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Induced mitotic crossing-over in *Ustilago maydis*

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Heterothallism in *Ustilago maydis* was first conclusively demonstrated by Hanna (1929). Infection and the production of diploid brandspores in the host, *Zea mays*, can normally only take place following the formation of a dikaryon from two compatible haploid strains. Rowell & De Vay (1954) and Rowell (1955*a*) showed that these processes were under the control of two loci, the *a* locus with two alleles and the *b* locus with multiple alleles. During the course of extensive investigations into the pathogenicity and mating system of *U. maydis*, several workers have encountered stable homothallic strains which were pathogenic when inoculated alone into the host (see Christensen & Rodenhiser, 1940; Holton, 1953). These strains appeared to be formed following the failure of reduction at meiosis during the germination of the brandspores. The frequency of their occurrence varied very considerably in different crosses. Strains derived from brandspores produced by solopathogenic strains often showed segregation for compatibility factors, which demonstrated that the solopathogens were heterozygous for such factors. Christensen (1931) found that the vegetative cells of sporidia were uninucleate, and considered that the strains were diploid rather than dikaryotic. Rowell (1955*b*) obtained further evidence for this by showing that somatic segregation and recombination of the two compatibility loci could be induced by alpha radiation. Had the sporidia of the solopathogen not been diploid, only the original parental genotypes should have been recovered as segregants.

Pontecorvo, Tarr-Gloor & Forbes (1954) and Pontecorvo & Kafer (1958) have demonstrated that segregation from heterozygous diploid strains of *Aspergillus nidulans* can take place as a result of two independent processes. The first is mitotic crossing-over, which had already been discovered and analysed in detail in *Drosophila* by Stern (1936). This process takes place at the four-strand stage and is followed by normal mitotic segregation of centromeres. It is detected by the fact that half the cross-overs result in the production of homozygosity from the point of exchange to the end of the chromosome arm. An analysis of mitotic crossing-over indicates the position of the centromere and the linear order of markers within each arm. The second is the process of haploidization, during which markers on different chromosomes may recombine, but markers on the same chromosome always segregate together. This allows markers to be assigned to specific linkage groups. Since the initial work on *Aspergillus*, somatic segregation (in which mitotic crossing-over and haploidization have not usually been distinguished)

has been demonstrated in a number of other Ascomycetes or imperfect fungi (see reviews by Pontecorvo, 1956, 1959).

If solopathogenic strains of *U. maydis* are indeed diploid it should be possible to obtain such strains which are heterozygous for several biochemical markers, and to follow the segregation of these. The present work was originally undertaken in order to examine the possibilities of using somatic segregation as a routine genetical technique in the construction of linkage maps in *U. maydis*; it has in addition suggested the means whereby an experimental investigation of the mechanism of mitotic crossing-over could be undertaken.

METHODS

Strains. The same stocks as those previously described (Holliday, 1961) have been used. Five biochemical markers have been employed: *ad-1*, *me-1*, *leu-1*, *pan-1* and *nic-3* (growth requirement of adenine, methionine, leucine, pantothenic acid and nicotinic acid respectively).

Media. The composition of the complete and minimal media previously used has been modified. The following components have been altered:

Agar: New Zealand Davis agar.

Salt solution: made up to twice the concentration used previously and 62·5 ml. added to each litre of medium.

Hydrolysed casein: 'Oxoid' hydrolysed casein, 2·5 g. per litre of complete medium.

Yeast extract: 'Oxoid' yeast extract, 1 g. per litre of complete medium.

The pH of media was adjusted to 7·0 before sterilization.

Ultra-violet light irradiation. In order to induce segregation, 10 ml. of a suspension of diploid sporidia in distilled water were exposed with continual agitation in an open petri dish 16 cm. from a 7-watt Hanovia low-pressure mercury lamp. Eighty-five per cent of its output was in the 2537 Å region, and the dose at this distance was 1300 ergs/cm^2/sec. Sporidia were either freshly grown on complete medium or had been stored in suspension at 3°C. for short periods.

The isolation and identification of auxotrophs. Sporidia were spread on plates on complete agar and the colonies replicated to minimal medium (Lederberg & Lederberg, 1952). Late-germinating or slow-growing colonies less than 0·5 mm. in diameter often failed to replicate and were therefore usually ignored. Auxotrophs were isolated by removing some of the non-growing cells on minimal medium and inoculating them on plates of complete medium. Their requirements were tested either by replication to plates of supplemented minimal medium or by auxanographic techniques.

Inoculation of maize seedlings. A new method of inoculation has been used which is considerably quicker and simpler than inoculating with a hypodermic syringe, or by the partial-vacuum method of Rowell & De Vay (1953). Seeds of maize variety Golden Bantam were dusted with mercury seed dressing and germinated on moist plastic foam at 25–30°C. Seedlings with a coleoptile length of 1–4 cm. were selected for inoculation. Inocula were prepared by streaking sporidia on

plates of complete medium (usually four streaks per plate) and incubating for 2 days. The coleoptile and young leaves of the seedlings were severed with a scalpel about 2 mm. above the node. The exposed surface of the shoot was jabbed successively into the appropriate inocula. Six seedlings were inoculated for each mating type test or for tests of solopathogenicity. The seedlings were grown under the same condition as previously described. This method of inoculation resulted in early death or extreme stunting of about 20 % of the seedlings, irrespective of the pathogenicity of the inocula. In tests between compatible haploid strains, as high a proportion of the surviving seedlings developed galls as with other methods of inoculation.

Other methods and techniques are the same as described previously or are given in the text.

EXPERIMENTAL RESULTS

(*a*) *The detection of solopathogenic diploid strains*

From analysis of tetrads (Holliday, 1961) it was known that the marker *ad-1* was situated between *me-1* and *leu-1*, about 30 units of recombination from the former and 20 from the latter. A fourth marker, *pan-1*, which is closely linked to *a*, was probably loosely linked to *leu-1*. A cross was set up with adjacent markers in repulsion and including a further marker, *nic-3*, unlinked with any of the others: *me-1 leu-1* a_2b_2 × *ad-1 pan-1 nic-3* a_1b_1. From this cross, wild-type prototrophic progeny should be obtained only as a result of a double cross-over, or triple cross-over if *leu-1* and *pan-1* are linked. If any diploid strains arose these should also be prototrophs since biochemical markers are recessive.

It was found that about 10 % of a random sample of progeny were in fact stable prototrophs. It was very unlikely that all of these could have arisen as a result of multiple exchanges. Several isolates were inoculated into maize seedlings and were found to be solopathogenic. This particular cross appeared to produce an unexpectedly high frequency of failure of reduction at meiosis, whereas other crosses between stocks derived from the same wild-type strains did not produce detectable abnormalities. The galls produced by these solopathogenic strains were rather small, very irregular in shape and green or translucent in appearance, and were quite distinct from the large smooth white galls which are usually produced when two compatible haploid strains are inoculated. Some of these differences are shown in Fig. 1, Plate I.

One solopathogenic strain designated Diploid I was selected for detailed examination. All five biochemical markers were recovered in the progeny of a sample of brandspores produced by this strain. The sporidia were stained with basic fuchsin after acid hydrolysis (DeLamater, 1948), and were found to be regularly uninucleate. The diameter of the conidia has been used in *Aspergillus* as a criterion of diploidy, but in *Ustilago* sporidia are too variable in size (depending on their state of division) for this to be possible. The diploidy of sporidia has, however, been confirmed by other means. In sporidia stained with orcein after cold acid hydrolysis La Cour (in the press, and unpublished) observed two chromosomes at

metaphase in a haploid strain and four chromosomes in Diploid I. Dr N. Sunderland, of this Institute, has measured the deoxyribonucleic acid (DNA) content of sporidia of Diploid I, of an artificially synthesized diploid (see below) and of two wild-type haploids, $a_1 b_2$ and $a_2 b_1$. Using the methods described by Sunderland & McLeish (1961) he found that the values for the diploids were 3·9 and 4·0 g. $\times$ 10^{-13} per sporidium and for the haploids 2·0 and 1·8 g. $\times 10^{-13}$ per sporidium.

There can be little doubt that solopathogenicity is a reliable criterion of diploidy in *U. maydis*.

(*b*) *The segregation of Diploid I:*

$$\frac{+}{nic\text{-}3} \quad \frac{me\text{-}1 \quad + \quad leu\text{-}1}{+ \quad ad\text{-}1 \quad +} \quad \frac{+ \quad a_2}{pan\text{-}1 \; a_1} \quad \frac{b_2}{b_1}$$

A search was made for auxotrophic segregants arising spontaneously from Diploid I. About 200 sporidia were spread on each of a series of plates of complete medium. On replication to plates of minimal medium, about 0·1 % of the colonies proved to be auxotrophic, and three recessive markers were recovered in the small sample of segregants which was obtained (see Table 1).

In order to increase the sample of segregants without the labour of scoring very large numbers of colonies, two procedures are possible. Special techniques can be used which automatically select rare spontaneous segregants. Several have been developed for this purpose in *Aspergillus nidulans* (Pontecorvo & Kafer, 1958). Alternatively the rate of production of segregants can be increased by artificial treatments. Ultra-violet light has been used by Ikeda, Ishitani & Nakamura (1957) with *Aspergillus sojae*, and by James (1955), James & Lee-Whiting (1955) and Roman & Jacob (1958) with *Saccharomyces*; X-rays and nitrogen mustard by Sermonti & Morpurgo (1959) and Morpurgo & Sermonti (1959) with *Penicillium chrysogenum*; alpha-particles by Rowell (1955*b*) with *Ustilago maydis*, and formaldehyde, as well as ultra-violet light and nitrogen mustard, by Fratello, Morpurgo & Sermonti (1960) with *Aspergillus nidulans*. The use of treatments such as these, combined with indirect selection by means of replica plating, appeared to be the most convenient method at least for initial experiments.

Three experiments were carried out with low, high and intermediate doses of ultra-violet light. The results are presented in Table 1. The proportion of segregants among the survivors of the irradiation rose with increasing dose. The increase could not be due to the preferential survival of segregants already present in the cell population prior to irradiation, since with the low dose of radiation the rate of segregation was seven times higher than the spontaneous rate, whereas only 40 % of the cells were killed. There was therefore an absolute increase in segregation frequency.

All five markers were recovered amongst the segregants. With the exception of a single methionine requirer, all the segregants fell into the five classes: *ad-1*, *leu-1*, *me-1 leu-1*, *pan-1* and *nic-3*. There appeared to be no significant differences in the frequencies of the types of segregant obtained in the different experiments. The fact that so few classes of segregant were obtained and that all except one had

Table 1. *The segregation of Diploid I:*

$$\frac{b_2 \quad me\text{-}1 \quad + \quad leu\text{-}1}{b_1 \quad + \quad ad\text{-}1 \quad +} \qquad \frac{+ \quad + \quad a_2}{nic\text{-}3 \quad pan\text{-}1 \quad a_1}$$

Treatment	Survival (%)	Segregants* (%)	Frequency of segregants with requirement for: *nic*	*pan*	*ad*	*leu*	*leu*+*me*	*me*
None		0·11 ± 0·04	6	1	2	0	0	0
U.V. light (min.)								
1	60	0·70 ± 0·10	41	3	8	4	0	0
2½	3	3·38 ± 0·72	22	9	12	1	4	1
4	0·10	6·50 ± 1·74						
4½	0·07	7·14 ± 1·24	27	8	5	3	7	0
5	0·02	9·11 ± 2·55						
		Total U.V. treated	90	20	25	8	11	1
0·1% caffeine medium (days)								
2		0·94 ± 0·32	1	0	6	0	0	0
3		1·34 ± 0·28	7	0	10	1	5	0
4		1·11 ± 0·42	1	0	4	0	4	0
11		13·91 ± 1·44	4	44	1	0	0	0
		Total caffeine treated	13	(44)	21	1	9	0

* Mosaic colonies (see text) scored as single segregants.

a single requirement, indicates that haploids were not being formed. Chromosomes assort at random during haploidization, therefore it this process was occurring both parental genotypes should appear amongst the segregants, and if the markers are distributed on at least two chromosomes, as seems probable, then some recombinant genotypes should be detected. There were no recombinants and only one class, *me-1 leu-1*, which could have been of parental genotype. The simplest interpretation of the results is that the segregants were diploid, and that each of the five types arose as a result of a single mitotic exchange leading to homozygosity from the position of crossing-over to the end of the arm. Since *leu-1* and *me-1 leu-1* segregants were obtained and these markers are linked, it appears that *me-1* is between *leu-1* and the centromere. The single *me-1* segregant could have arisen as a result of a double exchange. The marker *pan-1* had been thought to be distal to *leu-1*. If so, single mitotic exchanges should have given rise to *pan-1* and *ad-1 pan-1* segregants. The data indicate that the evidence previously obtained for loose linkage between *leu-1* and *pan-1* was misleading; and that *pan-1* is on a separate chromosome arm. *Nic-3* must be positioned on a third arm. Mitotic crossing-over does not indicate whether different arms belong to the same or different chromosomes. Since *leu-1* is further from the centromere than *ad-1*, there should have been a greater number of segregants with this marker, but evidence from meiotic analysis has consistently shown that progeny carrying *leu-1* tend to have low viability.

It was found that after the lowest dose of ultra-violet radiation several of the colonies were of mixed phenotype. Ten were observed which consisted of a mixture of auxotrophic and prototrophic sporidia. Their replicas on minimal medium consisted of a semicircle of growing cells next to one of non-growing cells. The latter in each case responded to nicotinic acid. Two auxotrophic colonies were detected which were found to consist of a mixture of *ad-1* and *leu-1* segregants. These mosaic colonies would have arisen if the reciprocal products of a single cross-over survived. They correspond to the twin spots in *Drosophila*, by means of which Stern (1936) was able to prove the occurrence of mitotic crossing-over. Similar mixed colonies have been observed in yeast after ultra-violet irradiation of heterozygous diploid strains (James, 1955; James & Lee-Whiting, 1955). Mosaic colonies are liable to remain undetected, so their true frequency is probably higher than that observed.

Witkin (1958) has shown in *Escherichia coli* that post-irradiation treatment with caffeine can greatly increase the mutagenic effect of ultra-violet light, and an experiment was planned to determine whether a corresponding increase of somatic segregation could be achieved by this means. It was found, however, that caffeine itself induced segregation. Sporidia grew very slowly on a complete medium containing 0·1 % caffeine. After two or more days' growth sporidia were harvested, suspended in water and plated on complete agar. Viability of sporidia was complete or nearly so. The results of these experiments are given in Table 1. The same types of segregants were obtained as previously, but the frequencies differed. There was no longer an excess of *nic-3*, and there were no *pan-1* segregants except after the 11-day treatment, when they became extremely frequent. Clearly some selective mechanism for this segregant was operating. This could have been due to partially recessive mutations at a locus in the same arm as *pan-1* which conferred some resistance to caffeine. Mitotic crossing-over could lead to homozygosity of this locus and *pan-1* together with greater resistance to caffeine.

(c) *The artificial synthesis of diploids*

It was considered necessary to confirm the above conclusions by obtaining a diploid with the same markers in different coupling and repulsion arrangements. Suitably marked haploid stocks were available but when these were intercrossed abnormal segregation at meiosis was not observed. Therefore any prototrophic solopathogenic strains among the progeny were likely to be too rare to be detected. Nor were the methods which had been used in other fungi possible in *U. maydis*, since balanced heterokaryons between different auxotrophs are not obtainable. A suggestion by my colleague Dr P. R. Day led to the discovery of a new method for selecting diploid strains.

Maize seedlings were inoculated with *ad-1 me-1* a_2b_1 and *leu-1 pan-1* a_1b_2. The *nic-3* marker was omitted since the majority of segregants from Diploid I were nicotinic-acid requirers, whereas interest was centred mainly on the behaviour of the other four markers. As soon as galls appeared, which was 5 days after inoculation, pieces of tissue were removed from the centre of the galls under aseptic

conditions and placed on plates of minimal medium. Brandspores are not present in galls of this age. The dikaryotic mycelium is unable to grow on minimal medium, and the chance of obtaining reversions to prototrophy in either of the inoculated strains was negligible. There should be very strong selection for any diploid nuclei which could initiate sporidial growth. Each of several pieces of gall tissue yielded one or more colonies of vigorously growing prototrophic sporidia. Twelve sporidial isolates from six such colonies were made and all were found to be solopathogenic. The galls were similar to those produced by Diploid I. One of the isolates was selected for further examination. It will be referred to as Diploid II.

This method has been subsequently used in the routine synthesis of various heterozygous diploids from auxotrophic haploids (unpublished data). For instance, several diploids were obtained by this means from the same strains which gave rise to Diploid I. The DNA content of one of these was measured and found to be the same as that of Diploid I (see above.)

(*d*) *The segregation of Diploid II:*

$$\frac{me\text{-}1 \quad ad\text{-}1 \quad +}{+ \quad + \quad leu\text{-}1} \qquad \frac{+ \quad a_2 \quad b_1}{pan\text{-}1 \quad a_1 \quad b_2}$$

No spontaneous segregation was detected in a sample of over 6,000 colonies. Four experiments with ultra-violet treatment and one with caffeine were carried out. In detecting and classifying segregants a careful search was made for any mixed colonies which could consist of the reciprocal products of crossing-over. The results are shown in Table 2.

Table 2. *The segregation of Diploid II:*

$$\frac{b_1 \quad me\text{-}1 \quad ad\text{-}1 \quad +}{b_2 \quad + \quad + \quad leu\text{-}1} \text{ o } \frac{+ \quad a_2}{pan\text{-}1 \quad a_1}$$

Treatment		Segregants* (%)	Frequency of segregants with requirement for: *pan*	*ad*	*ad* + *me*	*leu*	*me*	*me* + *leu*	Frequency of mosaic colonies† (reciprocal products of crossing-over) *pan*/+	*ad*/*leu*	*ad me*/*leu*
None		< 0·047§	none								
U.V. light (min.)	Survival (%)										
1	53	0·72 ± 0·12	20	9	4	7	1	0	12	3	2
2	10	2·10 ± 0·69	24	4	8	9	0	1	0	0	1
4	0·8	1·85 ± 0·41	41	14	14	10	2	0	1	1	2
5	0·08	3·06 ± 0·53	12	5	8	8	3	0	0	0	0
	Total	U.V. treated	97	32	34	34	6	1			
0·1% caffeine medium											
4 days		1·72 ± 0·44	0	1	4	6	0	0			

* Mosaic colonies scored as single segregants.
† Components included in totals in previous columns.
§ 5% upper fiducial limit.

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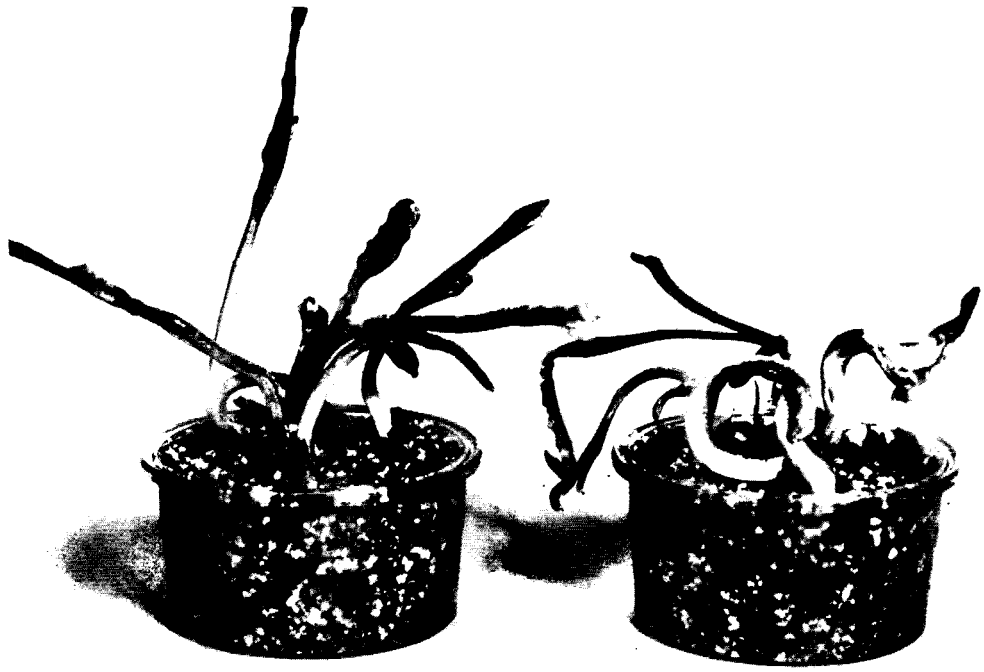
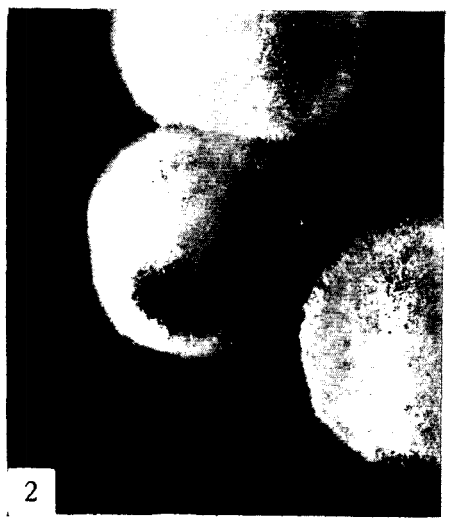
All the four markers were recovered amongst the segregants. Again the frequency of segregation increased with ultra-violet dose, and there were no conspicuous differences in the frequencies of each class of segregant with different doses. The caffeine treatment, as with Diploid I, did appear to have a different effect from ultra-violet in that it produced no *pan-1* segregants. There was also no evidence for the occurrence of haploidization. Since during this process chromosomes assort at random, if *pan-1* is on a different chromosome from the other three markers, half the haploids would have been *leu-1 pan-1* or *me-1 ad-1 pan-1*; if it was on the other arm of the same chromosome, half the haploids would have been *leu-1 pan-1*. The fact that segregants of this type were not observed, indicates that haploids must be rare, if not absent, in the sample of segregants obtained.

The majority of the segregants fell into four main classes: *leu-1*, *ad-1*, *ad-1 me-1* and *pan-1*, and each of these could have been derived from a single mitotic exchange. The position of the centromere in relation to the linked markers was confirmed; and *pan-1* lies on a separate arm from these markers. After the lowest dose of radiation a number of colonies were found which were half auxotrophic and half prototrophic. The auxotrophs always responded to pantothenic acid. Some colonies which were auxotrophic were clearly distinguishable into two halves of different pigmentation (Fig. 2, Plate I). When cells were isolated from each half and tested for their requirement, it was found that those from the non-pigmented half always required leucine, and those from the brown required either adenine or adenine and methionine. These mosaic colonies are those that would be expected to arise from a reciprocal mitotic exchange. A few similar colonies were found after higher doses of ultra-violet, but a high proportion of mixed colonies would not be expected when sporidial survival was low.

The *me-1* segregants could have arisen as a result of double crossing-over. The data suggests that this is more liable to occur after a high dose of radiation. The origin of the segregant *me-1 leu-1* was less easy to explain. It could have arisen as a result of three exchanges involving all four chromatids, but a more probable origin is as follows. A spontaneous exchange between *me-1* and *ad-1* with the incorporation of both recombinant strands into the same daughter cell would produce a prototroph of the genotype: $\frac{me\text{-}1 \quad + \quad leu\text{-}1}{+ \quad ad\text{-}1 \quad +} \frac{pan\text{-}1}{+}$. An exchange between the centromere and *me-1* in a later division, probably after irradiation, would produce the *me-1 leu-1* segregant. The occurrence of spontaneous rearrangement of markers emphasizes the importance of using freshly synthesized diploids for mitotic analysis if confusing results are to be avoided. It may have been that the shortage of *pan-1* segregants from Diploid I in comparison with their frequency from Diploid II was due to part of the cell population in the former becoming homozygous for the wild-type allele following spontaneous crossing-over at an early stage in the growth of this strain. Since different cell populations were usually used in the different irradiation experiments, any significant variation in the proportion of each type of segregant between experiments could perhaps be attributed to the same cause.





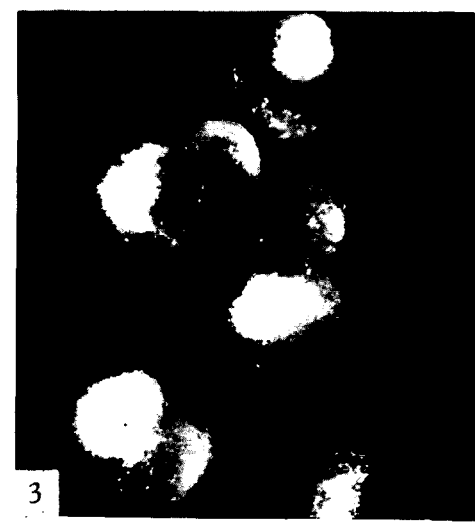


Fig. 1. Maize seedlings inoculated (a) with sporidia of Diploid I, and (b) with sporidia of compatible haploid strains.

Fig. 2. A colony consisting of the reciprocal products of an induced mitotic exchange in Diploid II. ×15.

Fig. 3. Stable diploids homozygous for one chromosome arising as sectors from the slow-growing pigmented monosomic strain. ×22.

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(*e*) *Confirmation of the diploidy of segregants*

A simple test can be carried out on the segregants to confirm that they are not in fact haploids. If haploid they should produce normal galls with one of the four wild-type tester strains: a_1b_1, a_1b_2, a_2b_1 and a_2b_2. If they are diploid they should be potentially solopathogenic, provided they are still heterozygous for the *a* and *b* loci; but their pathogenicity will be reduced or eliminated by their auxotrophy. It has previously been shown that when strains of opposite mating type which carried the same biochemical marker were inoculated into maize pathogenicity was impaired. With the markers *ad-1*, *me-1* or *leu-1*, none of the usual signs of infection were observed. With vitamin-requiring strains galls were formed, but these were very small and only appeared in a small proportion of the seedlings.

A random sample of segregants from Diploid I was tested for solopathogenicity and against the tester strains. This consisted of 13 *nic-1*; 10 *ad-1*; 9 *pan-1*; 1 *leu-1* and 5 *me-1 leu-1* segregants. In no case was a typical haploid reaction observed. All except a single *nic-3* and a single *me-1 leu-1* segregant either produced very small galls whether inoculated alone or with one of the testers, or were entirely non-pathogenic. The two exceptions produced white galls with a_1b_1 and a_2b_1, and no galls with the other testers. These strains appear to be homozygous b_2 whilst remaining heterozygous for the *a* locus. Strains with similar behaviour were found by Rowell (1955*b*). The *me-1 leu-1* segregant had other unusual properties which will be discussed below. Its behaviour showed that the common *b*, *nic-3* segregant probably arose as a result of two exchanges, each giving rise to homozygosity for a single locus. When Diploid I was itself inoculated with the haploid testers the reaction was always the same as when it was inoculated alone.

Pan-1 is closely linked to the *a* locus. Of 19 tetrads in which segregation for these loci was followed 17 were parental ditype and 2 were tetratype, and in 18 random progeny no recombination was observed (Holliday, 1961 and unpublished data). The majority, if not all, the segregants which are homozygous for *pan-1* must also be so for a_1, which is the allele in coupling with it; yet there was no evidence of increased pathogenicity when these segregants were inoculated with a_2b_1 or a_2b_2 as compared with the other tests. Common *a* diploids must therefore be solopathogenic. In order to test the possibility that a third *b* allele might interact with diploids heterozygous b_1/b_2, Diploid I and two *pan-1*, two *nic-3* and one *ad-1* segregant from it were tested against strains a_1b_3 and a_2b_3. In each case the reaction was the same as that with the other testers.

A second method for showing the diploidy of segregants is to demonstrate that they are still heterozygous for the markers which have not segregated. Three *pan-1* segregants which were obtained from Diploid II after 5 minutes' ultra-violet treatment were examined in this way. After irradiation with ultra-violet each showed segregation for the other three markers. The results of these experiments are shown in Table 3.

(*f*) *An unstable segregant*

The *me-1 leu-1* segregant which was found to be homozygous b_2 was recovered from Diploid I after 5 minutes' irradiation. It grew very slowly on complete

Table 3. *Second-order segregation from three* pan-1 *segregants from Diploid II*

Number of segregant	U.V. light (min.)	Survival (%)	Segregants (%)	Frequency of segregants with requirement for pantothenic acid and			
				ad	*ad* + *me*	*me*	*leu*
1	2	0·5	1·11 ± 0·27	7	7	2	10
2	2	0·5	0·96 ± 0·20	11	5	2	6
3	2	0·8	0·77 ± 0·19	7	4	2	4

medium but produced sectors which grew at the normal rate and were completely stable (Fig. 3, Plate I). Both the slow-growing cells and those from a number of different sectors responded to methionine and leucine. The tests for pathogenicity had been carried out on strains derived from two separate sectors.

If the sectors are homozygous *me-1 leu-1* and b_2, and heterozygous for the *a* locus, they are also likely to be heterozygous for *nic-3* and *pan-1* and lack the *ad-1* marker. Sporidia derived from one sector, were irradiated with ultra-violet for $2\frac{1}{2}$ minutes. Colonies derived from surviving sporidia were replicated to minimal medium supplemented with hydrolysed casein. Twenty-seven segregants were obtained; twenty had an additional requirement for nicotinic acid and seven for pantothenic acid. Two of the former were tested for their mating response, which was found to be the same as that of the parent strain. Two of the latter were also tested; they produced normal galls only with $a_2 b_1$. Since *pan-1* is closely linked to the *a* locus this result was expected. These two *me-1 leu-1 pan-1* segregants are homozygous for both *a* and *b* loci and give a mating response indistinguishable from a haploid $a_1 b_2$, although the genetic evidence shows them to be diploid.

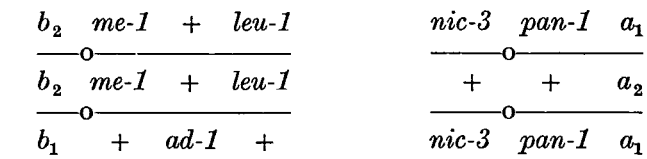
It seemed probable that the original unstable strain was aneuploid or monosomic, and that it gave rise to a stable diploid strain homozygous for one chromosome following nondisjunction of centromeres at mitosis. Such strains might be expected to lose a chromosome by the same process and become haploid (Pontecorvo & Kafer, 1958). Half the haploids should be *me-1 leu-1 pan-1 nic-3*, but no sectors with this genotype were detected. If the sectors were homozygous for one chromosome, this indicates that the *b* locus must be on the same chromosome as *me-1*, *ad-1* and *leu-1*, but on the opposite side of the centromere from these markers. (The possibility that it is on the same side and closely linked to the centromere seems less probable, since the *nic-3* segregant from Diploid I which was homozygous b_2 was not homozygous for the markers in coupling with b_2, as would have been expected following an exchange between the centromere and the *b* locus). If this is so, the loci *nic-3* and *pan-1* must be on opposite arms of the second chromosome.

(*g*) *Synthesis of a triploid*

Since a diploid homozygous *me-1 leu-1* b_2 was available, it was possible to use it to obtain a triploid strain. The diploid was inoculated with *ad-1 pan-1 nic-3* $a_1 b_1$. Six days after inoculation pieces of gall tissue were placed on minimal medium. After a lag of several days a single prototrophic colony appeared. This was found

to be weakly solopathogenic. No brandspores were formed in the very small galls. Unlike prototrophic diploid strains, this strain was rather unstable. Considerable morphological variation occurred and the rate of spontaneous segregation to auxotrophy was high. Of 1220 colonies replicated from complete to minimal medium, 17 were auxotrophic, a segregation frequency of $1{\cdot}39 \pm 0{\cdot}34\,\%$.

If this strain was triploid it should have the following genotype:



Mitotic exchanges should lead to homozygosity from the point of exchange to the end of the arm for two out of three homologous chromosome arms. It should therefore be possible to obtain segregation for all the markers except *ad-1*, since this could only become homozygous following two successive exchanges. One hundred and thirteen auxotrophic segregants which arose spontaneously or after ultra-violet treatment were identified. They had the following phenotypes:

nic-3	59
pan-1	39
leu-1	4
me-1 leu-1	7
nic-3 pan-1	3
me-1 leu-1 nic-3 pan-1	1
Total	113

It is not possible to determine by mating tests whether these segregants were all triploids or whether, as may be possible, diploids were also present. The fact that no *ad-1* segregants were obtained provides evidence for the triploid condition of this prototrophic strain, but the additional evidence which would be provided by showing that a single *ad-1* allele was present has not been obtained.

DISCUSSION

Mitotic crossing-over and chromosome mapping

Ustilago maydis has certain features which make genetic analysis outside the sexual cycle simple and profitable. Heterozygous diploids are readily obtained. The natural frequency of mitotic crossing-over can be greatly enhanced by artificial means, so that with the technique of replica plating large samples of segregants can be obtained, even without the selective techniques which must be used in *Aspergillus*. Reciprocal products of mitotic crossing-over can be detected. The haploid chromosome number of two simplifies the marking of each chromosome and the determination of the positions of new markers. The ploidy of segregants can be determined where necessary by pathogenicity tests.

For some purposes mitotic analysis can have considerable advantages over meiotic analysis. In earlier investigations numerous crosses were made between various markers, but linkage was detected relatively rarely (Holliday, 1961). It seems probable that meiotic crossing-over is very frequent. Mitotic crossing-over, on the other hand, occurs only in a small proportion of cells and most of the exchanges are single. The effect of this is greatly to increase the linkage between markers within chromosome arms. The linkage of such markers which assort at random during meiosis can be easily detected by mitotic analysis. The value of analysis of mitotic crossing-over in comparison with meiotic would tend to decrease with increasing chromosome number.

Although the types of segregant obtained from the diploids showed that the five markers were distributed on three chromosome arms, and indicated the position of the centromere in relation to the three linked markers, an examination of the relative frequencies of the various types does not appear to provide much useful information concerning the distance of the markers from their respective centromeres. There is considerable heterogeneity in quantitative results from the two diploids and also between the different treatments. It seems possible, for instance, that caffeine tends to induce crossing-over near the centromeres. This would explain the shortage of *nic-3* and *leu-1* segregants from Diploid I and of *ad-1* segregants from Diploid II after caffeine treatment (Tables 1 and 2). In the construction of accurate chromosome maps the methods which have been used would appear to be most valuable as a guide to crosses which could be most profitably examined by tetrad analysis.

The effect of ultra-violet light on crossing-over

Fratello *et al.* (1960) have pointed out that induced segregations from a heterozygous diploid which result in the phenotypic expression of recessive alleles in single chromosome arms could be due to terminal deletions rather than mitotic crossing-over. In *Ustilago* the evidence shows that segregation induced by ultra-violet light is not the result of chromosome breakage. The mosaic colonies which were detected after irradiation always consisted of the segregants which would be expected following the survival of the reciprocal products of crossing-over. The several *me-1* segregants which were obtained could have arisen either as a result of interstitial deletions, or from double crossing-over within an arm. Two terminal deletions in a single cell are more likely to be produced, and to be detected, than a single interstitial deletion; yet simultaneous segregation of loci in separate arms was only recorded once. On the other hand it is possible to account for the occurrence of double cross-overs within arms (see below). There is as yet no similar evidence to show whether the segregants which arose spontaneously or after caffeine treatment were produced as a result of terminal deletions or mitotic crossing-over.

In mitotic crossing-over two processes must be imagined: pairing of homologous chromosomes or parts of chromosomes, followed by crossing-over. Since low doses of ultra-violet induce crossing-over (see also Roman & Jacob, 1958) it seems

probable that only one of these processes is affected. There are therefore two possibilities: either ultra-violet induces pairing and crossing-over follows spontaneously, or somatic pairing is already present in a high proportion of cells and crossing-over is produced by the effect of ultra-violet. The stimulation of recombination by ultra-violet in bacteriophage (Jacob & Wollman, 1955), *Escherichia coli* (Jacob & Wollman, 1957) and yeast (Roman & Jacob, 1958) has been explained by supposing that the irradiation produces lesions in the genetic material (DNA), and that crossing-over occurs by the switch synthesis or copy choice mechanism, first postulated by Lederberg (1955). During duplication of the DNA a switch of synthesis from one chromosome to the other occurs when a lesion is reached. Therefore the greater the number of lesions produced the greater the amount of crossing-over. This model, at least in its simplest form, would not account, however, for the production of reciprocal cross-overs such have been observed after low doses of ultra-violet in *Ustilago*. It is also in disagreement with the results obtained by Levine & Ebersold (1958) with *Chlamydomonas*. They found that crossing-over was not increased at meiosis in zygotes formed from ultra-violet irradiated gametes.

If ultra-violet light induces crossing-over in chromosomes which are already paired, double exchanges should be distributed at random. This was not so. A number of double exchanges were observed within the only chromosome arm in which they were detectable, whereas only one segregant was obtained which could have been derived from exchanges in two different arms, although such segregants should have been detected much more readily than the former. If the effect of ultra-violet is to initiate pairing, this could spread from the point of contact and allow occasional double cross-overs in fairly localized regions to occur. Another point which may well be relevant is that whereas ionizing radiation has a relatively slight, if any, effect on crossing-over at meiosis in *Drosophila* females, when pairing is complete, it does induce some crossing-over in males and during non-meiotic divisions (Whittinghill, 1951). The effect of such radiation on mitotic crossing-over in *Ustilago* appears to be similar to that of ultra-violet light (unpublished data). The results with *Chlamydomonas* (Levine & Ebersold, 1958) are also consistent with the observations on *Drosophila*. The evidence therefore indicates that ultra-violet light initiates pairing of homologous regions of chromosomes and that the proportion of cells in which this occurs, and possibly the length of the paired region, increases with increasing dose.

It has frequently been observed that ultra-violet irradiation results in a lag in cell division in micro-organisms. Kelner (1953) and Kanazir & Errera (1956) demonstrated in *E. coli* that ultra-violet inhibited synthesis of DNA with little effect on that of RNA and proteins. In the same organism Witkin (1958) has shown that the length of the lag is proportional to the dose of ultra-violet irradiation, and that the addition of low concentrations of caffeine to the medium on which irradiated cells were plated increased the lag. According to Darlington (1932) the difference between mitotic and meiotic division lies in the timing of cell division and chromosome division. In meiosis the initiation of cell division is precocious

in comparison with that of mitosis, with the result that the chromosomes pair before they have divided in order to restore an equilibrium like that found at mitosis. If ultra-violet light (and perhaps also caffeine) inhibits DNA synthesis, but not the other processes of cell division, the same effect would be produced. The longer the lag in the division of the chromosomes the greater would be the chance of chromosome pairing.

The situation is more complicated if the mutagenic effect of ultra-violet light is considered. Diploid cells in fungi are several times more resistant to the lethal effects of ultra-violet light than haploid ones (see review by Pomper & Atwood, 1955). This can be most simply attributed to the fact that recessive lethals do not kill diploid cells. A diploid cell surviving a high dose of radiation might be expected to carry several recessive mutations scattered at random throughout the genome. If mitotic crossing-over occurred during the division of paired chromosomes by a copy choice or similar mechanism, then any segregant produced would also become homozygous for recessive lethals distal to the point of exchange, and would not survive. Therefore with high doses of radiation there should be automatic selection for cells in which crossing-over leading to homozygosity has not occurred. The proportion of segregants amongst the survivors should fall at high doses. This is not observed. Moreover, a cross-over near the centromere will be more likely to result in homozygosity for a lethal, than one nearer the end of the chromosome. In fact, the ratio of cross-overs between the centromere and *me-1* to those between *me-1* and *ad-1* (Table 2) or between *me-1* and *leu-1* (Table 1) does not increase with increasing dose.

These results can be explained in two ways. Either the killing of the sporidia with ultra-violet light is not primarily due to genetic damage, and the number of recessive lethals in a surviving diploid is small. Or crossing-over takes place by breakage and reunion of chromatids, and occurs only in those cells which are irradiated after the DNA has been duplicated. If so, recessive lethals will remain in a heterozygous condition. This last possibility is not of course in agreement with the hypothesis that ultra-violet light induces pairing before duplication of the DNA. It can, however, be tested experimentally. If the segregants obtained after a high dose of radiation are heterozygous for recessive lethals, they should segregate abnormally after a second dose of radiation. Any lethal in coupling with and distal to a marker will prevent that marker appearing among the segregants, unless double crossing-over occurs. Three survivors of a high dose of ultra-violet light were examined in this way (Table 3) and none of them appeared to carry lethals which prevented any of the three recessive markers from segregating. An examination of a larger sample of survivors should provide evidence as to which of the theoretical possibilities which have been considered is the correct one.

The mating system of Ustilago maydis

Rowell (1955*b*) found that after irradiation of a diploid solopathogenic strain of *U. Maydis* with alpha-particles, strains could be recovered which were no longer solopathogenic. These were of six types: two with parental and two with recom-

binant combinations of the *a* and *b* loci, and two which were heterozygous *a* but homozygous for one or other of the *b* alleles. He did not detect any strains which were homozygous *a* and heterozygous *b*. From his data it can be concluded that *a* and *b* are on different chromosomes, since during the process of haploidization chromosomes assort at random; and that common *b* strains arose as a result of mitotic crossing-over. The absence of common *a* segregants could have been due either to the close linkage of this locus to the centromere, or because such strains retain their solopathogenicity. It is now evident that the latter explanation is the correct one. *Pan-1* is closely linked to the *a* locus but not to the centromere. Diploids homozygous for *pan-1* and a_1 are solopathogenic. They are unable to fuse with a_2 haploids, even though the resulting heterokaryon would be prototrophic, and therefore might be expected to have a strong selective advantage in comparison with the weakly pathogenic auxotrophic diploid. This fusion must be prevented by the presence of the two different *b* alleles in the diploid nucleus, rather than the existence of a *b* allele in the haploid in common with one of those in the diploid; since a third *b* allele in the haploid still does not result in the formation of a heterokaryon. When only one *b* allele is present in the diploid, fusion with a haploid with a second allele readily takes place.

These results underline the functional distinction between the two loci. Whereas the *a* locus is responsible for the fusion of compatible haploid sporidia, it has no effect on the pathogenicity of the fungus, which is under the control of the *b* locus. This perhaps makes it possible to understand more easily the peculiar mating system in *U. maydis*. The multiple alleles controlling pathogenicity could have evolved quite separately from the two allele mating system which is common to most of the smut fungi (see Whitehouse, 1951).

A further point of interest concerns the difference in the behaviour of the dikaryon and the diploid. Whereas the dikaryon has complete pathogenicity and is non-saprophytic, the diploid with the same complement of genes is vigorously saprophytic and has reduced pathogenicity. This is an example of the novel type of position effect discussed by Pontecorvo (1952) and detected by him on one occasion in *Aspergillus*.

Aneuploidy

In a recent paper Kafer (1960) discusses the formation of unstable strains in *Aspergillus* following spontaneous or induced non-disjunction of chromosomes in diploids. She has detected trisomics and predicts that monosomics should also occur, although none were found in her material. Such monosomics would be expected either to break down further to haploids, or revert following non-disjunction to diploids homozygous for a whole chromosome (see also Pontecorvo & Kafer, 1958). In the single case analysed in *U. maydis*, a monosomic appeared to revert only to the diploid condition. Other examples of slow-growing unstable strains induced by ultra-violet light may well have escaped notice, since very small colonies could not on the whole be scored by replica plating and were ignored. In his experiments with alpha radiation Rowell (1955*b*) obtained a number of very

slow-growing colonies, which reverted to colonies with normal growth of different mating types. He claims that up to six strains with different mating behaviour could be obtained from a single colony, but he does not state what mating types were obtained in this way. It seems very probable that some of these unstable strains were trisomics which could segregate to form two diploids, one of which would be solopathogenic, and four different haploids. Other strains may well have been monosomic. As suggested by Kafer (1960), the unstable segregants produced by nitrogen mustard in diploids of *Penicillium chrysogenum* by Morpurgo & Sermonti (1959) almost certainly provide yet another example of chromosomal unbalance leading to the loss or gain of particular chromosomes, and the formation of more vigorous balanced strains. These authors, however, believed that their unstable strains were diploids which underwent mitotic crossing-over at a high rate in particular regions of the genome.

Since $n = 2$ in *Ustilago* it should be relatively easy to follow the behaviour of chromosomes in unstable strains and also use this behaviour to assign markers to particular chromosomes. With an adequate number of markers it should be possible to obtain genetic confirmation of the chromosome number.

SUMMARY

1. Two different methods of selection were used to obtain stable prototrophic solopathogenic strains of *Ustilago maydis* from haploids with different biochemical requirements. The strains were shown to be heterozygous diploids.

2. Two diploids which were examined in detail had four markers in common, but they were in different coupling and repulsion phases. Rare spontaneous segregation was detected in one of the diploids, but a high frequency of segregation was obtained in both after treatment with ultra-violet light. The proportion of segregants amongst the survivors increased with the dose. These auxotrophic segregants were detected by means of the replica plating technique.

3. The types of segregant which were obtained from both diploids were consistent with the view that they arose as a result of mitotic crossing-over. After low doses of radiation the reciprocal products of crossing-over were often detected. There was no evidence from the phenotypes of the segregants that haploidization was occurring. The diploidy of a sample of the segregants was confirmed by mating-type tests, and by the fact that they showed further segregation after another dose of radiation.

4. A slow-growing unstable segregant recovered after a high dose of radiation proved to be a monosomic strain which consistently reverted to a stable diploid homozygous for one chromosome. It was possible to use this auxotrophic diploid together with a haploid with different biochemical requirements, to synthesize a prototrophic triploid strain. The triploid was much less stable than the diploid strains.

5. By means of pathogenicity tests with certain segregants it was possible to distinguish the function of the two loci which control the mating system. The *a*

locus is responsible for the fusion of haploid sporidia and has no effect on the pathogenicity of the heterokaryon which is under the control of the *b* locus.

6. The effects of ultra-violet light on mitotic crossing-over do not seem to be easily compatible with a copy choice or similar mechanism of recombination.

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