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The production and behaviour of diploids of *Coprinus lagopus*

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1. INTRODUCTION

Since the parasexual cycle was first described in *Aspergillus nidulans* by Pontecorvo and Roper in 1952, reports of the complete or partial operation of this cycle in other fungi have continued to appear. An important aspect of parasexuality is that stable diploid strains of normally haploid fungi can be used for genetic studies. In particular it provides a unique opportunity to compare gene interactions in diploid nuclei with that between haploid nuclei in a heterokaryon. Already a few differences have been observed in *A. nidulans* (Luig, 1962; Roberts, 1962; Apirion, 1963, see Pontecorvo). The heterokaryon of *Aspergillus* can have varying ratios of the two component nuclei and also a very non-random distribution of the nuclei in the mycelium (Clutterbuck & Roper, 1964). This variation makes an interpretation of diploid-heterokaryon comparisons difficult. A feature of basidiomycete fungi such as *Coprinus lagopus* is the dikaryon in which a strict 1 to 1 ratio of the component nuclei is maintained, the two nuclei dividing close together in complete synchrony. This might make *Coprinus* particularly suitable for such comparative studies if diploid strains were available.

That it should be possible to obtain diploid strains of *C. lagopus* seemed likely in view of the fact that diploid strains have been synthesized in another basidiomycete fungus, *Ustilago maydis* (Holliday, 1961). There is also much evidence from both *Coprinus* (Buller, 1931; Quintanilha, 1938; Swiezynski, 1962, 1963) and the related fungus *Schizophyllum commune* (Papazian, 1954; Crowe, 1960; Ellingboe & Raper, 1962; Parag, 1962; Ellingboe, 1963) indicating somatic recombination in the dikaryon which suggests that diploid nuclei can be produced as a result of some kind of vegetative karyogamy. The technique of dikaryon–monokaryon matings (termed di-mon, Papazian, 1950), used by the majority of the above workers, selects for recombinant nuclei which may be haploid or aneuploid. This investigation was undertaken to develop a standard procedure for synthesizing stable diploid strains of *C. lagopus*.

2. MATERIALS AND METHODS

(*a*) *Life cycle of* C. lagopus

Germination of a single haploid sexual spore results in the formation of a monokaryon colony, the cells of which each contain a single nucleus. The monokaryon

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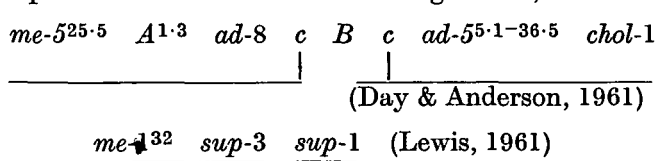
produces large numbers of uninucleate asexual spores, the oidia, which are somewhat cylindrical in shape and vary considerably in length. Sexual reproduction is initiated by the mating of two monokaryons of compatible mating-types to form a dikaryon. Compatibility is controlled at two independent loci referred to as *A* and *B*. Each of these loci has a number of alleles and a dikaryon is formed only between two strains having different alleles at both loci (tetrapolar mating). The dikaryon can be readily distinguished from the monokaryon by the regular formation of clamp connexions between each cell. The cells of the dikaryotic mycelium contain two nuclei, one derived from each of the participants in the mating. These two nuclei remain discrete until the formation of the fruiting body. Fusion then occurs in the basidium to produce a single diploid nucleus and is immediately followed by meiosis and the production of four haploid basidiospores. Apart from these sexual spores, produced by the fruiting body, the mycelium of the dikaryon produces chlamydospores asexually. Chlamydospores are irregular in size and shape but always contain both nuclear components of the dikaryon.

Anastomoses between monokaryons in which either or both the mating type loci have the same allele (incompatible matings) result in the formation of a heterokaryon. Heterokaryons are referred to as common *A*, common *B*, or common *AB* depending on which alleles are identical. The *A* gene controls clamp formation and synchronous nuclear division, and the *B* gene nuclear migration (Swiezynski & Day, 1960*a*). Thus in a common *A* heterokaryon nuclear migration is possible but clamps cannot be formed. In the common *B* heterokaryon where clamps are formed but nuclear migration is prevented, the clamps fail to fuse with the adjacent cell (false clamps) and the migrating nucleus is thereby trapped. When the alleles at both loci are similar neither nuclear migration nor clamp formation occurs. Although both nuclear types are present in these heterokaryons, there is not the strict 1:1 ratio characteristic of the dikaryon.

(*b*) *Strains*

The strains used are listed in Table 1. PR12614 (A_6B_6 *ad-8 me-5*) and 94226 (A_6B_3 *ad-5 chol-1*) were obtained from P. R. Day. SR strains were derived from these by crosses to other stocks in the culture collection. The linkage relations of the mutants present in these strains has been given as;



An additional growth requirement imposed by the *ad*-5 mutation in 94226 was identified as histidine by Cowan. This locus has therefore been designated *adhi*-1 rather than *ad*-5 (Cowan, 1964).

(*c*) *Culture of fungus*

Mycelial cultures were grown in an incubator at 37°C. on solid medium in 9 cm. petri dishes. The minimal and complete media were those used by Lewis (1961).

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Supplemented media contained individual requirements added in the following amounts per litre of minimal medium; adenine 200 mg.; histidine 200 mg.; methionine 100 mg.; choline 2 mg. For the production of sexual fruiting bodies sterile horse dung was used (Lewis, 1961).

Table 1. *Genotypes of strains of* Coprinus lagopus *referred to in the text.* ad-*8*, adhi-*1*, chol-*1*, me-*5 and* me-*1 determine requirements for adenine, adenine + histidine, choline and methionine* (*2*) *respectively.* Sup-*1 and* sup-*3 are recessive mutations of the* me-*1 gene*

(*a*) *Strains used for the synthesis of diploid strains*

PR12614	A_6B_6 *me-5 ad-8*
94226	A_6B_3 *adhi*-1 *chol*-1
SR52	A_6B_3 *me*-5 *adhi*-1
SR53	A_6B_6 *ad*-8 *chol*-1
SR60	A_6B_3 *me*-5 *adhi*-1 *me*-1 *sup*-3
SR61	A_6B_6 *ad*-8 *chol*-1 *me*-1 *sup*-1

(*b*) *Strains used as testers*

TC	A_2B_3 wild-type
12803	A_2B_6 ,,
H1	A_5B_5 ,,
H3	A_5B_5 ,,
H9	A_6B_6 ,,
CA2.36	A_2B_6 *adhi*-1
SR31	A_2B_3 *chol*-1

(*d*) *Experimental procedures*

The standard procedures were adopted for preparing and plating oidial and basidiospore suspensions. Viability of oidia was estimated by counting the number of visible colonies obtained on plates containing 200–300 spores. 0·01% furfurol was incorporated into the medium to increase the percentage germination of basidiospores (Emerson, 1954).

Genetic analyses of oidial and basidiospore colonies

Spores were sown at a concentration of 1×10^3 per plate on complete medium and incubated for 18 hours. Germinated spores were then isolated to fresh plates of complete medium. Gene mutations imposing biochemical requirements were identified by testing auxotrophic colonies for growth on media supplemented with different combinations of requirements to determine the individual requirements of each colony. Where two genes caused the same requirement, the specific locus was identified by a dikaryon complementation test. Complementation tests were also employed in cases of doubtful response to supplements. The *A* and *B* loci were identified by dikaryon tests with tester stocks having known *A* and *B* constitution.

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Dikaryon complementation tests

Monokaryons were mated to compatible stocks having known gene mutations. The dikaryons formed were tested for ability to grow on minimal medium. Growth of dikaryons represents complementation and therefore non-allelic mutations, no growth represents non-complementation between allelic mutations. The reverse situation is true in complementation tests involving methionine suppressor genes since complementation restores methionine requirement and non-complementation maintains methionine independence.

Growth experiments

Growth of individual strains was estimated by measuring colony diameter over a period of 5 days. Strains to be compared were standardized by growing on the same medium for 4 to 5 days. Inoculations of each strain were made in triplicate to minimize any effects owing to slight variations in inocula size. Two diameters at right angles were marked on the under surface of the growth plates and the diameter of each colony was measured along these lines. This gave a total of six diameter measurements per strain at each time interval. The initial diameter was subtracted from the mean of the six readings and these values expressed as radial growth (i.e. diameter/2), were plotted against time.

3. RESULTS

Production and identification of diploid strains

(*a*) *Technique*

Heterozygous diploids are readily obtained from balanced heterokaryons of *Aspergillus nidulans* either as sectors or as conidial colonies (Pontecorvo, Roper, Hemmons, MacDonald & Bufton, 1953). The essential features of the technique first described by Roper (1952) are a system which combines two nuclear types in the same cytoplasm, and a combination of genes which makes a heterozygous diploid phenotypically distinguishable from the haploid parental strains. A heterokaryon composed of two haploid strains carrying gene mutations imposing different biochemical requirements gives rise to rare prototrophic heterozygous diploid conidia with nuclei resulting from the fusion of two haploid nuclei, one from each parent. Diploid colonies can be selected by plating large numbers of conidia onto unsupplemented medium on which haploid conidia of either component genotype are unable to grow.

The above technique was adapted to produce diploid strains of *Coprinus* from common *A* heterokaryons. The common *A* heterokaryon was chosen because of its similarity to the ascomycete heterokaryon with respect to nuclear migration and the production of large numbers of uninucleate asexual spores (oidia) of both component types. In the initial experiment, selection for diploidy was made from a common *A* heterokaryon having the following composition,

(*me*-5 A_6 *ad*-8 B_6 + +) PR12614 + (+ A_6 + B_3 *adhi*-1 *chol*-1) 94226

There is complementation between the genes in these strains so that the heterokaryon was stable and grew on minimal medium, however it was also stable and

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grew faster on complete medium. Oidia harvested from this heterokaryon grown on complete medium were sown on minimal medium at concentrations ranging from 2×10^6 to 4×10^6 per plate. After 3 days ten to fifty prototrophic colonies had developed per plate indicating that diploid colonies might be present.

(*b*) *Oidial test*

Since oidia are uninucleate, haploid oidia from a heterokaryon would be unable to grow on minimal medium whereas in the case of a prototrophic diploid, equal numbers of colonies would be expected from oidia sown on complete and minimal medium. Small samples of oidia from a single colony were sown in equal numbers on complete and minimal medium and visible colonies were counted after 3 days. From a sample of prototrophic colonies isolated, three diploid colonies were identified by this oidial test. The three diploid colonies are referred to as Diploids

Table 2. *Results of oidial test*

Strain	Numbers of colonies on* Complete medium	Minimal medium
Diploid I $\left(\frac{me\text{-}5 \quad A_6 \quad ad\text{-}8 \quad B_6 \quad + \quad +}{+ \quad A_6 \quad + \quad B_3 \quad adhi\text{-}1 \quad chol\text{-}1}\right)$	47	29
Diploid II ,,	20	20
Diploid III ,,	82	10
Parent heterokaryon $\frac{(me\text{-}5 \quad A_6 \quad ad\text{-}8 \quad B_6 \quad + \quad +)}{+(+ \quad A_6 \quad + \quad B_3 \quad adhi\text{-}1 \quad chol\text{-}1)}$	32	0
TC $A_2 B_3$ wild-type	123	93
H9 $A_6 B_6$ wild-type	166	68

* Equal numbers of oidia were plated on each medium.

I, II, and III in Table 2. The table also includes control platings of oidia from two wild-type monokaryons (TC and H9), and from the parent heterokaryon.

Since the discrepancy between the numbers recorded on the two types of medium in the case of Diploids I and III was also true of the two wild-type monokaryons, it was assumed to be an effect of reduced or delayed germination on minimal medium. That this difference was not attributable to the presence of auxotrophic oidia from the two diploid colonies was shown by transferring samples of twenty colonies from complete to minimal medium. All colonies were fully prototrophic. Further proof of the diploid nature of these colonies is presented later.

(*c*) *Growth rates*

The growth of the diploid colonies was noticeably faster than that of the parent heterokaryon. To confirm this observation the growth of Diploids I, II, and III, and the parent heterokaryon were compared on minimal and complete medium, and were also compared with the growth of a wild-type monokaryon, H9. The

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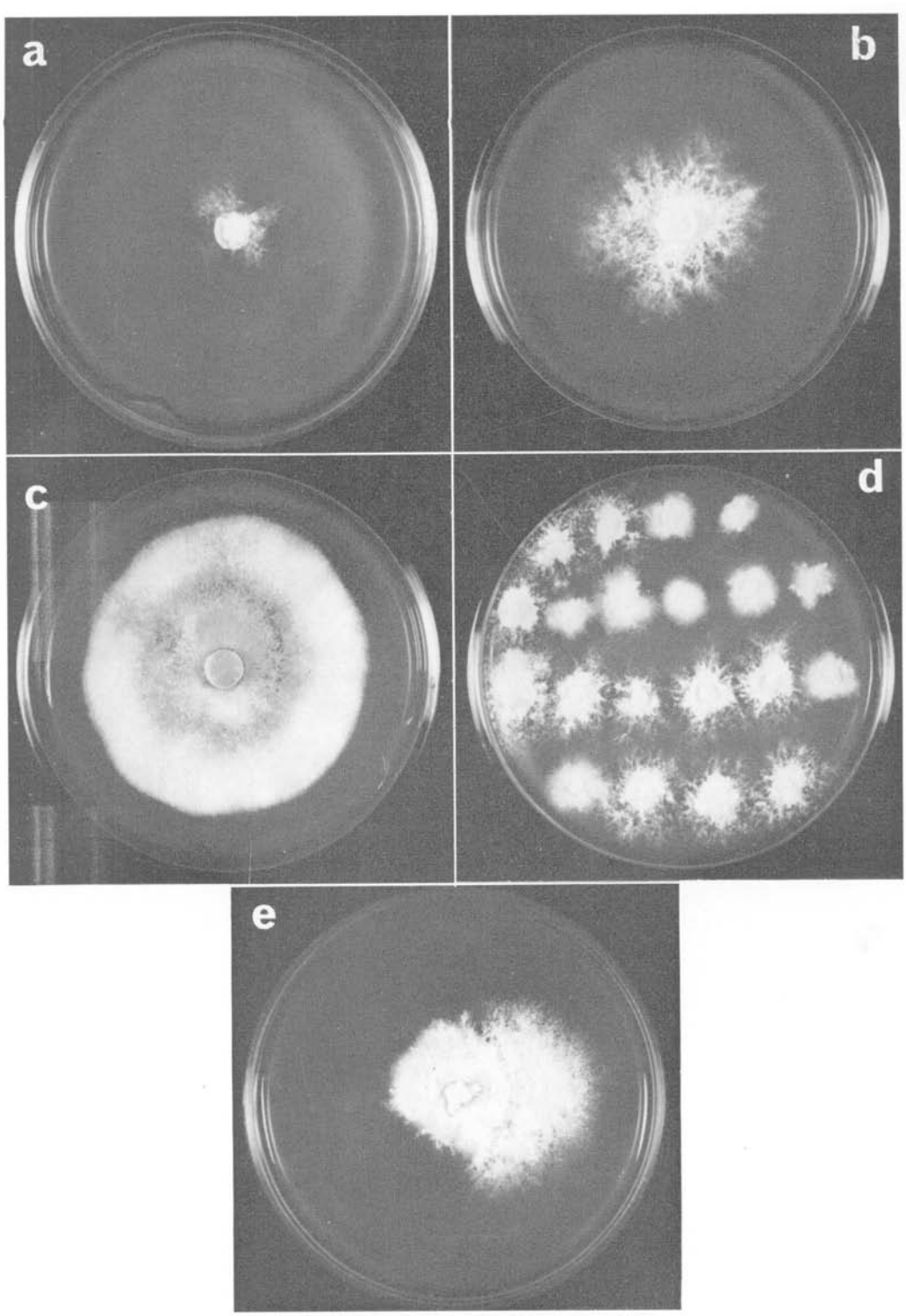

(a) Common *A* heterokaryon colony formed from two strains with different growth requirements growing on minimal medium. Growth is slow and irregular. (b) Diploid colony growing on minimal medium. Growth is faster and more uniform that that of the parent heterokaryon. (c) Wild-type haploid monokaryon colony growing on minimal medium. (d) Petri dish of minimal medium with eleven diploid colonies and nine heterokaryotic colonies. (e) Diploid sector in growing heterokaryon.

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results are shown graphically in text-fig. 1. The three diploid colonies gave identical readings at each stage of the experiment and are represented as a single line. The radial growth rate was 10 mm., 8 mm., and 4 mm. per day for the monokaryon, diploids, and heterokaryon respectively on minimal medium, and 8 mm. per day for all three types of colony on complete medium. The diploid thus grew at twice the rate of the heterokaryon on selective medium. Apart from the obvious difference in growth rate on minimal medium, the three types of colony exhibited a distinct morphology. The heterokaryon grew unevenly and the outline of the colony was irregular (Plate 1(a)). This probably reflected the difficulty of maintaining a balance between the two nuclear components. The diploid formed a much more uniform colony but had a feathered appearance caused by the production of long radiating hyphae with numerous short branches (b). The monokaryon grew evenly to form colonies with very regular outline, a characteristic of most *Coprinus* monokaryons (c). These morphological differences were not so obvious on complete medium.

The colonies illustrated were of equal age, and it is therefore clear that the combination of growth rate and colony morphology on selective medium can be used to distinguish between diploid and heterokaryon colonies. The results also show that on minimal medium the diploid has a distinct selective advantage over the parent heterokaryon.

(*d*) *Frequency of diploid production*

Using the criteria of growth rate and colony morphology to identify diploids it was possible to estimate the frequency with which diploid oidia were produced by common *A* heterokaryons. Estimates were made from two heterokaryons which give rise to prototrophic diploid colonies. These were, the original heterokaryon in which the mutant alleles were in the *cis* arrangement in each of two chromosome pairs, giving diploids

$$\left(\begin{array}{cccc|cccc} & \text{I} & & & & & \text{II} & \\ \textit{me}\text{-}5 & A_6 & \textit{ad}\text{-}8 & & B_6 & + & + & \\ + & A_6 & + & & B_3 & \textit{adhi}\text{-}1 & \textit{chol}\text{-}1 & \end{array}\right)$$

and a heterokaryon between strains SR52 and SR53 in which these same alleles were in the *trans* arrangement,

$$\left(\begin{array}{ccc|ccc} \textit{me}\text{-}5 & A_6 & + & B_3 & \textit{adhi}\text{-}1 & + \\ + & A_6 & \textit{ad}\text{-}8 & B_6 & + & \textit{chol}\text{-}1 \end{array}\right) \quad \text{Diploids IV, V, and VI.}$$

By using a concentration of approximately 1×10^5 oidia per plate it was possible to isolate all the prototrophic colonies which had developed after 3 days. These were transferred to fresh plates of minimal medium and diploid colonies were identified after a further 3 days. Plate I(d) illustrates a plate of twenty colonies, eleven of which are diploid and clearly distinguishable from the remainder which are heterokaryons.

It is not excluded from the results obtained with these two heterokaryons that some of the prototrophs are aneuploids disomic for the two selected chromosomes,

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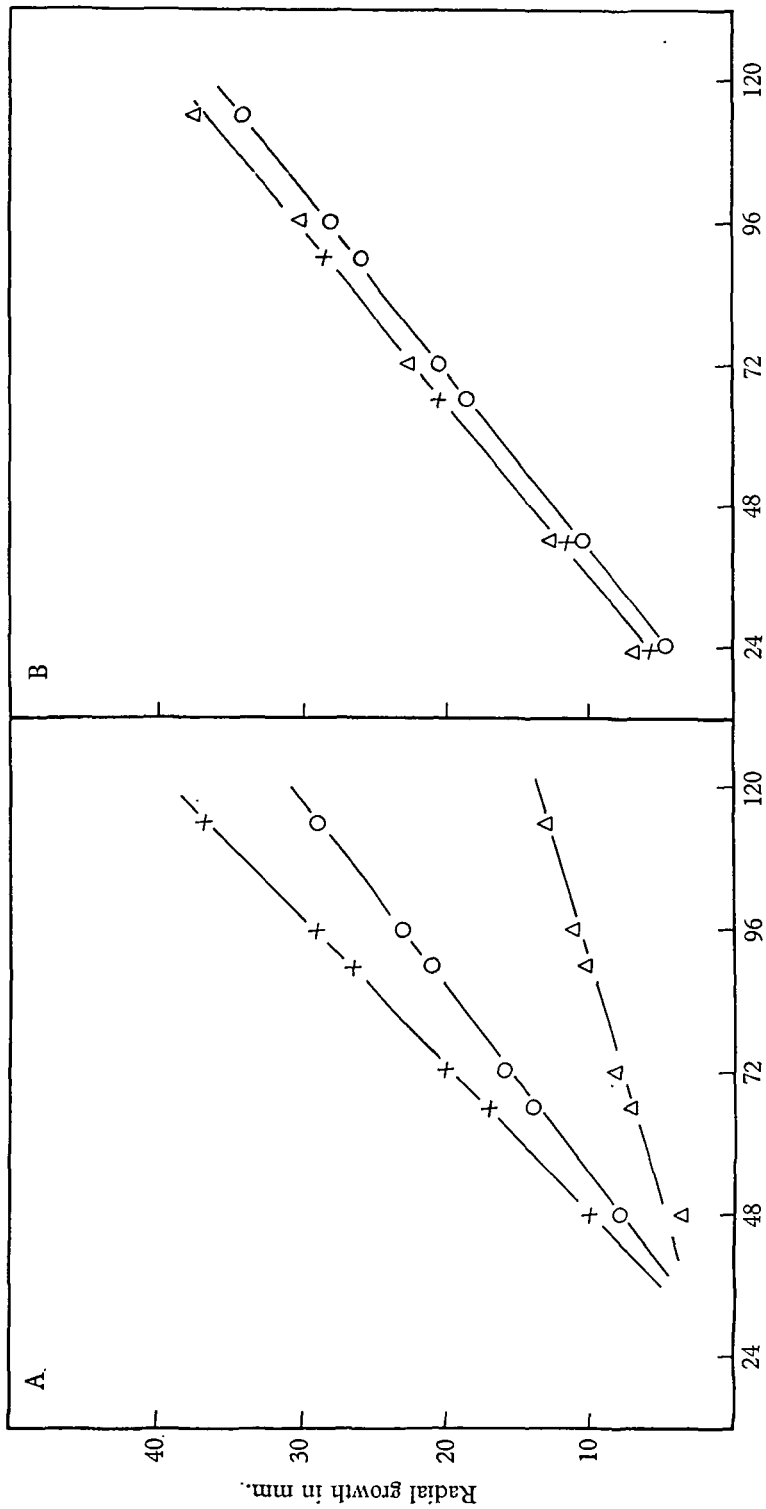

Text-fig. 1. Growth of a wild-type haploid monokaryon, diploid and heterokaryon on *A* minimal medium and *B* complete medium. × haploid, ○ diploid, △ heterokaryon.

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I and II, and monosomic for some or all of the other six chromosomes. To test this possibility, diploids were selected from a heterokaryon between SR60 and SR61 in which additional selective markers, *me*-1, and the recessive complementary suppressors of *me*-1, *sup*-3 and *sup*-1, were used to give methionine requiring diploids of the genotype,

$$\left(\begin{array}{cccccccccc} & \text{I} & & & \text{II} & & & \text{III} & & \text{IV} \\ \underline{me\text{-}5} & A_6 & + & B_3 & adhi\text{-}1 & + & me\text{-}1 & sup\text{-}3 & + & \\ + & A_6 & ad\text{-}8 & B_6 & + & chol\text{-}1 & me\text{-}1 & + & sup\text{-}1 & \end{array}\right) \text{ Diploid VII.}$$

Selection was made on both minimal medium and minimal medium supplemented with methionine. No prototrophic colonies were obtained on minimal medium alone as expected if only diploids are produced: the methionine requirement due to homozygosity for *me*-1 would not be suppressed in diploids heterozygous for the two suppressors. That methionine requirement was not due to the *me*-5 gene was confirmed by complementation tests with an *me*-5 stock. The loss of only one chromosome, either $\underline{me\text{-}1\ +}$ or $\underline{+}$, removing one of the genes dominant over the suppressor, would have made the resulting aneuploid prototrophic, therefore the complete lack of development of any colonies on minimal medium was good evidence that the technique yielded diploids and not an appreciable proportion of aneuploids.

The frequencies with which diploid oidial colonies were obtained from these three heterokaryons are given in Table 3. Occasional diploid sectors arise when hetero-

Table 3. *Frequency of diploid production from three heterokaryons*

Heterokaryon	Selective medium	Viable oidia plated × 10^4	Diploids obtained	Diploids per 10^4
PR12614 + 94226	Minimal	14·7	42	2·83
SR52 + SR53	Minimal	1·26	41	30·0
SR60 + SR61	Minimal + methionine	6·6	5	0·76
SR60 + SR61	Minimal	13·2	0	0

karyons are grown on selective medium. These sectors grow out rapidly from the margin of the colony. A sector produced in a 6-day-old culture is shown in Plate I(e).

(*e*) *Measurements of diploid and haploid oidia*

One of the diagnostic features of diploidy in *Aspergillus nidulans* is an increase in conidial diameter compared with the haploid resulting in a corresponding doubling of the volume (Pontecorvo, Tarr Gloor & Forbes, 1954). Oidia of *Coprinus* are extremely variable in size and it would be impossible to use this kind of test for identifying ploidy. It was possible, however, by measuring a random sample of oidia from the parental haploid strains and three of the diploid strains described, to demonstrate that the average diploid oidial size is significantly larger than that of the haploid. The length and width of 100 oidia from each strain were measured to the nearest 0·5 of a unit of an eye-piece micrometer. Values obtained were

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multiplied by 0·9 to convert measurements to μ. The mean values calculated from these measurements are given in Table 4.

Table 4. *Size of oidia from haploid and diploid strains*

Strain	Mean length in μ	Mean width in μ	Volume cu. μ
PR12614 (Haploid parent 1)	5·18 ± 0·155	1·93 ± 0·025	15·15
94226 (Haploid parent 2)	5·42 ± 0·153	1·98 ± 0·029	16·67
Diploid I	6·62 ± 0·179	2·19 ± 0·026	24·92
Diploid II	6·50 ± 0·137	2·18 ± 0·024	24·23
Diploid III	6·23 ± 0·163	2·21 ± 0·028	23·89

An approximate estimate of the volume was made by assuming that an oidium is a cylinder. Comparing volumes, the difference in size is more apparent and the figures obtained represent a ratio of diploid to haploid of 1·5. However, the actual length range, 2·7–13·5 μ, and width range 1·8–3·15 μ, apart from the shortest and longest classes of the former were exhibited by oidia from both types of strain.

(*f*) *Measurements of hyphal width*

All measurements were made on the terminal hyphal cell adjacent to the septum. The mean width values obtained showed a significant difference between haploid and diploid hyphae as would be expected from the previous measurements of oidia.

Table 5. *Hyphal width of haploid and diploid strains*

Strain	Mean width in μ
PR12614 (Haploid parent 1)	2·42 ± 0·042
94226 (Haploid parent 2)	2·58 ± 0·038
Diploid I	3·05 ± 0·045
Diploid II	3·05 ± 0·055
Diploid III	3·02 ± 0·047

Behaviour of diploid strains

(*a*) *Mating-type*

Each of the diploid strains ($A_6A_6B_3B_6$) was mated to monokaryons with A_2B_3 and A_2B_6 mating-types. In all instances dikaryons were formed with both the tester strains. It was apparent from this that the compatibility was not affected by the presence of common B alleles in the two nuclei. Moreover, dikaryons formed between haploid and diploid strains arose either at the junction of two strains, as a result of bilateral transfer of nuclei, or as unilateral transfer of nuclei by either the diploid or the haploid monokaryon. Thus all types of dikaryotization were observed. On the other hand, the heterokaryon generally behaved as a donor in dikaryon formation, although it can accept nuclei in regions which are monokaryotic (Swiezynski & Day, 1960*b*).

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(*b*) *Haploidization*

Evidence for haploidization of diploid strains was looked for by (i) analysing samples of oidial colonies and (ii) selecting for methionine-independent colonies from a methionine-requiring diploid strain heterozygous at two *me*-1 suppressor loci.

(i) *Analysis of oidial colonies.* Colonies were derived from oidia from Diploids I, II, and III, all three of presumably identical genotype, and from Diploid IV.

Haploidization, as expected from other organisms, turns out to be rare so that without selection very few haploids are obtained. One aneuploid was identified among 515 colonies from oidia of Diploids I, II, and III; one haploid and two aneuploids from 455 colonies of Diploid IV. Aneuploid colonies grew poorly, were unstable and readily broke down through oidia to give haploids with parental and recombinant genotypes.

(ii) *Selection for methionine-independent colonies from Diploid VII.*

$$\left(\frac{me\text{-}5 \quad A_6 \quad +}{+ \quad A_6 \quad ad\text{-}8} \quad \frac{B_3 \quad adhi\text{-}1 \quad +}{B_6 \quad + \quad chol\text{-}1} \quad \frac{me\text{-}1 \quad sup\text{-}3 \quad +}{me\text{-}1 \quad + \quad sup\text{-}1}\right)$$

The *sup*-1 and *sup*-3 genes which suppress the methionine requirement of the *me*-1 gene complement in the diploid to restore methionine requirement. Methionine-independent colonies could be produced by any one (or a combination) of four processes: (*a*) complete haploidization; (*b*) formation of monosomics exposing one or both of the suppressor genes; (*c*) non-disjunction of either or both suppressor chromosomes giving homozygosis for suppressor; (*d*) mitotic crossing-over to give homozygosis for suppressor.

Selection was made on three types of supplemented minimal medium. Selection was made on medium supplemented with adenine or choline to look for haploid colonies in which a single growth requirement would implicate mitotic crossing-over between genes linked to the *A* and *B* mating-type genes. The results are given in Table 6.

Table 6. *Selection and identification of methionine-independent colonies from Diploid VII*

Medium supplements	Number of oidia plated	Number of colonies obtained	Identification
Adenine, histidine and choline	$1{\cdot}32 \times 10^4$	7	2 prototrophic diploid A_6B_6/B_3 *me*-1 *sup*-1
			3 prototrophic aneuploid A_6B_6/B_3 *me*-1 *sup*-1
			1 aneuploid A_6B_6 *chol*-1 *me*-1 *sup*-3
			1 haploid (sector) A_6B_6 *me*-5 *chol*-1 *me*-1 *sup*-3
Adenine	$60{\cdot}2 \times 10^4$	14	10 prototrophic diploid A_6B_6/B_3 *me*-1 *sup*-1
			1 prototrophic diploid A_6B_6/B_3 *me*-1 *sup*-3
			3 prototrophic aneuploid A_6B_6/B_3 *me*-1 *sup*-3
Choline	$60{\cdot}2 \times 10^4$	24	14 prototrophic diploid A_6B_6/B_3 *me*-1 *sup*-1
			1 prototrophic diploid A_6B_6/B_3 *me*-1 *sup*-3
			9 prototrophic aneuploid A_6B_6/B_3 *me*-1 *sup*-3

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The majority of the colonies obtained were still diploid. These could have been produced by either process (*c*) or (*d*); absence of markers linked to the suppressor genes made it impossible to distinguish between the two. Only one haploid colony was identified and this sectored from an aneuploid. This suggests that haploids are difficult to obtain even when a selective technique is used. It is not surprising, therefore, that there was no evidence of mitotic crossing over between genes linked to *A* and *B*.

(*c*) *Crosses*

Attempts were made to fruit dikaryons formed between diploid strains and haploid monokaryons in order to identify random samples of the basidiospore progeny. From a total of thirteen matings all but four of the dikaryons produced fruiting bodies. Since these crosses resulted in a dikaryon with a haploid and a diploid nucleus in each cell, the fusion nucleus in the basidium presumably would be triploid. As a consequence, abnormal segregations characteristic of triploid meiosis were expected.

Maturation of fruiting bodies took 15–18 days compared with 10–12 days in normal haploid crosses, and the number of basidiospores produced was low. A striking feature was the aberrant appearance of the basidiospores. Plate II(a) and (b) illustrates a comparison between these spores and those from a normal haploid cross. Normal basidiospores are characteristically elliptical in shape, regular in size and have a single germ pore. Typical basidiospores from crosses involving diploids were irregular in size, and extremely variable in shape. The heart-shaped spores notably appeared to have two germ pores. This latter feature is similar to that observed in diploid pollen grains in many species of higher plants. Large numbers of aborted spores were also present. An examination of the gill surface of one fruiting body showed that abnormal tetrads were produced. From a random count of 378 tetrads, 73 'fives', 1 'six', and 4 'threes' were recorded, fives representing 19% of the sample. A typical area of gill is shown in Plate II together with that obtained from a normal haploid cross. It was found that many of the germinated spores isolated either failed to grow or grew poorly, indicating that a large proportion of unbalanced aneuploids were produced.

Crosses summarized in Tables 7 and 8 were between diploids and haploid tester strains having a common *B* allele (either B_3 or B_6). The results in Table 9 were obtained from one diploid strain crossed to two haploid monokaryons in which the *B* allele (B_5) was different from either of those present in the diploid nucleus.

All the genes present in the diploid nucleus were recovered from three of the crosses. However, from only one of these analyses was the expected type of segregation obtained (Diploid VI × 12803) and the homologous chromosomes of the diploid recovered in equal numbers. From other crosses, only some of the genes of the diploid nuclei were identified in the progeny. In spite of poor viability, large enough spore samples were analysed to discount inviability causing the non-recovery of other genes and it is probable, therefore, that the chromosomes on which these genes were located were not present at meiosis.

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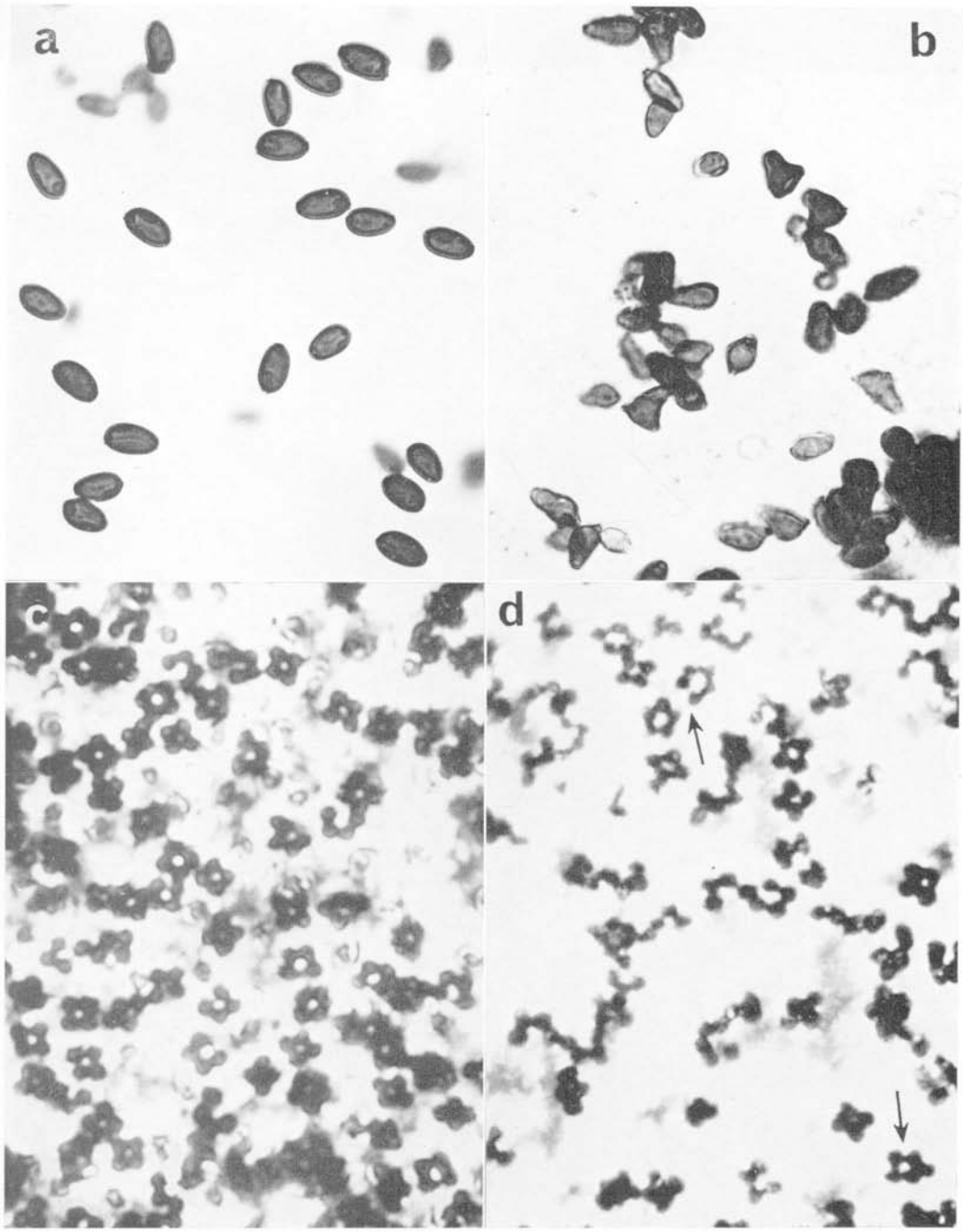

(a) Normal basidiospores from a cross between two haploid strains; × 750. (b) Aberrant basidiospores from a cross between Diploid I and a haploid strain; × 750. (c) Gill surface of a fruiting body derived from a cross between two haploid strains showing normal tetrads of basidiospores; × 450. (d) Gill surface of a fruiting body derived from a cross between Diploid I and a haploid strain. The number of tetrads is reduced; arrows point to 'fives'; × 450.

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Table 7. *Segregations in basidiospore progeny from crosses between TC* (A_2B_3 *wild-type*) *and Diploids I, II and III*

$$\left(\frac{\text{me-}5 \quad A_6 \quad \text{ad-}8}{+ \quad A_6 \quad +} \circ \frac{B_6 \quad + \quad +}{B_3 \circ \text{adhi-}1 \quad \text{chol-}1}\right)$$

and Diploids V and VI

$$\left(\frac{\text{me-}5 \quad A_6 \quad +}{+ \quad A_6 \quad \text{ad-}8} \circ \frac{B_3 \quad \text{adhi-}1 \quad +}{B_6 \circ + \quad \text{chol-}1}\right)$$

Expected random segregation ratios are 2:1 *for:* A_6:A_2, +:me-*5*, +:ad-*8*, +:adhi-*1*, +:chol-*1*

Cross	Segregation in linkage group I	Observed	Expected	Segregation in linkage group II	Observed	Expected	Remarks
Diploid I	**A*$_6$:*A*$_2$	104:32	(90·7:45·3)	*B*$_3$:*B*$_6$	61:74	(90:45)	Disturbed 2:1 ratio of chromosome I caused by unequal segregation. Partial haploidization before meiosis causes 1:1 ratio in group II and loss of *B*$_3$ *adhi*-1 *chol*-1 chromosome.
	+ :*me*-5	121:15	(,,)	+ :*adhi*-1	135:0	(,,)	
	+ :*ad*-8	119:17	(,,)	+ :*chol*-1	135:0	(,,)	
Diploid II and III	*A*$_6$:*A*$_2$	112:62	(116:58)	*B*$_3$:*B*$_6$	90:84	(116:58)	2:1 ratio in group I. Deficiency of *adhi*-1 and *chol*-1 indicates that the *B*$_3$ *adhi*-1 *chol*-1 chromosome was not present in all the meiotic nuclei.
	+ :*me*-5	142:32	(,,)	+ :*adhi*-1	155:19	(,,)	
	+ :*ad*-8	139:35	(,,)	+ :*chol*-1	155:19	(,,)	
Diploid V	*A*$_6$:*A*$_2$	41:41	(54·7:27·3)	*B*$_3$:*B*$_6$	63:19	(54·7:27·3)	1:1 ratios and loss of *me*-5 *A*$_6$ + and *B*$_3$ *adhi*-1 + in Diploid V and + *A*$_6$ *ad*-8 and *B*$_3$ *adhi*-1 + in Diploid VI indicate haploidization prior to meiosis.
	+ :*me*-5	82:0	(,,)	+ :*adhi*-1	82:0	(,,)	
	+ :*ad*-8	41:41	(,,)	+ :*chol*-1	41:41	(,,)	
Diploid VI	*A*$_6$:*A*$_2$	89:81	(113·3:56·7)	*B*$_3$:*B*$_6$	97:73	(113·3:56·7)	
	+ :*me*-5	79:91	(,,)	+ :*adhi*-1	170:0	(,,)	
	+ :*ad*-8	170:0	(,,)	+ :*chol*-1	98:72	(,,)	

* The calculation of observed and expected segregation in linkage group I includes one disomic A_6/A_2 colony. If a correction is applied for the probable occurrence of A_6/A_6 disomics the observed and expected ratios become 104·5:32 and 91:45·5 respectively. Since the correction applied is small, it has not been made here and in other crosses in which small numbers of disomic colonies were scored.

It is concluded therefore that diploid nuclei are unstable in this dikaryon system, and undergo haploidization sometime between dikaryon formation and fruiting. If haploidization occurred before the fruiting body was initiated, then obviously some chromosomes would not be represented at all in the basidiospore progeny, whereas haploidization at a later stage would result in a heterogenous nuclear population and unequal representation of homologous chromosomes as observed in crosses involving Diploids I, II, and III.

A correlation between haploidization and the *B* constitution of the haploid tester strain was apparent in crosses involving Diploids I, V, and VI. In those crosses in which the haploid nucleus had a *B* allele common to the diploid nucleus haploidiza-

Table 8. *Segregations in basidiospore progeny from crosses between* 12803 (A_2B_6 *wild-type*) *and Diploids V and VI*

$$\left(\frac{\text{me-}5 \quad A_6}{+ \quad A_6} \; + \; \text{ad-}8 \; \circ \; \frac{B_3}{B_6} \; \circ \; \frac{\text{adhi-}1}{+} \; + \; \text{chol-}1\right)$$

Expected random segregation ratios are 2:1 *for:* $A_6:A_2$, $B_6:B_3$, +:me-*5*, +:ad-*8*, +:adhi-*1*, +:chol-*1*

Cross	Segregation in linkage group I	Observed	Expected	Segregation in linkage group II	Observed	Expected
Diploid V	$A_6:A_2$	78:75	(102:51)	$B_6:B_3$	83:70	(102:51)
	+:*me*-5	153:0	(,,)	+:*adhi*-1	80:73	(,,)
	+:*ad*-8	75:78	(,,)	+:*chol*-1	153:0	(,,)
Diploid VI	$A_6:A_2$	42:21	(42:21)	$B_6:B_3$	51:17	(45·3:22·7)
	+:*me*-5	42:21	(,,)	*+:*adhi*-1	49:9	(38·7:19·3)
	+:*ad*-8	46:17	(,,)	*+:*chol*-1	45:13	(,,)

* Excludes five colonies disomic for linkage group II.

Remarks

Diploid V: 1:1 ratios and loss of chromosomes *me-5* A_6 + and B_6 + *chol-1* indicates haploidization prior to meiosis.

Diploid VI: Trisomic segregation in both linkage groups.

tion effected loss of the chromosome carrying the common *B* allele. However, the results in Table 9 showed that haploidization was not confined to dikaryons having nuclei with a common mating-type allele. The effect of haploidization in this latter type of dikaryon would naturally be selection against the common *B* allele, and other chromosomes would be affected randomly.

(*d*) *Haploidization of diploid nuclei in a dikaryon*

Two haploid monokaryons, A_2B_6 *adhi*-1 (CA2.36) and A_2B_3 *chol*-1 (SR31) were mated to Diploids IV, V, and VI. If the diploid nucleus underwent haploidization in these dikaryons a requirement for either adenine and histidine or choline would represent homozygosity for these genes, and therefore a loss from the diploid of the chromosome carrying the *B* allele common to the haploid nucleus. The di-

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Table 9. *Segregations in basidiospore progeny from crosses between H1 and H3* (A_5B_5 *wild-type*) *and Diploid III*

$$\left(\frac{\text{me-}5 \quad A_6 \quad \text{ad-}8}{+ \quad A_6 \quad +} \circ \frac{B_6 \quad + \quad +}{B_3 \quad \text{adhi-}1 \quad \text{chol-}1}\right)$$

Expected random segregation ratios are 2:1 *for:* $A_6:A_5$, +:me-5, +:ad-8, +:adhi-1, +:chol-1 *and* 1:1:1 *for* $B_3:B_5:B_6$

Cross	Segregation in linkage group I	Observed	Expected	Segregation in linkage group II	Observed	Expected	Remarks
Diploid III × H1	$A_6:A_5$	65:60	(83·3:41·7)	$B_3:B_5:B_6$	68:57:0	(41·7:41·7:41·7)	1:1 segregation and loss except one of *me*-5 A_6 *ad*-8 and B_6 + + indicate prior haploidization.
	+:*me*-5	124:1	(,,)	+:*adhi*-1	62:63	(83·3:41·7)	
	+:*ad*-8	124:1	(,,)	+:*chol*-1	61:64	(,,)	
Diploid III × H3	$A_6:A_5$	73:48	(80·7:40·3)	*$B_3:B_5:B_6$	95:32:0	(42·3:42·3:42·3)	2:1 segregation and double reduction of *adhi*-1 and *chol*-1 indicates trisomic segregation but there is complete absence of B_6, *me*-5, and *ad*-8.
	+:*me*-5	121:0	(,,)	+:*adhi*-1	28:99	(84·7:42·3)	
	+:*ad*-8	121:0	(,,)	+:*chol*-1	28:99	(,,)	

* Includes six colonies disomic for linkage group II $\left(\frac{B_5 \quad \textit{adhi}\text{-}1 \quad \textit{chol}\text{-}1}{B_3 \quad \textit{adhi}\text{-}1 \quad \textit{chol}\text{-}1}\right)$ which indicates double reduction.

karyons were grown on complete medium and subcultured at 3- and 4-day intervals. At successive subcultures each dikaryon was tested for growth on minimal medium.

Five of the six dikaryons became auxotrophic within 11 days. These results supported the evidence obtained from the basidiospore analyses, that haploidization of these diploid nuclei can be influenced by the *B* allele of the haploid tester nucleus.

4. DISCUSSION

The adaptation of Roper's technique for *Aspergillus nidulans* proved successful in selecting for diploid oidial colonies of *Coprinus lagopus* from common *A* heterokaryons. There is little doubt that the colonies obtained were indeed diploid. There are eight chromosomes in the haploid genome of *C. lagopus* (Lewis, 1957) and it has been possible at this stage to mark a maximum of four homologues in one diploid strain (Diploid VII). The chromosomes are small and it is difficult to obtain good photographs, but at least thirteen chromosomes have been observed in one diploid oidium. Apart from the ability to identify the genes present, either from crosses or haploidization, diploid colonies have a characteristic growth behaviour which is quite distinct from the unstable aneuploid colonies produced during the course of various experiments. On selective medium, diploids grew at twice the rate of the parent heterokaryon, haploidization was rare and sectoring was not observed. This is clearly indicative of a balanced chromosome complement. In contrast, aneuploid colonies grew very slowly and haploidized readily through oidia or sectors.

Diploid oidia were produced by common *A* heterokaryons with a frequency ranging from approximately 1×10^{-3} to 1×10^{-4}, a high frequency when compared with values which have been given for other fungi (Table 10). The selection technique is correspondingly very efficient. It is interesting that diploid sporidia of *Ustilago maydis* have been recovered as 10% of the progeny from a particular dikaryon (Holliday, 1961). This suggests that diploids can be produced at a higher frequency in the basidiomycetes than in other fungal groups.

The heterozygous *B* diploid strains described formed dikaryons with haploid monokaryons having either of the similar *B* alleles. This mating behaviour is similar to that described for both heterozygous *B* and heterozygous *A* disomic strains of *Schizophyllum commune* (Raper & Oettinger, 1962). As already suggested by Raper and Oettinger, the mating behaviour of these heterozygotes infers that the incompatibility mechanism in the basidiomycetes is not oppositional. In other words, the presence of common alleles does not prevent dikaryon formation. Obviously the mechanism is different from that already known in higher plants where the incompatibility reaction of common *S* alleles is actively oppositional (Lewis, 1954).

The characteristics of increased oidial size and mitotic haploidization by a stepwise process (as opposed to meiotic reduction) in diploid strains of *Coprinus* are comparable to those associated with diploidy in *Aspergillus nidulans* (Pontecorvo, Roper, Hemmons, MacDonald & Bufton, 1953; Pontecorvo & Käfer, 1958). In-

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ability to recognize mitotic crossing-over is probably due to the small number of markers used in the present system. However, it is clear that some form of a parasexual mechanism operates in *C. lagopus*. An interesting feature of this study has been the results obtained by crossing haploid and diploid strains. Good evidence was obtained for the fusion of the two nuclear types in the basidia prior to meiosis. When the diploid nucleus had not undergone partial haploidization it can be assumed that the fusion nucleus was triploid. Low spore yield, abnormal tetrads, and aberrant spore shape are all associated with triploid or partially triploid segregation. Whilst fruiting occurs in diploid strains of *A. nidulans*, there is no evidence to show that fusion of two diploid nuclei occurs to give tetraploid meiosis; the asci have been shown to develop apogamously from a single diploid nucleus.

Table 10. *Fungi in which stable diploid strains have been identified*

Fungus	Spontaneous frequency per 10^6	
ASCOMYCETES		
Aspergillus nidulans	0·1–1	Pontecorvo (1956)
FUNGI IMPERFECTI		
Aspergillus niger	35	Pontecorvo, Roper & Forbes (1953)
Aspergillus oryzae	< 20	Ikeda, Ishitani & Nakamura (1957)
Aspergillus sojae	0·2, 3	Ishitani, Ikeda & Sakaguchi (1956)
Penicillium chrysogenum	0·025, 0·36	Pontecorvo & Sermonti (1954)
Penicillium expansum	9·4	Barron (1962)
Fusarium oxysporum f. pisi	0·03	Buxton (1956)
Fusarium oxysporum f. cubense	0·6	Buxton (1962)
BASIDIOMYCETES		
Coprinus lagopus	100–1000	Casselton
Ustilago maydis	100,000	Holliday (1961)

The origin of asci with sixteen spores instead of the normal eight spores is therefore uncertain (Elliott, 1960). These features associated with triploid segregation in *Coprinus* possibly represent a difference between heterothallic (*Coprinus*) and homothallic (*A. nidulans*) systems.

In the reports of studies on somatic recombination in both *Coprinus* and *Schizophyllum* the inability to recover a diploid stage has generally been interpreted to mean that diploid nuclei are unstable and rapidly become haploid. But this is obviously not the case, as the present work has shown that common *A* diploid monokaryons of *Coprinus* are extremely stable and only rarely haploidize even when selective pressure is applied. Diploid strains of *Ustilago* are also stable and as yet mitotic haploidization has not been discerned. Heterozygous diploids of this fungus are solopathogenic and it requires the specialized conditions of the brandspores, in which meiosis occurs, for haploid nuclei to be produced. The finding that diploid nuclei of *Coprinus* are unstable when combined in a dikaryon with a haploid nucleus is in contrast to their stability in monokaryons.

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Both instability and the influence of the haploid nucleus in the dikaryon have important implications when the literature on somatic recombination in *Coprinus* and *Schizophyllum* is considered. In general this has been looked for in the dikaryon as such (Parag, 1962) or in di-mon matings. Under these conditions it would be expected that a diploid nucleus arising at random in a dikaryon would rapidly haploidize. For example, in the incompatible di-mon system, e.g. $A_1B_2 \times (A_1B_1)$ (A_2B_2) where selection is made for a recombinant nucleus (A_2B_1) compatible with the monokaryon, a diploid nucleus formed by fusion of the two nuclei of the dikaryon $\frac{A_1}{A_2}\frac{B_1}{B_2}$ would be compatible with the nucleus of the monokaryon, and a new dikaryon could be formed. In such a dikaryon, haploidization of this diploid nucleus would be influenced by the mating-type of the haploid nucleus (A_1B_2) and would result in a recombinant nucleus of the observed type (A_2B_1). Such a mechanism, combined with mitotic crossing-over during the diploid phase, could account for some of the results reported, particularly in view of the very high frequency with which diploid nuclei can arise in the basidiomycetes.

The reason for instability of diploid nuclei in a dikaryon with haploid and diploid nuclei is not clear, but it may be a lack of exact synchrony of a haploid and a diploid nucleus in the conjugate division in the dikaryon. Their instability is undoubtedly the reason why diploid nuclei have not been recovered from systems involving a dikaryon. Whether or not diploid nuclei would be unstable in a dikaryon in which both nuclear components are diploid has yet to be investigated.

SUMMARY

Diploid strains of *Coprinus lagopus* have been synthesized from common *A* heterokaryons either as oidial colonies or sectors. The criteria of growth rate and colony morphology on selective medium were used to distinguish between diploid and heterokaryon colonies. The average oidial size and hyphal width of diploid strains was significantly greater than that of the haploid parental strains.

Diploid monokaryons were very stable and only rarely produced haploid segregants. However, aneuploid intermediates in haploidization have been identified and these segregated further to give haploid monokaryons with recombinant genomes.

Dikaryons formed from diploid and haploid strains produced fruiting bodies. Meiosis and basidiospore production were irregular owing to the formation of triploid or partially triploid fusion nuclei in the basidia. In contrast to their stability in monokaryons, diploid nuclei tended to be unstable when combined in a dikaryon with a haploid nucleus, and often underwent partial haploidization before fruiting. Segregation of genes in the basidiospore progeny reflected whether haploidization had occurred before or after the formation of the fruiting body. If the haploid nucleus had a *B* mating-type allele common to the diploid nucleus, haploidization effected loss of the common allele.

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