3 replicate 28-day-old colonies and counting the number of mature perithecia (sexual reproductive structures) in them ; with each colony, 2 circles were taken near the inoculum and 2 near the growing margin. The same 12 circles were transferred

into measured amounts of Tween 80 (polyoxyethylene sorbitan monooleate, a wetting agent) and the number of conidia (asexual spores) in the suspensions were estimated from haemocytometer counts.

Spore-colourm utants were obtained by ultraviolet irradiation of wild-type conidia suspended in 1 per cent. physiological saline. Irradiated samples were plated on Plunkett's medium (Plunkett, 1953) of pH 5·2 at a density of about 10^6 per Petri dish and examined after 4 days for white or yellow conidial heads.

The Plunkett medium (PM) used has the following composition ; sucrose, 50·0 gm. ; dipotassium hydrogen phosphate, 5·0 gm. ; magnesium sulphate, 2·5 gm ; asparagine, 1·0 gm. ; thiamine, 50 μ gm. ; ferrous sulphate, trace ; New Zealand agar, 15 gm. ; distilled water, 1000 ml The pH is adjusted with 1·0M hydrochloric acid.

3. RESULTS

(i) Morphological differences between isolates

The main morphological characters of each strain are listed in table 2. In addition to the three comparative criteria used other

TABLE 2

Metrical characteristics of strains grown on MM and sporulation on PM, pH 5·2 measured on an arbitrary scale from 1-6

Strain	Growth-rate mm. per day	No. conidia per mm² × 10⁻³	No. perithecia per mm²	Sporulation on PM, pH 5·2
1	5·1	36·3	0·1	1
1w₃	4·8	22·9	1·7	1
2	5·0	28·0	0·1	1
2w	4·8	21·0	0·7	1
3	5·7	16·6	0·1	1
4	3·7	53·4	0·0	1
4w	3·6	27·4	1·2	1
5	1·6	32·3	0·8	2
5w	2·2	17·3	0·5	2
6	4·8	45·3	1·2	1
6w	4·5	39·7	0·8	1
8	3·4	2·0	0·8	2
10	4·2	20·4	1·9	1
10w	3·9	17·3	1·0	1
11	4·4	8·1	0·1	1
11y	3·9	6·8	0·2	1
12	4·6	6·1	4·2	1
12y	4·8	4·1	4·0	1
13	4·9	39·7	1·4	1
13w	4·7	27·1	1·2	1
14	5·0	20·2	1·0	1
14y	4·6	19·0	1·5	1
15	2·5	1·8	0·1	6
16	1·1	0·0	2·5	6
17	3·4	0·3	1·2	6
18w	5·9	19·3	0·0	1
19	4·7	2·4	4·5	1
20	4·7	1·0	1·5	3
21	5·6	1·3	2·0	3
22	4·5	6·5	7·1	1
23	3·0	0·0	0·2	4
24	3·9	0·0	0·6	5
25	3·2	3·0	0·9	2
26	5·4	38·7	0·8	1
26y	5·5	48·4	0·7	1

N

distinguishing features, including conidiophore size and shape, intensity of spore colouration and pigmentation both within the hyphæ and secreted into the medium were obvious. Certain groups of strains were either identical or basically similar in appearance but other isolates were unique. Some of these similarities and differences are outlined below.

Strains 1, 2, 3, 6, 10, 13, 14 and 26 : basically similar to type culture IMI 16040.

Strains 1, 2 and 3 : very similar in appearance but slight differences in surface morphology.

Strains 4 and 10 : colonies identical in appearance.

Strains 8 and 25 : colonies identical in appearance.

Strains 12, 19 and 22 : colonies identical in appearance.

Strain 5 : large conidial heads ; much more aerial mycelium produced than by all other strains.

Strain 18 : very small conidial heads.

Strain 15 : colonies yellow ; yellow pigment secreted.

Strain 16 : colonies covered entirely by large perithecia.

Although the differences between our isolates of *A. nidulans* were large and indicated a very variable species, it is difficult to assess how much of this variation would have been apparent if all strains had been freshly isolated from the wild. Some strains had been maintained in culture collections for many years on a variety of media, MM, malt agar or PDA (potato dextrose agar), and some had been preserved under mineral oil. The effects of successive propagations by mass hyphal transfer, of different media and of mineral oil must have led to an accumulation of nuclear gene mutations in many isolates and for these reasons it was not possible to make any meaningful correlations between morphological characters of these strains and their isolation source. Further work, however, involving only freshly isolated material from England is now in progress and all of the 18 isolates tested so far are basically similar in appearance to type culture IMI 16040. Nevertheless, they can be distinguished on various morphological and physiological differences particularly in sporulation, sexuality, pigmentation and rate of growth.

(ii) *Effect of different media on growth and sporulation*

One of the requirements for the heterokaryon tests is a medium which promotes maximum sporulation of the strains, so a number of available media were compared using isolates 1, 8, 17 and 22 as tests strains (plate I, figs. 1-4). Maximum production of conidia was obtained using PM (pH 4·6-5·2) followed by PM (pH 6·0-7·0), 2 per cent. malt agar (pH 5·4), PM (pH 3·2-4·4), 2 per cent. malt agar (pH 4·0), CM, MM (pH 5·8) or MM with additional phosphate and sucrose, PDA (4 per cent. dextrose) and MM (pH 7·0). On PM (pH 4·6-5·2) isolates 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 18, 19, 22, 25 and 26 were almost completely conidial (plate I, fig. 3). Malt

agar (pH 5·4) gave similar results except that the strains were more sexual and conidiophores were spaced further apart (plate I, fig. 6). Sporulation of some strains was only slightly better on these two media than on MM and others remained aconidial regardless of changes in media or culture conditions (*e.g.* strain 17, plate I, figs. 1-4).

Lowering the pH of any medium increased sporulation and also affected growth-rate, pigmentation and spore-colour so that many strains easily distinguishable on MM looked identical, especially when grown on PM or 2 per cent. malt agar.   Optimal pH for growth on any medium varied both between isolates and between the spore-colour mutants and the wild-type of the same isolate.

These tests showed that the medium most suitable for distinguishing between isolates is MM containing New Zealand agar.   Even the use of Oxoid No. 3 (Spanish) agar in place of N.Z. agar in MM increased growth-rate and sporulation and often affected pigmentation.

4. HETEROKARYON TESTS

Proof of heterokaryosis can be supplied by the formation of a " balanced heterokaryon " whose growth is an improvement on the two homokaryons from which it was obtained.   Typical examples of " balanced heterokaryons " are those in which (*a*) growth on any medium is normal whereas each homokaryon grows poorly on the same medium or (*b*) growth on MM is possible whereas neither homokaryon, each having a different nutritional requirement, can grow at all on MM.   In contrast, where naturally occurring heterokaryons have been obtained from the wild, some component homokaryons have been found to differ very little indeed in rate of growth (Jinks, 1952*b*).   Both of these proofs, however, require mutant strains whose derivation is difficult and time consuming.   Even more important, by using biochemically deficient mutants a heavy selection pressure is applied which favours heterokaryon formation and this may give a false indication of the potentiality of naturally occurring strains to heterokaryotise under natural conditions.

A simpler method for detecting heterokaryons in *A. nidulans* requires only that the two component homokaryons differ by major genes affecting spore-colour.   If two parental strains have green spores and white spores respectively then a heterokaryotic mycelium formed after hyphæ of the homokaryons have anastomosed will contain nuclei from both green-spored and white-spored parents. During conidiophore formation in such a heterokaryon, both types of nucleus may be present in each vesicle but each sterigma, from which chains of conidia arise, is homokaryotic.   Hence, all conidia in any one chain have the same nuclear type and will be either green or white, but in any single conidial head both green and white spores will be present.   Thus, the production of mixed conidial heads (plate II, figs. 4-6) by a culture consisting of two homokaryons differing in

respect of spore colouration indicates that the two strains are hetero-karyon compatible. By this method, even when a mixed culture consists almost entirely of homokaryotic hyphæ, the presence of a few heterokaryons can be detected despite the fact that they are not at a selective advantage in rate of growth. Observation of hyphal anastomosis has been used as a method of testing strains (Ishitani and Sakaguchi, 1956) but this is, in itself, probably an unreliable criterion of heterokaryon compatibility since it has been shown in *Neurospora crassa* that hyphal fusion occurs readily between non-compatible strains (Wilson *et al.*, 1961).

Spore colour mutants were obtained in the way already described. With most strains, as many as 24 independent mutations resulting in a non-green spore colour were obtained in each 10^6 irradiated spores plated, but in each of isolates 1 and 2 only one spore-colour mutant (white) was recovered from more than ten times as many spores. No spore-colour mutants at all of strain 3 have been observed on the 24 plates examined. Altogether, slightly more yellow than white spore-colour mutants were seen but in nearly all strains both colours were common. Most non-white colour variants had deep yellow spores but pale yellow, pale green (grey) and olive green conidial heads also occurred in small numbers.

When testing any two isolates for heterokaryon compatibility, the green-spored wild-types and their spore-colour variants were inoculated together in all combinations involving both green and non-green conidia. For each test, both PM (pH 5·2) and 2 per cent. malt agar (pH 5·4) were used (plate I, figs. 5 and 6) except where auxo-trophic strains were involved in which case CM was used instead. To estimate the frequency of mixed conidial heads in a mixed culture, several areas of 2·5 sq. mm. in which both parental conidial types were intermingled (plate II, figs. 1-3) were selected at random. These were examined at 100 × magnification (plate II, fig. 3) and the number of mixed conidial heads (plate II, figs. 4-6) was determined as a proportion of the total number of conidial heads in the field. The mean frequency of mixed heads in a number of different areas from each of at least three mixed cultures was taken as an indication of the degree of heterokaryon compatibility of any two strains (table 3). Numerous factors are known to affect hyphal anastomosis (Raper and Fennell, 1953 ; Ishitani and Sakaguchi, 1956) and these would, in turn, affect the frequencies of mixed conidial heads. However, since mixed heads were produced readily on PM (pH 5·2) and only slightly less so on 2 per cent. malt agar (pH 5·4) these two media were used in parallel and under controlled conditions of environment (25° C.) and pH (any pH below 5·0 greatly reduced the proportion of mixed heads ; a pH of about 5·6-6·2 seemed optimal for heterokaryon formation, giving a slight increase in mixed heads, but sporulation of many strains at these pH's was poor).

The results of these tests, summarised in table 3, show that all

TABLE 3

Frequencies of mixed conidial heads in intra- and inter-strain combinations.

Spore-colour mutants

Green-spored wild-type strains	1w	2w	3†	26y	4w	10w	5w	6w	7y* pro	13w	8w	25y	11y	12y	19y	22w	14y	18w
1	0·5[20]	3·6[6]	…	0·4[3]	0	0	0	0	0	0	0	…	…	…	…	…	0	0
2	1·1[6]	4·3[20]	…	0·3[7]	0	0	0	0	0	0	0	…	0	…	…	…	0	0
3	0·2[4]	3·0[3]	…	0·1[4]	0	…	…	0	…	0	…	…	…	0	…	…	0	0
26	1·4[3]	0·1[3]	…	3·6[18]	…	…	…	…	…	0	0	…	0	…	…	…	0	…
4	0	0	…	…	1·8[16]	2·6[5]	…	…	0	0	0	…	…	0	…	…	0	0
10	0	0	…	…	3·1[5]	4·0[11]	0	0	0	0	0	0	…	0	…	…	…	…
5	0	0	…	…	0	0	4·9[13]	0	0	0	0	0	0	0	…	…	0	0
6	0	0	…	…	0	0	0	1·6[16]	0	0	…	0	…	0	…	…	0	0
*7bi⁻	0	0	…	0	0	0	0	0	3·8[11]	3·6[4]	…	…	…	…	…	…	0	…
13	0	0	…	0	0	0	0	0	4·1[4]	4·6[13]	…	…	…	0	…	…	0	0
8	0	0	…	…	0	0	0	0	…	…	5·4[9]	0·1[3]	…	0	…	…	0	0
25	…	…	…	…	…	…	…	…	…	…	0·1[3]	4·8[15]	…	0	…	…	0	…
11	…	…	…	…	0	0	0	0	0	0	…	…	3·0[10]	0	…	…	0	0
12	0	0	…	0	0	0	0	0	0	0	0	0	…	3·4[17]	3·8[3]	3·0[3]	0	0
19	0	…	…	…	…	…	0	…	0	…	…	…	…	3·9[4]	5·0[7]	3·8[3]	0	0
22	…	…	…	…	…	…	0	…	0	0	…	…	…	2·8[3]	3·5[3]	3·5[9]	0	0
14	0	…	…	…	0	…	0	0	0	0	…	…	0	0	…	0	3·9[27]	0

(Superscripts, denote number of replicate tests on which frequencies are based; w = white, y = yellow, bi⁻ = biotinless, pro⁻ = prolineless.) Some combinations, denoted by a … sign, have not been tested. A zero score means that at least 3 and as many as 6 replicate tests of that combination failed to produce a single mixed conidial head. Frequencies are given as percentages (see Text).

* This strain supplied only as an auxotroph.   † No spore-colour mutant could be derived from this strain.

wild type isolates are compatible with their own respective spore-colour mutants but few examples of inter-strain, and none of inter-species, compatibility were found.   Furthermore, the examples of inter-strain compatibility involved isolates which are either believed or are known to have a common source and should, therefore, be classed as identical or near-identical strains.   Strains 7 and 13 both came originally from Glasgow ;   strains 19 and 22 were recovered from soil samples taken from the same locality and sent together to the CMI, and strain 12 is probably a subculture of one of these since it came originally from CMI and is, like 19 and 22, a strain of *A. quadrilineatus* (a member of the *A. nidulans* group).   Isolates 4 and 10 are ex-Baarn strains and they produce colonies which are identical in appearance ;   it is suggested, therefore, that Henrard's mutant (isolate 10) is a derivative of isolate 4 or it is perhaps the same strain. This leaves only the two isolates of *A. nidulans* var. *echinulatus* and four isolates of *A. nidulans* in which compatibility of different strains is reasonably certain.   All of these were isolated in the same area, Birmingham.

5. DISCUSSION

(i) *Use of heterokaryon tests for determining species relationships*

Although every one of our wild-type strains formed heterokaryons readily with their own spore-colour variants very few of them were compatible with different strains of the same species and in all examples of inter-strain compatibility, mixed heads were much less common than in comparable intra-strain combinations.   Estimates of the frequencies with which different strains heterokaryotise may, therefore, be a measure of the genic similarity of those strains.   Thus, where the frequencies of mixed conidial heads both within and between strains are almost identical (2 and 3 ;   4 and 10 ;   12, 19 and 22) the strains concerned differ probably by only a few genes.   In other instances, where strains form heterokaryons more rarely with each other than they do with their own spore-colour mutants (2 or 3 and 26 ;   8 and 25) the differences in genotype are presumably more extensive.   On this assumption, strain 18w which arose spontaneously from the wild-type strain NRRL·194 should have been compatible with strains 7 and 13 ;   their failure to form heterokaryons is presumed to be due to an accumulation of gene mutations in the white spored strain, 18w, since it first arose because strains 13w and 18w differ substantially in many respects (see section 3 (i)).

The nature and number of genic, and perhaps also cytoplasmic, differences required to prevent, or reduce the frequency of, hetero-karyon formation between strains is difficult to assess.   Heterokaryosis between strains of *Fusarium oxysporum* (Buxton and Ward, 1962) and strains of *Aspergillus fonsecaeus* (Raper and Fennell, 1953) seems to be affected by the nature of the nuclear mutations induced in the parent homokaryons, and even single gene differences appear to determine

the presence or absence of heterokaryons. On the other hand, numerous derivatives of strain NRRL 194 of *A. nidulans*, differing by a large number of major gene mutations which arose in the laboratory, are compatible (Kafer, 1958) although the frequencies of mixed heads and ease of heterokaryon formation in the various crosses have not been compared. Recent work with English isolates of *A. nidulans* (Grindle, unpublished) has shown that there are many " compatibility groups " within which heterokaryons are formed easily but between these groups, heterokaryons occur with a much reduced frequency or not at all. It seems, therefore, that a complex system of incompatibility factors is responsible.

Initial work on English strains suggests that they are (*a*) very similar in appearance but can still be distinguished on a number of morphological characters, and (*b*) sometimes inter-strain compatible, but not always so since even isolates from the same soil sample may be incompatible. The work of Jinks (1952*a*) indicates that a similar situation exists among English strains of the *Asymmetrica* group of the genus *Penicillium*. He found heterokaryon compatibility between some, but not all, wild isolates and observed that although his isolates were similar in appearance, they could usually be distinguished on a number of morphological and physiological characters.

The ability of different strains to form heterokaryons may prove to be clinal. If so, gene flow within localised populations will be relatively unrestricted but between two spatially isolated populations it will be either interrupted or occur only gradually through other intermediary compatible strains. It is hoped that further work now in progress will show whether such gradients or clines exist in English populations of *A. nidulans*, and also reveal the nature of the incompatibility system.

The application of heterokaryon compatibility tests as an aid in fungal taxonomy and in determining species relationships is limited technically by the necessity for good sporulation media and spore-colour mutants (two different colours may be required in each strain for species with multinucleate spores) or other marked strains (morphological or biochemical-requiring mutants). As in Taschdjian and Muskatblitt's (1955) proposed system of hyphal fusion analysis, most strains, even though similar in appearance, are not compatible so the absence of heterokaryons may not imply a species difference ; the issue is complicated further when both hyphal and mating type incompatibility systems are superimposed. Conversely, compatibility of different isolates may be evidence for their close relationship so the heterokaryon technique should be useful particularly in assessing the genic similarity of morphological variants and physiological races of the same species—it has already been shown in the case of the Birmingham isolates that morphological similarities are, by themselves, not necessarily reliable indications of the degree of relationship between strains. By using mutant strains with nutritional requirements,

genetic relationships have been shown between formæ of *Fusarium oxysporum* (Garber *et al.*, 1961), between formæ of *F. oxysporum* and *Gibberella fujikuroi* (Dhillon *et al.*, 1961) and between formæ of *F. oxypsorum* and *F. solani* and *Nectria hæmatococca* (Buxton and Ward, 1962).

(ii) *Validity of different criteria for detecting heterokaryons of filamentous fungi*

The evidence for heterokaryosis as presented by various workers is of two main types, cytological and genetical. Cytological criteria are seldom used, however, and where they involve the observation of anatomosis between hyphæ of different parents the tests are unreliable for the reasons outlined in section 3.

Genetical evidence depends on two basic phenomena, segregation and complementation.

1. *Segregation.* (*a*) In species with uninucleate spores, heterokaryons derived from two homokaryotic strains which differ in the colour of their asexual spores can be detected if they produce conidiophores in which both parental spore types (distinguished by their different colours) are present side by side.

(*b*) Heterokaryons derived from two strains differing in certain measurable phenotypic characters (*e.g.* growth-rate, pigmentation, spore density) can be detected by re-extracting both homokaryotic parental strains from single conidiophores.

2. *Complementation.* (*a*) In species with multinucleate spores, heterokaryons derived from two parents, each with a different spore colour, can be distinguished if conidia with wild-type spore colouration are produced.

(*b*) In mixed cultures composed of two homokaryotic strains having (i) different nutritional requirements or (ii) different morphology, heterokaryons arising from them can be detected if they (i) can grow on a minimal medium lacking both of the specific nutrients required by the homokaryotic components, or (ii) arise as single hyphæ or sectors which grow at a different rate to that of either parent. These are usually referred to as " balanced heterokaryons ".

Our proof of heterokaryosis (method 1 (*a*)), which involves the segregation of spores with different colours in single conidiophores, is based solely on visual evidence but it is possible that heterokaryons are formed so infrequently by some strains that mixed conidial heads may be overlooked. It is also possible, but we think improbable, that within a heterokaryotic mycelium nuclei of the same genotype might associate and remain together so that the chance of different genomes occurring in the same conidiophore would be remote. Where species with multinucleate spores are involved (method 2 (*a*)), however, the observation of conidial heads with wild-type colouration can be very misleading ; conidial heads composed entirely of spores with the wild-type colour which were obtained from mixed cultures of two
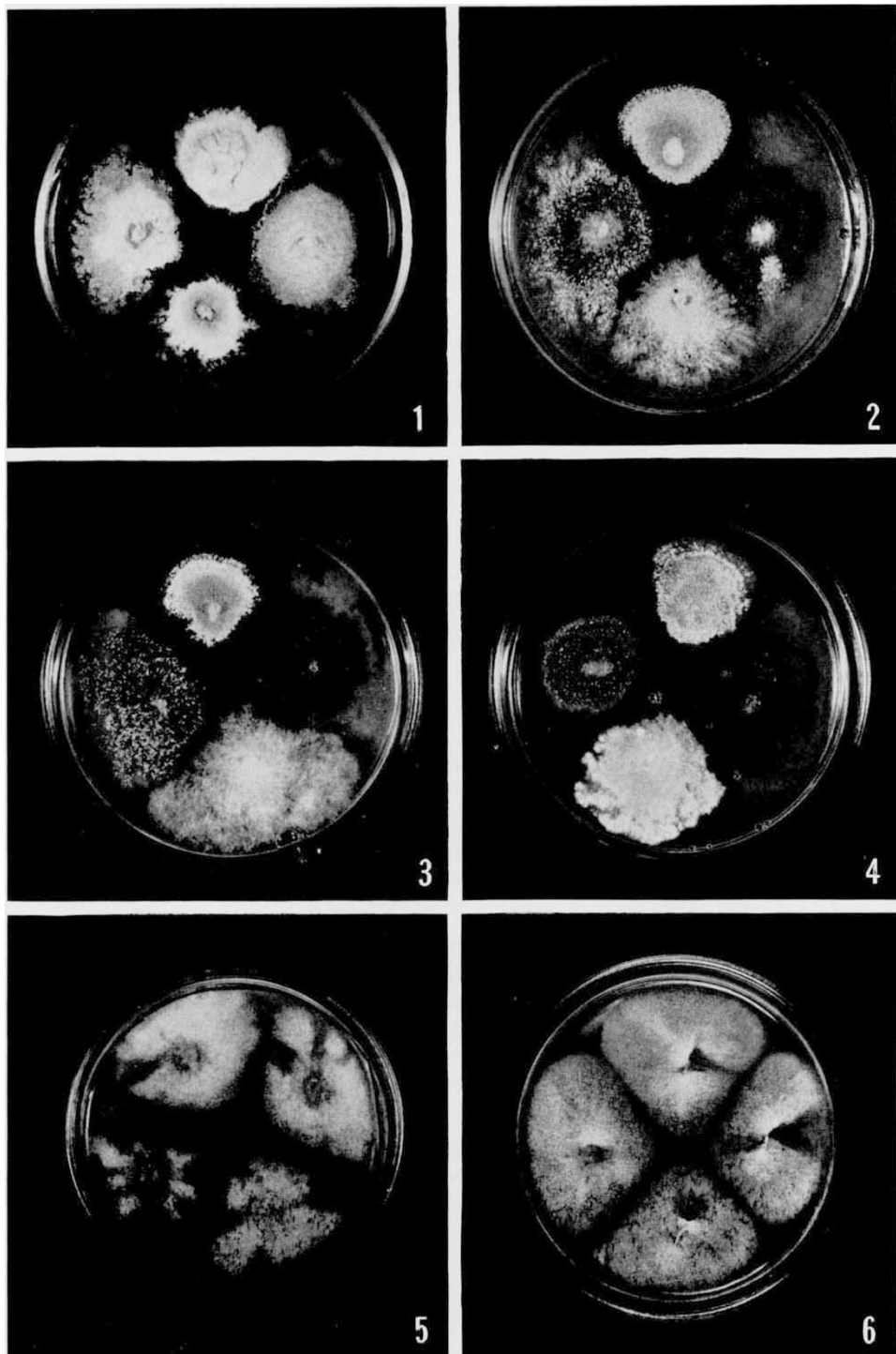
Plate I

FIGS. 1-4.—Sixteen-day-old cultures of *A. nidulans* var. *echinulatus* strain 8 (top), *A. quadrilineatus* strain 22 (left), *A. nidulans* strain 1 (right) and *A. nidulans* var. *acristatus* strain 17 (bottom) growing on (1) MM, *p*H 7·0, (2) PM, *p*H 7·0, (3) PM, *p*H 5·6 and (4) PM, *p*H 4·4. Plates show the variation encountered in the *nidulans* group and the different responses to changes in medium. They also show the usual increase in sporulation resulting from the use of PM and lowering the *p*H, strain 17 being one of the very few exceptions. All heterokaryon tests were carried out on PM with a *p*H slightly below that illustrated in fig. 3.

FIGS. 5 and 6.—Strains 4 and 13 and their spore colour variants growing together in all combinations on (5) PM, *p*H 5·2 and (6) 2 per cent. malt, *p*H 5·0 for determining their heterokaryon compatibility and frequencies of mixed conidial heads. Plate show the superiority of PM over malt agar as a sporulation medium.

Plate II
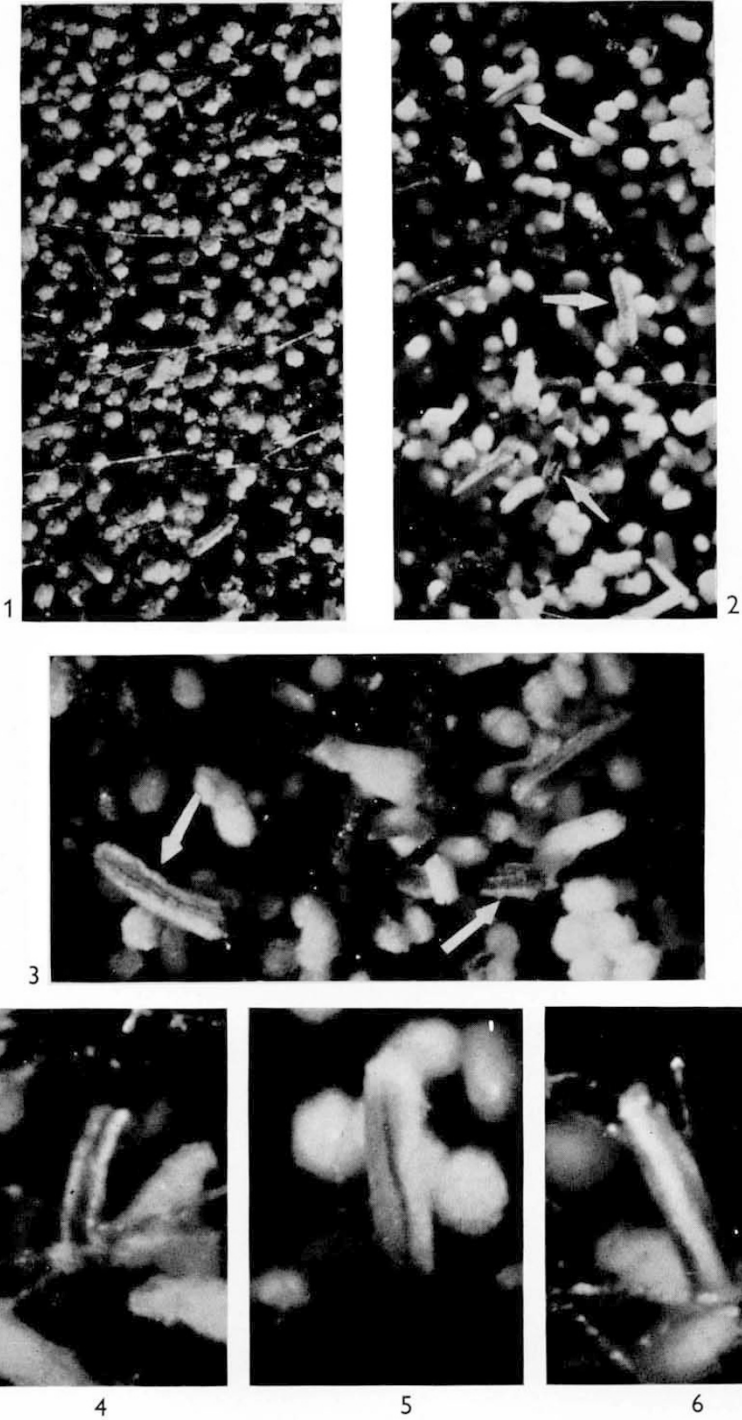
Figs. 1-3.—Conidial heads in a mixed culture of *A. nidulans* strains 2 and 2w showing (1) density at centre of colony (×40), (2) density at edge of colony (×40), and (3) density at edge of colony (×100). Arrows indicate mixed conidial heads.

Figs. 4, 5 and 6.—Typical mixed conidial heads of *A. nidulans* showing (4) chains of green and yellow spores (5 and 6) chains of green and white spores.

different spore-colour mutants of *Aspergillus fonsecæus* have, in some instances, been found to be wild-type in phenotype only since, on progeny testing, only one of the mutant parents was recovered (Raper and Fennell, 1953). Ishitani and Sakaguchi (1956) obtained similar results with other *Aspergilli* and found also that a yellow-spored mutant of *A. sojæ* developed yellow-green spores if grown on agar containing culture filtrates of other species of *Aspergillus* as well as of a white-spored strain of the same species. This suggests that some strains produce a substance, or substances, which can be absorbed into the mycelium of another strain and can be transported in the cytoplasm to the site of conidium formation where it affects the pigmentation of the spores. Presumably, whether the wild-type colouration is produced depends on the nature of both the donor and the recipient strains. These same authors have observed green *A. sojæ*-like conidial heads in mixed cultures of a yellow-spored *A. sojæ* and a host of other species and genera but in view of their own tests with culture filtrates, any inference that heterokaryons were formed between species and genera is an extremely misleading one.

It is equally misleading to assume that the absence of spores with wild-type colouration proves automatically the absence of hetero-karyons since different mutations affecting spore colour need not necessarily complement each other to give phenotypically wild-type conidial heads. Hence, it is possible that Raper and Fennell's (1953) data on *A. fonsecæus* which indicate that mutant strains vary greatly in their capacities to form heterokaryons may, in some cases, reflect not the ability of isolates to heterokaryotise but simply their ability to complement each other and produce spores with wild-type colouration. When using species with multinucleate spores as test organisms, therefore, the choice of spore-colour mutants seems to be critical and in any event it would appear advisable to progeny-test (method 1 (*b*)) any conidial heads with the wild-type colour from presumptive heterokaryons before the heterokaryotic nature of those spores can be assured. If, however, spores with wild-type colouration occur alongside both mutant spore-types in the same conidial head, evidence for heterokaryosis is based not only on complementation (method 2 (*a*)) but also on segregation (method 1 (*a*)) and such a combination of different criteria must provide undeniable proof that the component strains are heterokaryon compatible.

Two different criteria (methods 1 (*b*) and 2 (*b*)) were used in series by Jinks (1952*a*) to investigate wild heterokaryotic isolates of *Penicillium* spp. After sampling conidia from some wild strains he obtained two distinct types of asexual progeny which differed from each other and from the parent heterokaryon in their rate of growth. By inoculating the two components together and sampling single hyphal tips from the colony so formed, he then recovered the resynthesised heterokaryon which could be recognised since its rate of growth approximated very closely to that of the original wild

heterokaryon. This technique provides unambiguous proof of hetero-
karyosis and should be suitable particularly for detecting wild hetero-
karyotic isolates or for testing the compatibility of different wild-type
strains without first introducing gene mutations which might con-
ceivably effect their ability to heterokaryotise.

Most work done since 1952 has used the criterion of comple-
mentation as described in method 2 (*b*), but when using strains with
nutritional requirements or testing for " balanced heterokaryons "
and meiotic or mitotic recombinants, a heavy selection pressure in
favour of the heterokaryon is applied. This technique may be
important as a mechanism for producing new strains of imperfect
fungi with applications in industrial microbiological work, in which
case no comparison or correspondence with events under natural
conditions is presumably intended. Such a selection pressure should
be relevant and of biological importance under conditions in which
heterokaryosis can either confer on otherwise non-infective strains
the ability to attack specific host plants or endow the heterokaryon
with the ability to grow well on substrates which are less suitable for
the homokaryons. However, it is doubtful whether strains differing
by many major genes for biochemical requirements would, in fact,
be involved to any great extent in nature.

" Balanced heterokaryons " are usually recognised only if they
show an improvement in growth over the component homokaryons.
Unless other morphological characters are considered, therefore, it
must be difficult to distinguish those heterokaryons, should they occur,
having a rate of growth which is the same as the parental homo-
karyotic strains. Likewise, if two mutant strains with different
nutritional requirements do not complement each other and are thus
unable to grow on MM, they will be overlooked. It seems also that
the compatibility as well as the complementarity of different mutants
depends on the nature of the nuclear gene mutations. For example,
Buxton and Ward's (1962) data on heterokaryosis in *Fusaria* suggest
that the major gene mutations used in an attempt to produce stable
heterokaryons had more effect on heterokaryon compatibility than
any of the naturally occurring differences which might have been
present in the wild strains.

In view of the technical problems inherent in each of the different
methods, caution is required when extrapolating from laboratory
data to possible events under natural conditions. This applies
especially where a single isolate is used as the test strain and all
heterokaryons are produced by using mutants derived from that one
isolate. It seems, however, that of the various techniques described,
the observation of mixed conidial heads by strains with uninucleate
spores, although subject to errors, incurs the fewest disadvantages for
five reasons. Firstly, mutations affecting spore-colour are easy to
induce and only one such mutation in each isolate is required.
Secondly, since only one major gene mutation is needed, the possibility

of introducing incompatibility genes into the strains is minimised— so far, no spore-colour mutant induced in Birmingham has failed to produce mixed conidial heads with its own wild-type strain. Thirdly, many thousands of conidiophores can be scanned rapidly and mixed heads among them can be distinguished without much difficulty, even when they occur with a very low frequency. Fourthly, the formation of chains of asexual spores with different colours must be unambiguous evidence for the heterokaryotic condition of the mycelium from which such a conidiophore arose ; all mixed heads that have been progeny tested in Birmingham have yielded both homokaryotic parental strains. Finally, by using a minimum of induced nuclear gene mutations, results from laboratory tests are more likely to have some bearing on the behaviour of most isolates in the wild.

It seems clear from this investigation that in the sexual species *A. nidulans*, nuclear and cytoplasmic exchange between different isolates is not prevalent and is perhaps confined to strains of closely allied genotype. If similar barriers to heterokaryon formation exist in fungi having no perfect stage, their opportunities under natural conditions for outbreeding by mitotic recombination must be very restricted.

6. SUMMARY

Twenty-five different isolates of *Aspergillus nidulans* and related species were compared on different media and tested for their ability to form heterokaryons.

When grown on Czapek minimal medium (MM) containing New Zealand agar there were large differences between strains in rate of growth, sexuality and sporulation, but when Spanish agar was used in MM, isolates could not be distinguished so easily, and on Plunkett medium or 2 per cent. malt agar many of the differences disappeared. Production of asexual spores by most isolates was increased substantially on any medium by lowering the pH, the best results being obtained on Plunkett medium.

Each wild isolate formed heterokaryons with the spore-colour mutants derived from itself but few examples of inter-strain, and none of inter-species, compatibility were found. Inter-strain compatibility was confined to strains from the same locality but even these formed heterokaryons much more frequently with their own spore-colour mutants than they did with those derived from the other strains.

This heterokaryon test has advantages as a taxonomic tool and as a means of determining strain and species relationships. The significance of our findings for the outbreeding, gene recombination and variation of filamentous fungi is discussed.

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