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The production and characteristics of diploids in *Ustilago violacea*

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1. INTRODUCTION

Holliday (1961*a*) has discussed the advantages of the Ustilaginales for genetical studies. Many species in this order have the technical advantage that at one stage of the life cycle there are separate, yeast-like cells which reproduce by budding. These cells or sporidia, which are uninucleate and normally haploid, form compact colonies on artificial media and can be handled efficiently by the use of bacteriological techniques such as replica plating (Lederberg & Lederberg, 1952). Biochemical mutants have been induced and standard methods of genetic analysis have been devised for *Ustilago maydis* by Perkins (1949) and Holliday (1961*a*). A sexual cycle occurs in most species, and techniques for the production of vegetative diploid strains of *U. maydis* have been developed (Holliday, 1961*b*). Segregation due to mitotic crossing over has been analysed, but the other type of parasexual segregation resulting from haploidization of vegetative diploids has not been reported for this species (Holliday, 1961*b*, 1964).

The success of genetical studies with *U. maydis* suggested that other species of the genus might be equally useful and might possibly have additional technical advantages. After consultation with Dr R. Holliday, *U. violacea* was chosen as a technically suitable species. The growth of this species on artificial media and its cytology have been studied extensively by earlier workers (Fischer & Holton, 1957). One particular advantage was that Bauch (1922) had reported that haploid sporidia of this species fused readily on certain media to form dikaryons and that fusion could be controlled by changing the conditions of culture. This fusion, which is very rare in *U. maydis*, appeared to offer possibilities for the completion of the life cycle in culture. This paper reports the development of new techniques for genetical studies with *U. violacea*, including the production of diploid sporidia and their haploidization. These techniques could be applied to the analysis of other species in the Ustilaginales.

2. LIFE-CYCLE

The diploid resting spores or brandspores develop in the anthers of host plants, which are restricted to the Caryophyllaceae. Zillig (1921) listed 68 host species in this family and used these to define several physiologic races of the pathogen. The brandspores germinate on artificial media and produce three-celled hyphae or promycelia. The three cells of a promycelium and the brandspore each

bud off spherical, uninucleate haploid sporidia which can reproduce indefinitely by further budding. The promycelium and brandspore thus form a tetrad from which haploid clones are budded off. According to Brefeld (Harper, 1898) one promycelium only is produced from each brandspore on nutritive media, but on water agar several promycelia may be produced in succession from the same brandspore. This indicates that the first division of the diploid nucleus in the brandspore is mitotic, and that meiosis occurs in each promycelium as it forms.

The sporidia are the only stage in the life cycle which can grow on artificial media. This growth is by yeast-like budding and requires certain salts, a sugar and the vitamin thiamine (Blumer, 1937). Sporidia were shown by Kniep (1919) to fall into two mating types, designated here as a_1 and a_2. Extensive studies by Kniep (1919) and other workers (Whitehouse, 1951) have established that the mating system is truly bipolar, and Kniep (1926) also showed that the two mating types were homologous in the related species, *U. scabiosae*, *U. cardui*, *U. utriculosae*, *U. vinosa*, *U. anomala*, *U. tragopogonis pratensis* and *U. violacea*. Sporidia of opposite mating type conjugate under conditions of good aeration and low nutrition but nuclear fusion does not occur (Kniep, 1919; Bauch, 1922).

Under cultural conditions sporidial fusion is followed by a reversion to haploid budding, and dikaryotic hyphae are not formed (Harper, 1898). However, under natural conditions a binucleate mycelium is formed, which infects a host plant through the young shoot tissues, and becomes systemic, even reaching the roots (Fischer & Holton, 1957). According to Wang (1932) the hyphae do not always maintain a strictly binucleate condition in the host plant, but sometimes have several nuclei. Simple haustoria are produced with one or two nuclei. Although no clearly defined symptoms in the host plant are evident until flowering, Baker (1947) has reported that infected plants are usually shorter and more branched, and develop less anthocyanin than infected plants. The mycelium in the young anthers of the flower becomes regularly binucleate and develops thick gelatinous cell walls. The cells then round off in clusters, the nuclei fuse, and a thick reticulated wall is laid down (Wang, 1932). The pollen in the anthers is thus replaced by the developing brandspores, and the anther becomes dark purple, When the host is a female plant of a dioecious species, such as *Silene alba* or *S. dioica*, the plant branches in the same way as male plants (Baker, 1947), the ovary aborts, and the anthers which develop contain brandspores. This phenomenon of induced sex change has been the subject of much study (Fischer & Holton, 1957).

Several cytologists have examined the chromosome number in *Ustilago* species. Harper (1898) reported a haploid chromosome number of 6–8 in *U. scabiosae*, a species which Kniep (1926) showed to be closely related to *U. violacea*. Most other cytologists (review in Fischer & Holton, 1957; Person & Wighton, 1964) have claimed a haploid chromosome number of two in several species including *U. violacea*.

3. MATERIALS AND METHODS

(i) *Stocks*

Brandspores were collected from an infected plant of *Silene alba* growing near Burghfield, Berkshire. Several monosporidial colonies were isolated from the sporidia produced by one brandspore. Two clones which gave abundant fusions when mixed together on water agar were used as the standard wild-type isolates of each mating type. All mutants were derived from one or other of these two stock cultures.

(ii) *Media*

Minimal medium: glucose, 10 g; salt solution, 50 ml; distilled water to 1 l.; pH 5·8 after autoclaving.

Salt solution: sodium nitrate, 20 g; potassium dihydrogen phosphate, 10 g; magnesium sulphate, 5 g; potassium chloride, 5 g; calcium chloride, 1 g; ferrous sulphate, trace; thiamin 10 mg; distilled water to 1 l.; stored at −3 °C.

Supplemented minimal medium: individual growth factors were added to minimal medium when necessary; ammonium nitrate, 1 g; sodium sulphite, 0·3 g; amino acids, 100 mg; adenine, 10 mg; vitamins, 1 mg/l.

Complete medium: glucose, 10 g; 'Yeastrel' yeast extract, 3 g; 'Lemco' beef extract, 1 g; peptone, 10 g; malt extract, 3 g; distilled water to 1 l.; pH 5·5 after autoclaving.

All media were solidified by the addition of 2 % 'Oxoid' no. 3 agar and sterilized by autoclaving at 15 p.s.i. for 15 min.

(iii) *Incubation and storage of cultures*

All cultures were incubated at 22 °C. Stock cultures in frequent use were maintained on complete medium at 2 °C and subcultured after 6 weeks. All stocks were also maintained as dried sporidia in screw topped Bijou bottles containing silica gel (B.D.H. 30–120 mesh) which had been dried and sterilized at 150 °C for 4 h. Such dried sporidia germinated readily when transferred to a nutrient medium. Colonies on complete medium reached a diameter of about 2 mm, in about 6 days and were rose pink in colour. Colonies on minimal medium grew slightly more slowly and were much paler.

(iv) *Plating and counting*

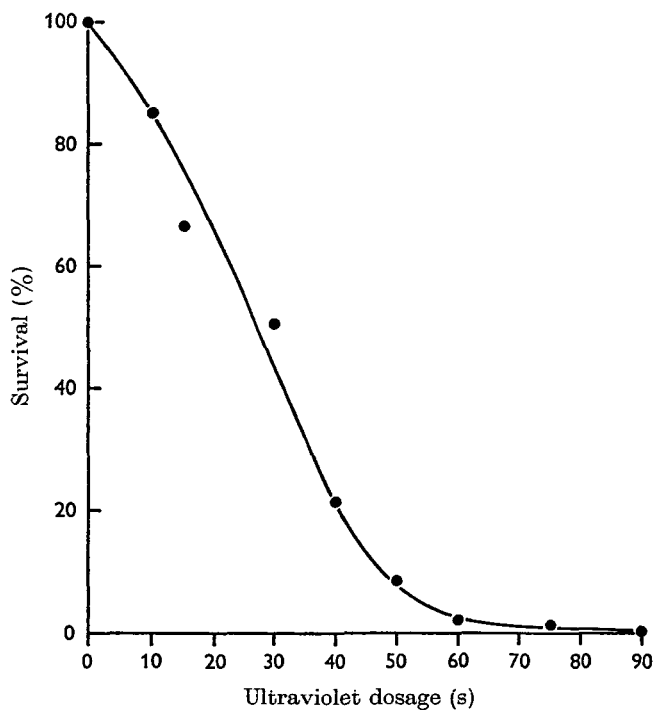
Sporidia were readily dispersed in sterile water, without the use of wetting agents, to form uniform suspensions of single cells. Estimations of the concentration of cells were made with a Neubauer haemocytometer. Suspensions were diluted and dispersed in, or spread on, agar using standard microbiological techniques. The viability of sporidia in suspensions was 90–100 % on complete and minimal medium.

(v) *Mating-type tests*

A simple technique was developed to identify the mating type of cultures. A Petri dish containing 1·5% water agar was marked off into six sections on its undersurface. Small quantities of sporidia from the wild-type a_1 isolate were deposited in three of the sections, and sporidia from the a_2 wild-type stock culture in the other three sections. Sporidia from the culture to be tested were then transferred to one of the sections containing a_1 sporidia and to one of the sections containing a_2 sporidia. It was thus possible to identify the mating type of three new cultures on one plate. The sporidia in each section were mixed together thoroughly with a sterile wire. After incubation overnight a coverslip was placed over the sporidia in each section of the plate and the cultures were examined under the high power of the microscope for sporidial fusions. In all cases in which fusions occurred with one stock culture, but not with the other, the new isolate was assigned to the mating type of the stock culture with which no fusions were observed. Plate 1, fig. 3, illustrates the fusion of compatible sporidia.

4. THE PRODUCTION AND IDENTIFICATION OF BIOCHEMICAL MUTANTS

Ultraviolet light was chosen as the mutagenic agent. The lamp was a Camag TL 900, and contained an 8 W fluorescent tube (G8T5), which emitted radiation in a very narrow band with a mean of 254 mμ. The filter, absorbing radiation outside the u.v. range, was left in place during all the experiments.



Text-fig. 1. The effect of u.v. dosage on survival of haploid sporidia.

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A sterile Petri dish was filled with 10 ml of a suspension of sporidia. The lid of the Petri dish was removed and the suspension was then agitated in a horizontal plane 12 cm under the lamp. Aliquots of this supension were removed at different time intervals and plated on complete medium to determine a survival curve (Text-fig. 1). The sporidia were stored at 3 °C for several weeks before irradiation, as Holliday (1961*a*) had observed that mutants were obtained from such sporidia at a significantly greater frequency than from freshly grown sporidia. After irradiation for 50 s about 200 viable sporidia were spread on each of a series of plates of complete medium. Auxotrophic mutants were isolated by the replica plating technique as used by Holliday (1961*a*). Fifty-two mutants were obtained at a frequency of about 0·5 % of the colonies surviving irradiation, a rather greater frequency than the value of 0·277 % recorded for *U. maydis* (Holliday, 1961*a*).

The biochemical requirements of the mutants were identified by auxanography (Pontecorvo, 1949). A preliminary investigation into the site of the genetic block in certain groups of mutants was attempted by testing the response to possible precursors. A summary of the growth responses of the auxotrophs is shown in Table 1.

The commonest mutants in *U. violacea* were those requiring arginine, lysine, methionine or histidine. A comparison of the mutants listed in Table 1 with those isolated in *U. maydis* (Holliday, 1961*a*; and personal communication) revealed several differences. Mutants unable to reduce nitrate and vitamin-requiring mutants were less frequent in *U. violacea* than in *U. maydis*, but lysine and histidine-requiring mutants were more common. It is probable that some of this apparent variation between the two species is due to differences in the composition of the complete media used for the isolation of the mutants. However, real differences between the two species were found in the types of arginine and methionine mutants.

In *U. maydis* only two types of arginine mutant were found: one responded to arginine only, and the other to arginine or ornithine but not to citrulline. These mutants resemble some of those reported for *Aspergillus nidulans* (Pontecorvo *et al.* 1953; but see Weglenski, 1966). The arginine mutants in *U. violacea*, however, resemble those in *Neurospora crassa* (Srb & Horowitz, 1944; Fincham & Day, 1965) and *Penicillium* (Bonner, 1946) in that citrulline promotes growth of many of the mutants whether they respond to ornithine or not. Although none of the arginine mutants responded to arginino-succinic acid, thin-layer chromatographic studies of extracts of the mutants which could utilize only arginine revealed that such mutants fell into two classes. One class accumulated citrulline, the other accumulated arginino-succinic acid (J. Evans, A. W. Day & J. K. Jones, unpublished). There is therefore good evidence that arginino-succinic acid is an intermediate in the conversion of citrulline to arginine in *U. violacea* and that the arginine pathway in *U. violacea* is very similar to that in *Neurospora* and other organisms (Fincham & Day, 1965).

The one proline mutant obtained also responded to glutamic acid, unlike many *Neurospora* mutants (Fincham & Day, 1965). This mutant was competitively

Table 1. *The types of mutants obtained following u.v. irradiation of wild-type sporidia*

Basic requirement	No. obtained	Response to related substances: Growth	Response to related substances: No growth	Remarks
Adenine (*ad*)	1	—	Hypoxanthine, inosinic acid, guanine, cytidine, uracil	Dark brown pigment accumulated when adenine limiting
Adenine (*ad*)	2	Hypoxanthine, inosinic acid, guanine	Cytidine, uracil	—
Ammonium ions (*nir*)	2	—	NO_2^-, NO_3^-	—
Arginine (*arg*)	6	—	Arginino-succinic acid, citrulline, ornithine, glutamic acid, proline	Some mutants accumulated citrulline, and others arginino-succinic acid, when arginine was limiting (see text)
Arginine (*arg*)	5	Citrulline	Arginino-succinic acid, ornithine, proline, glutamic acid	All mutants accumulated ornithine when arginine or citrulline was limiting
Arginine (*arg*)	4	Citrulline, ornithine	Arginino-succinic acid, proline, glutamic acid	—
Arginine or proline (*pro*)	1	Citrulline, ornithine, glutamic acid	Arginino-succinic acid	Severe competitive inhibition by several amino acids especially histidine
Histidine (*his*)	6	N.T.*	N.T.	—
Inositol (*inos*)	1	N.T.	N.T.	—
Lysine (*lys*)	11	N.T.	N.T.	—
Methionine (*me*)	2	—	Homocysteic acid, cystathionine, cysteine, $S_2O_3^{2-}$, SO_3^{2-}	—
Methionine (*me*)	3	Cysteine, $S_2O_3^{2-}$	Homocysteic acid, cystathionine, SO_3^{2-}	—
Methionine (*me*)	3	Cysteine, $S_2O_3^{2-}$	Homocysteic acid, cystathionine	—
Pyridoxin (*pdx*)	1	N.T.	N.T.	—
Sulphite ions (*sul*)	1	—	$S_2O_3^{2-}$, SO_4^{2-}; cysteine, methionine, homocysteic acid, cystathionine	—
No requirements (colony-colour mutants)				
Cream (*c*)	2	—	—	Pale cream colonies
Yellow (*y*)	1	—	—	Bright yellow colonies
Total	52			

* N.T. not tested.

inhibited by several amino acids, especially histidine. The competitive inhibition between lysine and arginine reported in *Aspergillus* (Pontecorvo *et al.* 1953) and *Neurospora* (Doerman, 1944) was not noticeable in arginine and lysine mutants of *U. violacea*. Lysine-arginine diauxotrophs grew well on minimal medium supplemented with widely differing proportions of lysine and arginine.

Some of the methionine mutants responded to methionine only and others to methionine, cysteine, thiosulphate ions, and sulphite ions. The latter mutants did not respond to homocysteic acid or cystathionine, which are usually immediate precursors of methionine (Smith & Childs, 1966). Similar mutants in *U. maydis* (Holliday, 1961*a*) responded to these intermediates, again suggesting differences either in metabolic pathways or in cell-wall permeability, between the two species of *Ustilago*.

During the examination of the replica plates for the presence of auxotrophs, several colony colour mutants were isolated. Wild-type colonies were pink, but these mutants formed cream or yellow colonies. The yellow colony marker has proved particularly useful for the visual demonstration of segregation. Analysis of meiotic segregation ratios has established that this marker and the auxotrophic markers are monogenically controlled. A methionine mutant has been obtained by means of the inositol starvation technique using the method described by Holliday (1962).

5. SYNTHESIS OF DIPLOID STRAINS

Earlier attempts to obtain diploid strains of fungi were usually based on the use of polyploidogenic agents and were generally unsuccessful (Pontecorvo *et al.* 1953). The first critically established instance of the synthesis of diploid strains was reported for *Aspergillus nidulans* (Roper, 1952) and was based on the fusion of genetically different haploid nuclei. This entirely different method had the advantage of producing heterozygous diploids. In this method the rare diploid nuclei which arise spontaneously in a balanced heterokaryon were selected either by colour or by prototrophic growth on minimal medium. This method has been followed more or less closely by most subsequent investigators, but it was not successful in *U. maydis* and other methods for the production of diploids were devised (Holliday, 1961*b*). A routine method for the isolation of diploids on artificial media, using the balanced heterokaryon technique, has however been developed for *U. violacea*.

The sporidial pairs after conjugation can be considered to be dikaryons. These dikaryons are obligately parasitic as they do not grow vigorously on artificial media under any conditions yet tested, and normally revert to the haploid condition. However if sporidia with different auxotrophic genes are allowed to fuse and the dikaryotic fused sporidia are plated on minimal medium, the reversion to the auxotrophic haploid state is prevented, and only the diploid cells produced by occasional nuclear fusions can grow.

Sporidial fusion in *U. violacea* was completely inhibited on both minimal and complete media. Present evidence indicates that this inhibition is due to certain cations present in these media. It was therefore necessary to mix complementary

auxotrophic lines of opposite mating type on water agar to induce fusion. The sporidia began to fuse after 3–6 h incubation and after 24 h about 60–70 % had fused. In some cases where lines carrying several non-leaky mutants were used, it was necessary to supplement the water agar with one or more of the required nutrients to allow sufficient growth of the fusion tubes. After incubation overnight the mixture of fused and unfused sporidia was scraped off the water agar with a sterile wire loop and spread on minimal medium.

After about 2 weeks incubation many prototrophic colonies were present, scattered over the plate. These colonies were more slimy and translucent than the colonies produced by haploids on minimal medium and so could readily be distinguished. The sporidia in these colonies were more than twice as large as typical haploid sporidia (see Table 2). They were dumb-bell shaped and filled with oily cytoplasm, quite distinct from the ellipsoid haploid sporidia. The large-celled isolates were formed only after fusions between compatible complementary lines, and thus could not have originated either by back mutation to prototrophy or by cross-feeding between the haploid lines. The development of sporidial pairs after conjugation on minimal medium has been followed by microscopic observation. Some of these fused pairs produced short hyphae from one or both of the fusing cells, or from the fusion tube connecting them. These hyphae, which were presumably dikaryotic, occasionally reached a length of 200 μ, but more often stopped growing when 20–60 μ in length and budded off a number of sporidia (Plate 1, fig. 4). Most of these sporidia were small and did not divide further, but some were large and divided vigorously (Plate 1, fig. 5). The small sporidia were presumed to have arisen from the dissociation of the nuclei in the dikaryon, and would therefore be haploid with parental auxotrophic genotypes, whereas the large sporidia which divided rapidly on minimal medium must have had balanced diploid genotypes produced by the fusion of haploid nuclei.

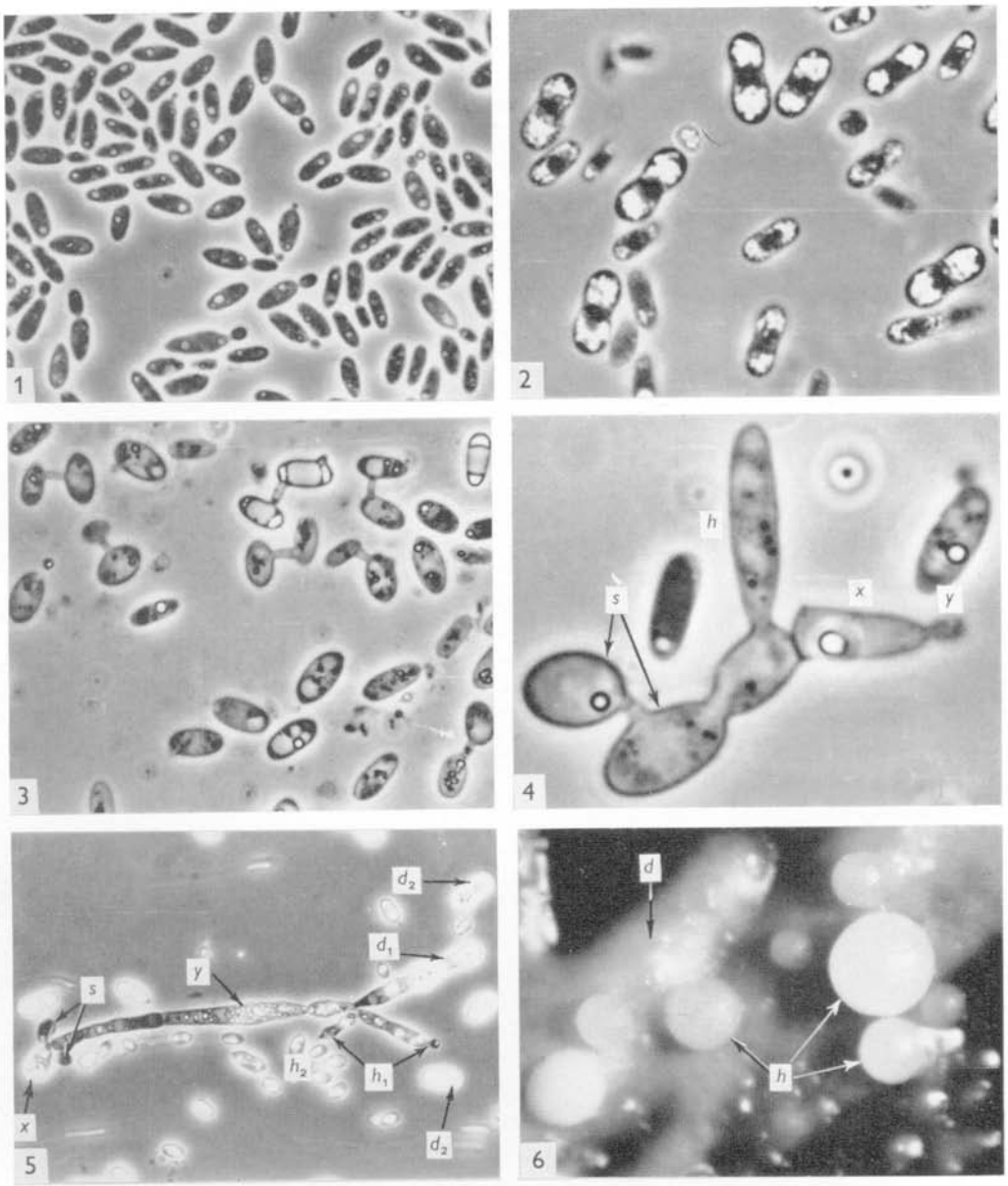
Sporidia from haploid, wild-type stocks and from large-celled isolates were stained with acetic-orcein (Elliott, 1960) and observed under a phase-contrast microscope. All isolates studied were uninucleate, indicating that the large-celled colonies were diploid and not dikaryotic. The genetical behaviour of these isolates provided more critical evidence for diploidy.

6. CHARACTERISTICS OF DIPLOID STRAINS

(i) *Sporidial size*

The lengths and widths of 100 random sporidia from cultures of haploid and diploid strains grown under identical conditions on complete and minimal medium were measured with an eyepiece micrometer under a × 100 oil-immersion objective (Table 2). The mean volumes were calculated from the mean lengths and widths for each treatment assuming that the sporidia were cylindrical.

The diploid sporidia were longer than the haploids, but were not much wider. It was very easy to identify diploid cultures, as the mean length of diploid cells was greater than the maximum length attained by haploids. Diploid sporidia of



Figs. 1 and 2. Differences in size, shape, and budding between haploid (1) and diploid (2) sporidia from 5-day-old colonies on minimal medium. Phase contrast, × 1200.

Fig. 3. Fusion between compatible haploid sporidia on water agar. Phase contrast, × 1200.

Fig. 4. The development of a short hypha (*h*) from a fused pair of sporidia (*s*) with reversion to haploid sporidial budding (*x*) and (*y*): 48 h after fusion. Phase contrast, × 3000.

Fig. 5. Short (*x*) and long (*y*) hyphae growing from a fused pair of sporidia (*s*). The long hypha has reverted to sporidial growth with a large diploid sporidium (d_1) budding off one branch, and others floating freely nearby (d_2). Haploid sporidia are budding off from other branches (h_1) and (h_2): 96 h after fusion. Phase contrast, × 500.

Fig. 6. The development of vigorously growing haploid colonies or papillae (*h*) from a background of diploid cells (*d*), plated on complete medium + *p*-fluorophenylalanine. × 10.

U. violacea cease to divide on minimal medium after 3–4 days, and then swell into a characteristic dumb-bell shape with very refractive cytoplasm. Under the same conditions, haploid cells continue to divide for much longer (10–15 days) and do not swell or attain a dumb-bell shape, though eventually the cytoplasm does become refractive. Plate 1, figs. 1 and 2 illustrate these differences in size, shape and division between haploid and diploid cells from 5-day-old colonies on minimal medium. These physiological differences are the reason why diploid cells are so much larger than haploid cells on minimal medium (Table 2). Diploid cells on complete medium, and on fresh minimal medium, resemble haploid cells closely except that they are longer and have double the volume.

Table 2. *The effect of ploidy and growth medium on the size of sporidia in 5-day-old colonies*

		Length (μ)		Width (μ)		Approx. mean vol. (μ^3)*	Vol. ratio diploid: haploid
Medium	Strain	Range	Mean ± S.E.	Range	Mean ± S.E.		
Complete	Haploid	2·9– 6·7	5·0 ± 0·79	1·9–4·8	3·5 ± 0·54	49·9	2·0:1
	Diploid	3·9–13·4	8·2 ± 1·97	2·9–4·8	3·8 ± 0·50	99·5	
Minimal	Haploid	3·4– 7·2	5·5 ± 0·72	1·9–4·3	3·0 ± 0·49	40·4	3·6:1
	Diploid	4·8–19·2	9·9 ± 3·04	2·4–6·7	4·0 ± 0·90	143·1	

*Assuming sporidia are cylindrical.

(ii) *The frequency of diploid formation*

A direct estimate of the frequency of diploids in the fused sporidial pairs was obtained. Sporidia of opposite mating type which had been mixed on water agar and incubated overnight were scraped off and suspended in sterile water. The concentration of fused sporidia was estimated with a haemocytometer, and approximately 3×10^5 fused sporidial pairs were spread over each of a series of plates of minimal medium. The plates were incubated for 2 weeks and the number of prototrophic diploid colonies was counted. These colonies could clearly be distinguished from occasional haploid revertants by their translucent appearance. Table 3 summarizes the results obtained with several combinations of haploid strains. Combinations which were known to be allelic and combinations of auxotrophic/prototrophic and prototrophic/prototrophic lines were included.

The mean frequency of diploids in the non-allelic combinations was $3{\cdot}4 \times 10^{-4}$. No diploids were formed when the combinations were allelic or when either of the fusing sporidia was not auxotrophic. In the latter case, where there is no selection against haploid growth, the fused sporidia continue to bud off haploid sporidia, and therefore the opportunity for nuclei of opposite mating type to fuse is lost. Diploids were not less frequent when pairs of leaky mutants were used (combinations 8 and 12 in Table 3), as might have been expected since such cells can bud slowly on minimal medium.

(iii) *The mating type reaction*

The diploid sporidia, which because of the method of formation were heterozygous for mating type did not fuse with either of the haploid lines a_1 or a_2 on water agar. These sporidia were therefore described as neutral.

Table 3. *The frequency of diploid formation*

Combination		Total no. of fused sporidial pairs on minimal medium (plasmogamy)	No. of diploid colonies formed (karyogamy)	No. of diploids per 10^4 fusions
Genotype of a_1 sporidium	Genotype of a_2 sporidium			
	(a) Prototrophic/prototrophic			
(1) +	+	$9{\cdot}00 \times 10^4$	0*	0*
	(b) Prototrophic/auxotrophic			
(2) +	*arg* (M 15)	$5{\cdot}20 \times 10^5$	0*	0*
	(c) Allelic combinations			
(3) *arg* (M 15)	*arg* (M 15)	$8{\cdot}00 \times 10^5$	0	0
(4) *arg* (M 15)	*arg* (M 15), *inos* (M 729)	$1{\cdot}34 \times 10^6$	0	0
(5) *lys* (M 716)	*lys* (M 716)	$2{\cdot}75 \times 10^5$	0	0
(6) *inos* (M 729)	*inos* (M 729)	$5{\cdot}00 \times 10^5$	0	0
	(d) Non-allelic combinations			
(7) *lys* (M 716)	*arg* (M 15)	$1{\cdot}12 \times 10^6$	468	4·2
(8) *arg* (M 32)	*arg* (M 15)	$5{\cdot}40 \times 10^5$	84	1·6
(9) *me* (M 11 C1)	*arg* (M 15)	$2{\cdot}70 \times 10^5$	140	5·2
(10) *arg* (M 11 C6) *inos* (M 729)	*arg* (M 15)	$3{\cdot}75 \times 10^5$	105	2·8
(11) *lys* (M 716)	*arg* (M 15), *inos* (M 729)	$1{\cdot}17 \times 10^6$	507	4·3
(12) *arg* (M 11 C6)	*arg* (M 15), *inos* (M 729)	$1{\cdot}72 \times 10^6$	602	3·5
(13) *lys* (M 716)	*lys* (M 84)	$4{\cdot}18 \times 10^6$	1222	2·9
(14) *lys* (M 11 C3)	*lys* (M 716)	$1{\cdot}20 \times 10^4$	5	4·2
(15) *lys* (M 84), *his* (M 11 C2)	*lys* (M 123)	$8{\cdot}40 \times 10^5$	188	2·2
(16) *lys* (M 11 C3)	*lys* (M 124)	$6{\cdot}00 \times 10^5$	310	5·0
(17) *pdx* (M 182)	*inos* (M 729)	$1{\cdot}71 \times 10^6$	336	2·0
(18) *lys* (M 716)	*his* (M 151)	$5{\cdot}00 \times 10^5$	165	3·3
(19) *arg* (M 15), *inos* (M 729)	*lys* (M 716)	$6{\cdot}40 \times 10^5$	180	2·8
(20) *arg* (M 15), *inos* (M 729)	*arg* (D 716a), *lys* (M 716)	$7{\cdot}60 \times 10^5$	270	3·1
			Mean	3·36

*Diploid colonies were not observed, but if present would have been difficult to see amongst the dense prototrophic haploid growth.

(iv) *Pathogenicity*

Pure isolates of diploid sporidia were inoculated into wounds in the axils of young leaves of seedlings of *Silene alba* with a mounted needle. This relatively

crude technique had been found to give 80–100 % infection when two compatible haploid strains were used. A similar frequency of infection was found with the diploid strains which are therefore described as solopathogenic, i.e. capable of infection as a pure culture (Christensen, 1931).

(v) *Segregation in diploid strains*

Segregation of the diploid strains has been followed during the sexual cycle, and also during the parasexual cycle (Pontecorvo, 1956). A qualitative demonstration of these segregations is presented to provide more critical evidence that the large-celled sporidia were diploid. A full description of techniques together with a quantitative analysis will be presented in a subsequent paper.

(*a*) *The sexual cycle*

Analysis of random sporidia derived from brandspores produced by the solopathogenic diploid strains confirmed that all markers used in the synthesis of diploid sporidia were present in the heterozygous state in these brandspores. The morphological and cytological details of the germination of the brandspores from the solopathogenic lines were typical for this species.

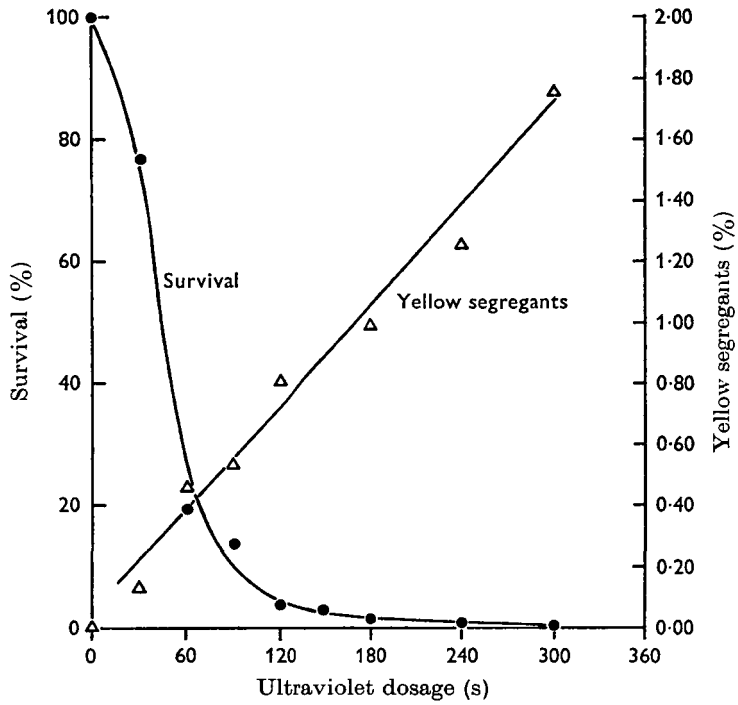
(*b*) *The parasexual cycle*

Mitotic crossing-over. About 100 diploids heterozygous for various markers were synthesized. In a very few cases spontaneous segregants expressing one of the recessive mutant genes in the diploid were isolated. These segregants were large-celled and presumably still diploid. Many more segregants were obtained after treatment with ultraviolet light by a technique identical to that used to obtain mutants in haploid strains. The colonies surviving the radiation were scored for colour segregants. The results for a diploid heterozygous for the recessive yellow colony marker, and three auxotrophic markers which were not scored in this experiment, are summarized in Text-fig. 2.

The frequency of yellow segregants increased with increasing dose of u.v. radiation. All the segregants so far obtained in this way have had larger cells than the haploid strain. In a few segregant clones the sporidia were intermediate in size, and may have been aneuploids, but the rest were almost certainly diploid, as their cells were of full diploid size and they were neutral in mating type. The segregants occurred far more frequently than could be explained by mutation, and the simplest explanation is that each arose from a single mitotic cross-over, which would produce homozygosity for the chromosome arm distal to the point of exchange. A few of the segregants were no longer neutral in mating type, but could fuse with one or other of the haploid stocks a_1 or a_2. It is probable that these segregants were homozygous for one of the mating type alleles, although monosomy of the chromosome carrying the mating type gene is a possible explanation.

Mitotic haploidization. Only one example of spontaneous haploidization has been observed during the analysis of many diploids. DL-*p*-fluorophenylalanine (PFP) has been used to induce haploidization (Morpurgo, in Lhoas, 1961) in several

species of the Aspergillaceae, e.g. *Aspergillus niger* (Lhoas, 1961), *A. fumigatus* (Strømnaes & Garber, 1963), *A. nidulans* (McCully & Forbes, 1965) and *Penicillium expansum* (Garber & Beraha, 1965). but attempts with *U. maydis* (R. Holliday, personal communication) and *C. lagopus* (L. A. Casselton, personal communication) have been unsuccessful. PFP readily produces haploids in *U. violacea*, and a routine technique has now been developed (Day & Jones, 1966).



Text-fig. 2. The effect of u.v. dosage on survival and mitotic crossing-over in a diploid strain of *Ustilago violacea*, heterozygous for a recessive yellow-colony marker.

Genotype of diploid: $\frac{y}{+}\ \frac{lys}{+}\ \frac{+}{arg}\ \frac{+}{inos}\ \frac{a_1}{a_2}$

Diploid sporidia were streaked over the surface of plates of complete medium containing 300 mg PFP/l. The sporidia budded at the normal rate for 2–3 days, then gradually ceased to divide. After 2–3 weeks, most of the sporidia stopped growing and became disorganized, but isolated cells grew vigorously, and formed scattered spherical colonies (*papillae*) above the medium (Plate 1, fig. 6). Many of these papillae were judged to be haploid, as they had small haploid-sized sporidia and a positive mating type reaction with one of the stock haploid lines. When these small-celled papillae were replicated to a series of test media, a wide range of genotypes, both recombinant and parental for the markers in the original diploid, was found.

Haploidization provides the most efficient means of assigning genes to linkage groups, as genes on the same chromosome remain linked whereas genes on different

chromosomes recombine randomly (Pontecorvo & Käfer, 1958). This method is being applied to the analysis of large numbers of diploids in order to ascertain the number of linkage groups, and hence the minimum chromosome number. This analysis is still in progress, but preliminary evidence indicates that there are at least seven linkage groups, and therefore a haploid chromosome number of not less than seven.

7. DISCUSSION

(i) *The synthesis and identification of diploids*

The direct synthesis of diploid sporidia on artificial media has not been reported in other species of the Ustilaginales. Diploids were produced readily by an adaptation of the balanced heterokaryon technique (Roper, 1952). Selection of the diploid cells was especially convenient as the dikaryon is obligately parasitic and cannot grow on such media. The same technique can be used as a complementation test to determine whether auxotrophic mutants are allelic. The ready fusion of sporidia on media therefore makes this species particularly suitable for genetic analysis using diploid strains.

Balanced dikaryons of *U. maydis* do not form on artificial media, probably because, under such conditions, compatible sporidia rarely fuse. A few workers have observed sporidial fusions on artificial media, but many others have been unable to confirm this (Fischer & Holton, 1957). The most successful attempt was reported by Bowman (1946) in which under optimum conditions less than 5 % of the sporidia had fused after 72 h. There is thus a physical barrier to the formation of dikaryons and hence of diploids of *U. maydis* on artificial media which is not present in *U. violacea* where at least 60–70 % of the sporidia fuse after about 24 h on water agar. The inhibitory effect of cations on fusion noticed in *U. violacea* is not the barrier in *U. maydis* as many workers have observed that fusion does not occur on water agar in this species.

Diploid strains of *U. maydis* were obtained by two methods both of which involve inoculation of the host (Holliday, 1961*b*). It is fortunate that such indirect methods are not required in *U. violacea*, since the dikaryon phase is of long duration and there are no obvious symptoms in the host until flowering.

There seems no doubt that the colonies obtained were diploid and not dikaryotic, as mitotic segregation was demonstrated. The characteristic translucent appearance of these diploid strains on minimal medium, compared with the more opaque haploid colonies, is a useful feature. The dikaryotic hyphae or sporidia do not grow vigorously on artificial media. A similar difference in growth behaviour between strains of different nuclear organization is also found in *Coprinus lagopus* (Casselton, 1965), and in both *U. violacea* and *C. lagopus* the differences are less obvious on complete medium. It was also possible to identify diploid and haploid strains by the length of sporidia. Cell size has been used as a test for ploidy in *Aspergillus nidulans* where the volume ratio is 2:1 (Roper, 1952), *Penicillium expansum* (Barron, 1962) and *Verticillium albo-atrum* (Hastie, 1964), but has not proved to be a useful test in the other basidiomycete species, *U. maydis* (Holliday,

1961*b*) and *C. lagopus* (Casselton, 1965) where the cells are very variable and the size overlaps. In *C. lagopus* the volume ratio of diploid to haploid is about 1·5:1 (Casselton, 1965) compared with 2–3·6:1 for *U. violacea*. The reasons for the different physiological behaviour between diploid and haploid cells on minimal medium, which leads to the high volume ratio of 3·6:1, are not known.

(ii) *The frequency of diploid formation*

Casselton (1965) has suggested that diploids are formed more frequently in the basidiomycetes than in the ascomycetes and Fungi Imperfecti. The evidence from *U. violacea* supports this suggestion, as the frequency of diploids was 3×10^{-4}, very similar to the value of 1×10^{-3} to 10^{-4} quoted for *Coprinus* and much greater than the values of 1×10^{-5} to 10^{-8} reported for various lower fungi (Casselton, 1965). Diploids of *U. maydis* were as frequent as 10% in one experiment (Holliday, 1961*b*), but this figure is not strictly comparable with the frequencies obtained in *Coprinus* and *U. violacea* as the diploids were obtained through failure of meiosis and not by nuclear fusion in a dikaryon.

These differences in frequency of nuclear fusion may reflect the difference in nuclear organization between dikaryons in the basidiomycetes, and heterokaryons in the ascomycetes and Fungi Imperfecti. The closer proximity of the nuclei in a dikaryon may be more favourable for fusion than the scattered distribution noticed in many heterokaryons. Another factor may be selection against diploid nuclei in *Aspergillus* and other fungi with heterokaryons. The frequency of diploid nuclei in heterokaryons has generally been estimated from the conidia, so that selection against the entry of diploid nuclei into conidia may affect these differences in frequencies. Clutterbuck & Roper (1966) have used fluorescence microscopy to observe nuclei directly in diploid–haploid heterokaryons of *Aspergillus nidulans* and have shown that there were approximately twice as many diploid nuclei in the hyphae as in the conidia. The estimates of the frequency of diploid formation in ascomycetes and Fungi Imperfecti may therefore be too low, because of the indirect method of sampling; but even when they are corrected for this error, the estimates are still much smaller than those reported for the basidiomycetes. The sampling technique in *U. violacea* permits direct measurements of the frequency of nuclear fusion, as the dikaryon cannot grow on an artificial medium and therefore either dies or produces a diploid. *U. violacea* is therefore a very suitable organism for studies of the factors affecting nuclear fusion in vegetative cells of fungi.

(iii) *The incompatibility mechanism*

The ready fusion of sporidia after only 3 h on water agar makes *U. violacea* a particularly convenient organism for studies of incompatibility mechanisms and the mating type reaction (Kniep, 1919; Bauch, 1922). The neutral behaviour of $a_1 a_2$ diploid sporidia to either a_1 or a_2 haploids provides evidence for the type of incompatibility mechanism found in *U. violacea*. Mechanisms involving complementation between unlike alleles can be eliminated; instead the system seems to

be one of positive incompatibility, i.e. opposition between like alleles, as found in higher plants and also in the B gene of *Coprinus* (Casselton & Lewis, 1966). The oppositional mechanism found in *U. violacea*, where diploids are neutral to haploids containing a common allele, resembles closely the gametophytic incompatibility system of higher plants, e.g. *Nicotiana sanderae* (East & Mangelsdorf, 1925; Lewis, 1954). It differs from that in *Coprinus* where diploids are not neutral, but are compatible with haploids, and with diploids having one but not both of the B alleles in common.

(iv) *Segregation in diploid strains*

Diploid strains were solopathogenic as has also been reported for *U. maydis* (Christensen, 1931; Holliday, 1961*b*). In both species of *Ustilago*, the diploid combines the pathogenicity of the dikaryon with the saprophytic ability of the haploid. As in diploid *Aspergillus* (Elliott, 1960), there is no evidence that fusion of two diploid nuclei occurs to give a tetraploid meiosis. The tetrad of sporidia therefore develops apogamously from a single diploid nucleus, and the segregation ratios are typical of diploid meiosis. Analysis of genetic segregation during meiosis can therefore be obtained from host plants inoculated either with two haploid lines of opposite mating type or with one diploid line.

Genetic analysis can also be obtained from the parasexual cycle, which was shown to occur in this species. Spontaneous segregation in diploid strains by mitotic crossing-over or by haploidization is very rare, which seems to be typical of the basidiomycetes. In *Coprinus*, the diploid nuclei are stable in monokaryons, but partly or completely unstable in diploid–haploid and diploid–diploid dikaryons respectively (Casselton & Lewis, 1966). The neutrality of the mating type of diploid sporidia of *U. violacea* prevents any comparable analysis of stability in similar dikaryons. Segregation due to mitotic crossing-over has not yet been observed in *Coprinus* (L. A. Casselton, personal communication) and haploidization has not been demonstrated for *U. maydis*, except for one doubtful report (Gattani, 1946). Both of these segregation mechanisms have been observed in diploid strains of *U. violacea*.

Spontaneous haploidization is very rare in *U. violacea* but large numbers of haploids can be induced by treatment with PFP, from 200–500 haploid papillae occurring on one plate of PFP medium. The rarity of spontaneous haploidization and the compact, non-spreading growth of the sporidial colonies ensures that each haploid papillum is derived from a separate haploidization event. These features, and the convenience of replica plating for the identification of the genotypes of large numbers of segregants, makes this species particularly suitable for the genetic analysis of the parasexual cycle.

It has been proposed (Day & Jones, 1966) that the vigorously growing haploid papillae originate after mutations to PFP-resistance have occurred in some of the diploid sporidia. As the majority of these mutations are probably recessive, there is a strong selection favouring the survival of cells which have become monosomic for the chromosome carrying the resistance gene, and which can therefore express resistance. These monosomic cells then presumably become fully haploid by a

stepwise loss of chromosomes (Käfer, 1961). It is probable that, in addition to selecting resistant genotypes, PFP actively induces haploids by causing irregularities in nuclear division. Some evidence that the haploid segregants from diploids treated with PFP are more resistant to PFP than the parental haploid stocks has been obtained. The resistance of these haploid segregants seems to resemble that of some mutants which have been isolated recently from haploid lines grown on PFP medium. It is hoped to use these mutants to investigate PFP-induced haploidization more extensively. Mutants resistant to PFP have been isolated in several other fungi, including *U. maydis* (Holliday, 1964), *N. crassa* (Kinsey, 1967), and *A. nidulans* (Warr & Roper, 1965). McCully & Forbes (1965) have reported, however, that the segregants obtained from PFP-induced haploidization in *A. nidulans* did not have increased resistance to PFP, and Lhoas (1967) has suggested another explanation for the action of PFP on *Aspergillus*. It is possible therefore that the mechanism by which PFP induces haploidization is different in *Aspergillus* and *U. violacea*.

As in diploid *Aspergillus*, PFP does not induce mitotic crossing over (Putrament, 1964) and haploidization and crossing-over remain separate events, rarely coinciding (Pontecorvo & Käfer, 1958). Thus genes on the same chromosome do not reassort, and linkage groups can be readily identified by the absence of recombinant classes.

Preliminary evidence from such analysis of PFP-induced haploids indicates that the haploid chromosome number is not less than seven. The results obtained by meiotic segregation have been consistent with this estimate. This result is in conflict with cytological observations that the haploid chromosome number is two in several species of *Ustilago* including *U. violacea* and *U. maydis* (Wang, 1932; Fischer & Holton, 1957; Person & Wighton, 1964). However, one early count of the chromosome number in *U. scabiosae* (a close relative of *U. violacea*) gave $n = 6$–8 (Harper, 1898). Since the nuclei are very small and chromosomes difficult to distinguish, these reports must be accepted with some reservations. The genetic evidence in *U. violacea* that n is greater than two is also supported by genetic evidence that there at least five chromosome arms in *U. maydis* (Holliday, 1964).

(v) *The importance of the parasexual cycle in Ustilago*

There is evidence from both *U. maydis* and *U. violacea* that diploid nuclei arise at a high frequency from failures in meiosis. Thus Christensen (1931) found that seven brandspores of *U. maydis* of a total of 275 examined gave rise to solopathogenic lines. Holliday (1961*b*) also reported that in one particular cross in the same species diploid strains arose at a frequency of about 10%. However, Schmitt (1940) found only three such strains in over 4000 isolates. In *U. violacea* Bauch (1922) and Zillig (1921) have found naturally occuring large-celled isolates of *U. violacea* at about the same frequency as Schmitt (1940). No explanation was offered for the origin of these lines. However, these isolates resemble closely the diploid strains reported above and it seems likely that they were diploid.

Diploid strains of *Ustilago* therefore arise relatively frequently by failures of

meiosis and by nuclear fusion in the dikaryon. Mitotic segregation either by crossing-over or by haploidization would lead to homozygosity in the diploid nucleus of the brandspore. Such mechanisms could therefore aid in the retention of favourable mutations, or in the elimination of unfavourable ones. However, as diploids are reduced to haploids by meiosis in each generation, and as spontaneous mitotic segregation has been shown to be very rare, at least under artificial conditions of culture, parasexual recombination, is likely to be of limited significance in the production of new and more virulent races.

SUMMARY

1. Forty-nine auxotrophic mutants were isolated after irradiation with ultraviolet light. The biochemical requirements were identified and the response to possible precursors tested in some of them.

2. Diploid colonies were synthesized from compatible, auxotrophic haploid strains on an artificial medium by an adaptation of the balanced heterokaryon technique. Selection of diploid cells was especially convenient as the dikaryon cannot grow on such media, and is therefore only a transitory stage under these cultural conditions. Diploid cells were different in shape and size from haploids, and gave rise to colonies which could be distinguished by eye from haploids. The way in which diploid colonies arose from fusions between haploid cells was followed by microscopic observation. When known numbers of fused haploid cells were plated on minimal medium, diploid colonies occurred at a frequency of 3×10^{-4}.

3. Diploid cells, heterozygous for mating-type alleles, were incompatible with either haploid mating-type (i.e. neutral).

4. Diploid cells could infect the host plant as a pure culture (i.e. they are solopathogenic).

5. Meiotic and mitotic segregation of the large-celled strains was used to confirm diploidy. Spontaneous mitotic segregation was very rare. Mitotic crossing-over was induced at a high frequency by irradiation of diploid cells with ultraviolet light. A convenient technique for induced haploidization was devised using *p*-fluorophenylalanine. Preliminary evidence using this technique indicates a haploid chromosome number of at least seven.

6. It is considered that *U. violacea* has many advantages as an organism for genetical research, especially for analysis of the parasexual cycle.

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