HETEROKARYON FORMATION AND GENETIC RECOMBINATION WITHIN ONE ISOLATE OF *THANATEPHORUS CUCUMERIS*

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Summary

Pairs of morphological mutants, of which six were obtained from single basidiospores of one homothallic wild stock, were found to anastomose readily and to form heterokaryons.

With some combinations of mutants a killing reaction followed anastomosis, yet this did not prevent heterokaryon formation. A number of heterokaryons produced were stable, but others were unstable. Some heterokaryons were fertile, others apparently were not. There is insufficient evidence to explain the killing reaction, the lack of stability, and the infertility in some combinations.

Recombination studies were carried out with three fertile heterokaryons that were stable. Tetrad and random-spore analyses showed that four genetic factors were segregating, all independently of one another. Each mutation affected cultural appearance, pathogenicity, and fertility.

I. Introduction

Field isolates of *Thanatephorus cucumeris* (Frank) Donk are usually heterokaryotic for a range of factors (Whitney and Parmeter 1963; Flentje and Stretton 1964; Garza-Chapa and Anderson 1966). In recent years a number of these field isolates have been induced to form the perfect stage either on agar (Whitney and Parmeter 1963), or on soil (Stretton *et al.* 1964), or on other substrates (Sims 1956). The single-basidiospore cultures obtained have almost always varied greatly in cultural appearance (Exner and Chilton 1943), growth rate (Flentje and Stretton 1964), enzyme activity (Papavizas and Ayers 1965), survival ability (Papavizas 1964; Olsen, Flentje, and Baker 1967), virulence (Garza-Chapa and Anderson 1966), and other characters. It is most likely that this variation is due to recombination and segregation in the perfect stage of genetically different nuclei in the original field isolate.

Critical investigation of anastomosis, heterokaryosis, and recombination in relation to variation has been impeded by the lack of suitable genetic markers. Whitney and Parmeter (1963) obtained evidence that a factor affecting the ability of single-basidiospore cultures to grow on Czapek–Dox agar appeared to be simply

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inherited. With this marker they demonstrated that heterokaryons could be synthesized between single-basidiospore cultures from a single parent. Garza-Chapa and Anderson (1966) also studied heterokaryon formation between single-basidiospore isolates, and although it appears from general cultural characters that some pairs of isolates did form heterokaryons, because of the lack of appropriate genetic markers they were unable to fulfil the rigid tests usually required (Davis 1966).

Recently Flentje, Stretton, and McKenzie (1967) obtained a series of spontaneous and induced mutants from a fertile, homothallic, homokaryotic parent isolate. These mutants, which were stable and self-sterile, differed from the parent isolate both morphologically and pathogenically, and have thus provided valuable material for a study of anastomosis, heterokaryosis, and recombination. This paper records the study which was carried out using these mutants.

II. Materials and Methods

The wild-type culture and the mutants used in this investigation have been described previously (Flentje, Stretton, and McKenzie 1967). They are isolate G3-56 (a wild-type single-basidiospore culture), four induced mutants (*sparse*, *stumpy*, *fleecy*, and *curly*), and two spontaneous mutants (*rusty* and *ropy*) which were all derived from G3-56. These all differed from the parent isolate in their growth habit on agar media and, apart from *fleecy*, were non-pathogenic.

The general medium used was potato–Vegemite–dextrose agar (P.V.D.A.), with Vegemite substituted for the Marmite in the original description (Flentje 1957) and instant mashed potatoes (Lacy and Bridgmon 1962) used instead of fresh potatoes. Soil agar, supplemented with antibiotics (10 p.p.m. aureomycin, 10 p.p.m. neomycin, and 10 p.p.m. streptomycin) when there was a risk of bacterial contamination, was used for collecting, isolating, and germinating basidiospores.

Methods have been described previously for determination of anastomosis and formation of heterokaryons (Flentje and Stretton 1964), for pathogenicity tests (Flentje, Stretton, and McKenzie 1967), and for fruiting (Stretton *et al.* 1964). In the following work mycelia from different mutant cultures were macerated together to encourage the formation of heterokaryons, viz: small blocks approximately 3 mm^3 of agar culture were pulped together with a glass rod and the pulp was then transferred to a P.V.D.A. plate. Whenever two cultures were macerated together in this way, the individual cultures were also macerated and cultured separately for comparison with the heterokaryon. Both random basidiospores and tetrads were isolated. Spores were shed on to Cellophane over soil agar and then isolated with a glass needle carrying a small piece of agar. For tetrad isolation direct from the hymenium, a micromanipulator was commonly used.

In recombination studies, progeny were classified according to morphology, pathogenicity, and fertility, whilst a heterokaryon test with each parent mutant was used to determine homology, viz: if the resulting heterokaryon had a wild-type growth habit, the progeny was non-homologous with the parent mutant.

III. Experimental Details and Results

(*a*) *Anastomosis and Heterokaryon Formation between Mutants and G3-56*

The six mutants and G3-56 were opposed in pairs in all possible combinations on Cellophane over agar. Pairings between all combinations resulted in anastomosis but the cell reactions following anastomosis varied (Table 1). Pairing of G3-56 with any mutant resulted in anastomosis without subsequent cell death (S reaction). However, because any wild-type heterokaryon produced from these pairings between G3-56 and a mutant would be culturally indistinguishable from G3-56, investigations

to establish heterokaryosis in these pairings were not continued. Pairings of *sparse*+*fleecy*, and *rusty*+*ropy* also gave S reactions. In all others, cell death occurred within 12–24 hr of the demonstration of cytoplasmic connections. The killing reaction was either complete (K reaction), where 100% of the anastomoses resulted in cell death, or intermediate (I reaction) with 70–90% of the anastomoses resulting in cell death while the remaining 30–10% were apparently unaffected. Four to five days later, a zone of dead cells was clearly visible at the line of junction between the mutant colonies in those pairings in which the killing reaction had occurred.

The killing reaction, however, did not prevent heterokaryon formation. Wild-type mycelium, as shown in Figure 1, grew out from all 15 combinations of paired mutants, including those where 100% of the anastomoses had resulted in a killing reaction.

TABLE 1

ANASTOMOSIS REACTIONS OF MUTANTS AND G3-56 WHEN OPPOSED ON CELLOPHANE OVERLYING AGAR

S = anastomosis with no subsequent cell death; K = anastomosis followed by cell death in 100% of cases; I = anastomosis followed by cell death in 70–90% of cases

Culture	Culture					
	sparse	*stumpy*	*curly*	*rusty*	*ropy*	*fleecy*
G3-56	S	S	S	S	S	S
sparse		K	I	I	K	S
stumpy			I	K	K	K
curly				I	I	I
rusty					S	I
ropy						K

The extent and rate of the killing did not appear to be influenced by temperature as the same results were obtained with mutants opposed at 5 and 25°C on Cellophane. However, temperature apparently affected heterokaryon formation. When the pairs of mutants were incubated at 5°C for several days over the period of anastomosis and then incubated at 25°C, wild-type sectors developed only from the pairs which anastomosed without cell death, viz. *sparse*+*fleecy*, and *rusty*+*ropy*. The rate of growth of mutants and wild-type was much slower at 5 than at 25°C. For example, wild-type hyphae elongated by 1–2 cells every 12 hr at 5°C whereas at 25°C 40–59 new cells were formed. It is probable that temperature affected heterokaryon formation through growth rate rather than through anastomosis.

(*b*) *Heterokaryosis and Recombination*

All 15 combinations of the six mutants produced wild-type growth when macerated in pairs and grown on agar. But only the four combinations *curly*+*rusty*, *curly*+*ropy*, *stumpy*+*ropy*, and *fleecy*+*ropy* were fertile and therefore suitable for recombination studies. However, the growth from the combination of *fleecy*+*ropy* was unstable, and will be discussed in a separate paper. The other three fertile combinations are discussed below.

(i) *The* curly+rusty *Heterokaryon*

Hyphal tips, taken from the putative heterokaryon *curly+rusty* (Fig. 2D), produced cultures indistinguishable from one another, wild-type in appearance and growth rate, and which anastomosed successfully with one another without cell death. Stability was evidenced by lack of cultural change after regular subculturing for 6 months.

TABLE 2

CLASSIFICATION OF SINGLE-BASIDIOSPORE PROGENY (SHED SPORES) FROM THREE HETEROKARYONS

Genes in the mutants *curly*, *rusty*, *ropy*, and *stumpy* are designated *cu*, *ru*, *ro*, and *st* respectively

Heterokaryons	Progeny				
	Wild-type	Parental Types		Double Mutant	Dead	Total
curly+rusty						
Genotype	+ +	*cu* +	+ *ru*	*cu ru*		
Frequency	12	19	19	8	9	67
curly+ropy						
Genotype	+ +	*cu* +	+ *ro*	*cu ro*		
Frequency	17	82	4	7	36	146
stumpy+ropy						
Genotype	+ +	*st* +	+ *ro*	*st ro*		
Frequency	18	37	18	7	54	134

The heterokaryon was indistinguishable from G3-56 in cultural appearance (Fig. 2), growth rate, pathogenicity (Fig. 4), and fruiting ability. Four cultural types of single-basidiospore progeny were recovered from this heterokaryon (Table 2), viz: wild-type (indistinguishable from G3-56), the two mutant types (*curly*, *rusty*), and a very slow growing "button"-type. Each wild-type was subsequently induced to fruit and the resultant basidiospores were 95–99% viable, yielding only wild-type progeny. Thus the wild-type was presumably the non-mutant recombinant. The *curly*- and *rusty*-type progeny were culturally identical and genetically homologous with *curly* and *rusty* respectively. Macerations of the button-type progeny with either *curly* or *rusty* produced only *curly* or *rusty* growth respectively, but with a third mutant (*sparse*, *stumpy*, or *ropy*) a wild-type heterokaryon was established, suggesting that the button progeny were double-mutant recombinants. These four phenotypes were recovered in frequencies not significantly different from equality ($\chi^2_3 = 6{\cdot}138, P = 0{\cdot}1\text{–}0{\cdot}05$) (Table 2); they may all have resulted from heterozygotes or may have included some selfed progeny. A second sample, however, yielded only *curly* progeny presumably resulting from basidia homozygous for *curly*. From these data it was postulated that *curly* and *rusty* phenotypes are each due to a single gene difference from wild-type and that these genes segregate independently. If the gene in the *curly* mutant is designated *cu* and that in *rusty ru*, then the genotypes of the progeny of this cross may be written: + + (wild-type), *cu* + (*curly*), + *ru* (*rusty*), and *cu ru* (double mutant). The *cu ru* colony on P.V.D.A. is small (2·0 cm diameter in 6 days) and dense, initially white but rapidly turning dark brown in colour. Hyphal branching is irregular and similar to that of *curly* but septation is regular like that in *rusty*. It is non-pathogenic to radish, and self-sterile.

Analysis of tetrads of basidiospores verified this hypothesis, as the results in the following tabulation show:

	Selfed Progeny		Hybrid Progeny				
	curly	*rusty*	Parental Ditype	Non-parental Ditype	Tetratype	Incomplete Progeny	Aberrant Progeny
Tetrad frequency	16	0	3	5	6	10	5

One group of tetrads was from the same fructifications as the shed basidiospores; another group was from fructifications resulting from reconstitution of the heterokaryon 7 months later. Of the total of 35 complete tetrads examined, 16 had resulted

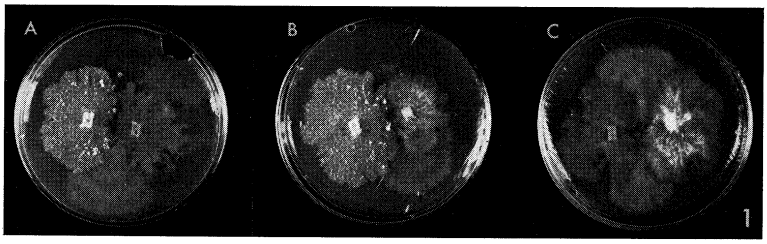

Fig. 1.—Sectors of wild-type mycelium formed from the zones of cell-death between paired mutants. A, *curly*+*rusty*; B, *curly*+*sparse*; C, *ropy*+*sparse*.

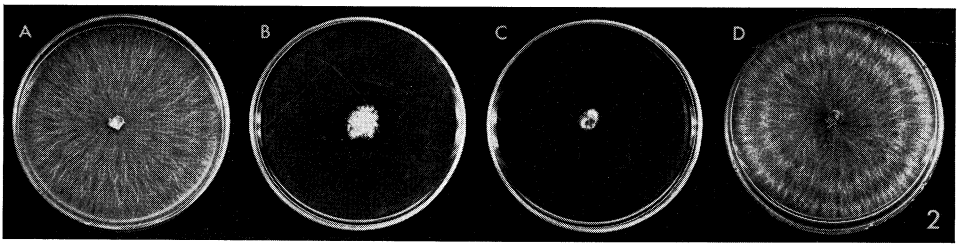

Fig. 2.—Cultural characteristics of wild-type parent G3-56 (A); mutants *curly* (B) and *rusty* (C); and heterokaryon resulting from maceration of *curly*+*rusty* (D).

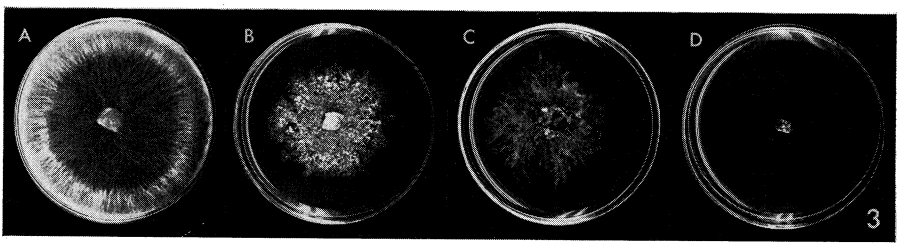

Fig. 3.—Single basidiospore progeny from the heterokaryon *curly*+*ropy*, showing four cultural types: wild-type (A); *curly* type (B); *ropy* type (C); and slower-growing type (double-mutant recombination) (D).

from selfing of *curly*; no selfed tetrads of *rusty* were recovered. There were 14 complete tetrads which must have resulted from basidia containing genetically different nuclei before diploidization. The frequency of parental ditype and non-parental ditype tetrads does not differ significantly from equality, indicating that the genes are unlinked (Perkins 1953). Five tetrads did not fit the hypothesis but may have resulted from errors in isolation from a dense hymenium actively shedding basidiospores.

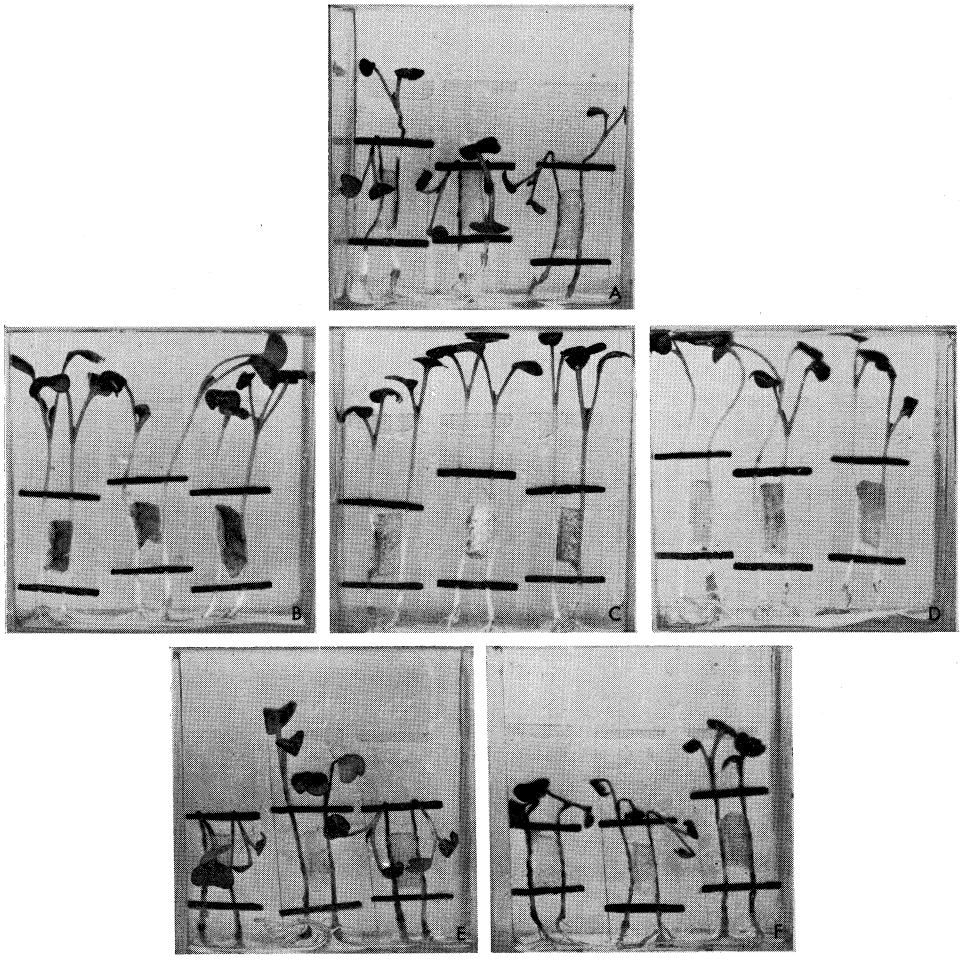

Fig. 4.—Pathogenicity tests on radish seedlings of G3-56 (A); mutants *rusty* (B), *curly* (C), and *ropy* (D); and heterokaryons *curly*+*rusty* (E) and *curly*+*ropy* (F).

Strands of hymenium including basidia with sterigmata, prebasidial cells, and vegetative runner hyphae were obtained, divided into the vegetative and reproductive components, and plated on Cellophane over soil agar containing antibiotics. Mycelial growth arising from basidial and prebasidial cells was either wild-type or *curly*; from each vegetative hypha it was wild-type; no *rusty* hyphae were detected. It would

thus appear that either the heterokaryon contained a greater proportion of *curly* than *rusty* nuclei, or there was a selection for *curly* nuclei in the prebasidial cells.

(ii) *The* curly+ropy, *and* stumpy+ropy *Heterokaryons*

Similar results were obtained from these heterokaryons as with *curly+rusty*, viz. wild-type heterokaryons yielding progeny of four morphological types (Fig. 3; Table 2). High percentages of *curly* and *stumpy* produced by these respective heterokaryons is probably due to the formation of homozygous as well as heterozygous basidia. However, selfing was not demonstrated as no tetrads could be isolated from the heterokaryon.

Although from each heterokaryon the frequency of the two recombinant types, wild-type and double mutant, differed significantly from equality with the wild-type in excess, one might expect the wild-type to be more viable than the double mutant.

TABLE 3

CLASSIFICATION OF SINGLE-BASIDIOSPORE PROGENY FROM HETEROKARYONS BETWEEN SINGLE-MUTANT AND DOUBLE-MUTANT NUCLEI

Genes designated as in Table 2

Phenotype	Heterokaryon *cu ru+stumpy*		Heterokaryon *cu ru+ropy*							
	Genotype	Shed Spores	Genotype	Shed Spores	Tetrads 1 and 2	3 and 4	5	6	7*	8†
Wild type	+ + +	9	+ + +	13	—	1	2	—	1	—
curly	*cu* + +	3	*cu* + +	10	1	1	—	1	—	1
rusty	+ *ru* +	11	+ *ru* +	14	1	—	—	—	—	2
stumpy or *ropy*	+ + *st*	7	+ + *ro*	13	—	—	—	1	(1)	—
Double mutant	*cu ru* +	6	*cu ru* +	30	—	—	—	1	1	—
Double mutant	*cu* + *st*	44	*cu* + *ro*	11	1	—	—	—	—	1
Double mutant	+ *ru st*	7	+ *ru ro*	13	1	1	—	1	—	—
Triple mutant	*cu ru st*	8	*cu ru ro*	9	—	1	2	—	1	—
Dead		98		67						
Total		193		180						

* Three-spored basidium (see text).

† Aberrant tetrad (see text).

(iii) *Heterokaryons Involving Single-mutant and Double-mutant Nuclei*

To establish the identity of the *cu ru* double mutant derived from the heterokaryon *curly+rusty*, it was combined with other mutants. In combinations of *cu ru* with *ropy*, and *cu ru* with *stumpy*, each gave wild-type growth which was culturally identical with G3-56, as well as being fertile and pathogenic to radishes. Progeny (shed spores) of each of these two combinations were classified into eight cultural types, namely: wild-type, the three single mutants, and four other types (Table 3). All the wild-type cultures, with one exception discussed below, yielded uniform wild-type progeny. The four new cultural types were identified by heterokaryon tests to be the three possible double mutants and the triple mutant. No mutant colony was self-fertile. Pathogenicity was not determined for the triple mutant or double mutants other than *cu ru*, which is non-pathogenic to radish, due to their

slow growth. The triple- and other double-mutant colonies reached maximum diameters of less than 12 mm after 3 weeks on P.V.D.A.

Progeny from shed spores of the heterokaryon *cu ru*+*ropy* appear to be the result both of selfing of *cu ru* and of crossing. The six recombinant types of progeny occurred with frequencies not significantly different from equality ($\chi^2_5 = 1{\cdot}657$, $P = 0{\cdot}9$–$0{\cdot}8$), indicating independent segregation of three single-gene differences. The spores from one three-spored basidium and each of seven four-spored basidia were isolated. The colonies produced from the three-spored basidium were one wild-type, one double mutant (*cu ru* +) and one triple mutant (*cu ru ro*), where *ro* designates the gene in the *ropy* mutant. The wild-type culture fruited, and a sample of spores yielded 41 wild-type and 7 *ropy* progeny plus 5 non-viable spores; this segregation suggests that the wild-type culture had been derived from a basidiospore which contained two nuclei, one + + + and one + + *ro*. For the purposes of subsequent tetrad analysis, the nuclei produced in the three-spored basidium were considered as four types, the fourth being *ropy*. All tetrads appeared to have been derived from hybrid basidia, and when classified according to tetrad types, seven fitted the hypothesis of three single-gene differences. With this small number of tetrads for analysis, the recovery of so many non-parental ditypes is evidence against linkage. There is not enough evidence to conclude that the spores from the aberrant tetrad (Table 3) came from more than a single basidium.

The results from the *cu ru*+*stumpy* heterokaryon were similar to those above.

IV. Discussion

The above studies show that heterokaryons all formed between mutant cultures derived from one homokaryotic parent isolate. Recombination occurs consequent to the formation of heterozygous nuclei in the basidia.

Tetrad and random-spore analyses of the various combinations of mutants showed that the four mutant types involved (*curly*, *rusty*, *stumpy*, and *ropy*) resulted from non-linked single gene mutations. Their specific effects on cultural appearance and pathogenicity have been described previously (Flentje, Stretton, and McKenzie 1967). In view of their effects on pathogenicity, and also the fact that *rusty* and *ropy* were spontaneous mutants, it is surprising that such a small percentage of the first generation single-basidiospore cultures obtained from field isolates in this laboratory and by other workers have been non-pathogenic. It is possible that under conditions of natural competition in the field, nuclei carrying mutations for avirulence are not maintained in the heterokaryon. As the heterokaryons were all wild-type in cultural characters and pathogenic, it was concluded that these are dominant characters.

The colonies which developed from the progeny of a three-spored basidium produced by the heterokaryon *ropy*+*cu ru* were: one wild-type, one double mutant, and one triple mutant. On fruiting the wild-type culture, both wild-type and *ropy* progeny could be recovered. This is the first genetic evidence in *T. cucumeris* of a culture derived from a binucleate basidiospore and supports earlier evidence of Flentje, Stretton, and Hawn (1963) that two nuclei may migrate from a basidium and enter a single basidiospore. It also emphasizes the importance of cytological studies as a complement to genetical studies in *T. cucumeris*.

Several problems regarding anastomosis, heterokaryon compatibility, and fertility emerged during these studies.

In the anastomosis studies, the killing reaction which occurred between paired combinations of mutants has also been observed in pairings of non-mutant progeny and of different field isolates of *T. cucumeris* (Flentje and Stretton 1964; Stretton, Flentje, and McKenzie 1966). In the present studies it appeared to vary in severity; some pairings resulted in death at every point of anastomosis while in others only 70–90% of anastomoses were affected. In all cases cell death occurred 12–24 hr after anastomosis, and involved not only the anastomosed cells, but also up to six contiguous cells on either side.

However, this killing reaction did not prevent the formation of heterokaryons at 25°C, where the growth rate (40–59 new cells in 24 hr) was apparently sufficient for the terminal cells to escape this reaction. In contrast, at 5°C the slower growth rate (3–5 new cells in 24 hr) allows for all the cells of the developing heterokaryon to be killed.

It thus appears that although there is some incompatability between the cytoplasm of the hyphae when they anastomose, modification to overcome this incompatability may occur rapidly in the cells of the heterokaryons formed by pairs of mutants.

The killing reaction was not mentioned by Whitney and Parmeter (1963) or by Garza-Chapa and Anderson (1966) in their studies of heterokaryon formation. Each found, however, that certain combinations of single-spore cultures failed to form heterokaryons, and proposed a hypothesis of compatability types, analogous to mating types, operating. It is difficult, however, to see how the pattern of heterokaryon formation described above between cultures differing by a single gene mutation can fit such a hypothesis and there is an obvious need for further detailed investigation.

Also, Flentje and Stretton (1964) suggested, partly on the basis of the killing reaction, that heterokaryon formation between different wild-type strains in the field probably may be very restricted. The present results throw doubt on this suggestion.

Only some of the heterokaryons appeared to be fertile. Environmental conditions are known to influence the development of the perfect state but in all fruiting experiments with these heterokaryons, G3-56 was included for comparison, and each time G3-56 fruited. No heterokaryon which was formed from two irradiated mutants produced the basidial stage, but neither did the heterokaryon which was formed from the two spontaneous mutants. The effect on fertility is unlikely to be due to cytoplasmic factors alone as mutant progeny from several crosses remained infertile. That six different mutations affected fertility suggests that there may be different stages in the sexual progression at which blockages may occur, as has been postulated by Raper (1960) for some Ascomycetes. This suggestion is supported by the isolation of *curly* hyphae from the basidial cells of the *curly*+*rusty* heterokaryon and by the tendency of *curly*, *stumpy*, and *cu ru* to self in some crosses. This indicates that a blockage may occur with these mutants prior to basidial formation. As there were no *rusty* hyphae isolated from the basidial cells of the *curly*+*rusty* heterokaryon and apparently no selfing with *rusty*, it is likely that the blockage in this mutant was in basidial development. There is, however, insufficient evidence to indicate precisely

where the blockage occurs, or to explain the apparent infertility of the majority of the heterokaryons formed by pairs of mutants. As fertility is a characteristic of wild-type, infertility may be due to incomplete complementation of mutants even though cultural characters and pathogenicity are completely restored. Alternatively, the nuclear ratio may affect fertility. It is very likely, however, that mutations such as those described above are at least partly responsible for the infertility of single-basidiospore cultures reported by a number of workers.

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