Heterozygous diploid analyses via the parasexual cycle and a cytoplasmic pattern of inheritance in *Verticillium spp.*

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Combining data from mitotic crossing over in, and haploidization of, heterozygous diploid strains, 33 markers have been mapped successfully in *Verticillium* spp. One marker (*Hyl*), which determines the absence of darkly pigmented resting structures in many stable hyaline strains, showed an irregular segregation. In view of previous evidence from acriflavine treatment and heterokaryon analyses in the fungus, this further substantiated indications of a cytoplasmic pattern of inheritance for this marker. The phenotypic expression of dark pigment genes showing nuclear inheritance, such as *Sot.*, depends upon the cytoplasmically determined (*Hyl+*) presence of resting structures.

1. INTRODUCTION

The only alternative to sexual recombination in fungi is somatic recombination which allows organisms unable to reproduce sexually to produce new combinations of their genetic material. Genetic investigation of the imperfect fungi became possible when Pontecorvo & Roper (1952), Pontecorvo (1954, 1956*a, b*) and Pontecorvo, Roper & Forbes (1953) conducted genetic analysis through the parasexual cycle with *Aspergillus niger* and *A. nidulans*. The techniques developed with these species were widely adapted for studies with many plant pathogens which, like *Verticillium*, have no known sexual stage (Pontecorvo & Sermonti, 1954; Buxton, 1956; Tuveson & Coy, 1961; Strømnaes, Garber & Beraha, 1964; Tinline & MacNeill, 1969). The essential features for genetic analyses through the parasexual cycle are mitotic crossing over and haploidization which are both rare and independent in *Aspergillus nidulans* (Pontecorvo, Tarr-Gloor & Forbes, 1954).

Haploidization apparently occurs via aneuploidy following non-disjunction (Käfer, 1961) in a diploid nucleus; this results in the segregation and reassortment of whole chromosomes. Markers of the same linkage groups therefore almost always remain together during mitotic division and haploidization. In contrast, markers of different linkage groups combine at random during the reduction from diploid to haploid chromosome complement, i.e. half are parental combinations

and half are recombinants. In practice, only approximate 1/1 segregation ratios are attained when analysing haploids produced via the parasexual cycle, probably mainly due to the differential clonal multiplication rates of haploid nuclei carrying different alleles.

Robinson, Larson & Walker (1957) suggested genetic recombination as a possible explanation for the wide variability of their monoconidial cultures of the uninucleate fungus *Verticillium albo-atrum*. Many workers have encountered hyaline variants of *Verticillium* characterized by the absence of either darkly pigmented resting mycelium (produced by *V. albo-atrum*) or microsclerotia (*V. dahliae*); such variants are difficult to classify since this normally depends largely on the type of resting structure produced by these imperfect plant pathogenic fungi.

Genetic recombination and the parasexual cycle in *V. albo-atrum* were first described by Hastie (1962) and Fordyce (1962) and have been reported on in detail later for both *V. albo-atrum* and *V. dahliae* (Hastie, 1964, 1967, 1968). In a comparison of the parasexual systems of *A. nidulans* and *V. albo-atrum*, Hastie (1970) found that the main difference is in the higher frequencies of mitotic crossing over and haploidization in the latter. He reported a frequency of haploidization in *Verticillium* as much as 0·2 per diploid nuclear division. This creates a complication when analysing *Verticillium* haploids in that they could have been derived from a diploid in which crossing over has occurred already. Typas & Heale (1977) have similarly noted a marked instability of heterozygous diploid cultures of *Verticillium* such that after 3 weeks, 90 % of the conidia sampled were haploid, whereas the corresponding figure for *A. nidulans* was 0·1 % (Pontecorvo, 1959). Although it is very difficult to isolate aneuploids following heterozygous diploid analyses, the slight irregularities in spore size distribution curves (obtained from a Coulter Counter Channelyzer) of segregating heterozygous diploids indicated the presence of such aneuploids (Typas, 1976; Typas & Heale, 1977).

Evidence for the possible operation of cytoplasmic factors in the expression, and perhaps the inheritance of, dark pigmentation in *V. albo-atrum* was first suggested by Hastie (1962) and was later supported by heterokaryon tests which Heale (1966) performed. The effects of acriflavine, in increasing the spontaneous rate of hyaline (*Hyl*) and partially hyaline (*Hyl+.p*) variants (Typas & Heale, 1976*a*) together with the detailed conidial analysis of heterokaryons of *V. albo-atrum* and *V. dahliae* (Typas & Heale, 1976*b*), contributed substantial evidence in support of cytoplasmic inheritance of characters associated with the development of dark resting structures.

The present study was undertaken to further investigate the mechanisms which control production of dark resting structures in *Verticillium* by analysis through the parasexual cycle. At the same time, combining data from mitotic crossing over in, and haploidization of, heterozygous diploid strains, 33 markers, most of them auxotrophic, have been mapped.

2. MATERIALS AND METHODS

(i) *Strains*

Auxotrophic and morphological (affecting pigmentation) mutants of *V. albo-atrum* and *V. dahliae* were isolated after treatment of conidia with u.v.-light or acriflavine (Typas & Heale, 1976*a*, *b*). Table 1 details the heterozygous diploids used for genetic analysis via the parasexual cycle. Gene symbols used throughout this work are based on the Demerec system (Demerec, Adelberg, Clark & Hartman, 1966) and mutant alleles with the phenotypes they determine are as follows: *arg* = arginine, *pab* = *p*-amino benzoic acid, *ino* = inositol, *cho* = choline, *trp* = tryptophan, *nic* = nicotinic acid, *met* = methionine, *ane* = aneurine, *asp* = asparagine, *urc* = uracil, *ade* = adenine, *gua* = guanine, *leu* = leucine, *pro* = proline, *bio* = biotin, *lys* = lysine, *phe* = phenylalanine, *pyr* = pyridoxin *amm* = ammonium.

(ii) *Media*

Czapek-Dox agar served as minimal medium (MM) (Typas & Heale, 1976*a*). Complete medium (CM) contained the constituents of MM with the addition of casein hydrolysate, yeast extract, malt extract, and peptone at 2 g/l. Solid media contained 2 % (w/v) Japanese 'Davies' agar. Addition of supplements to MM was as follows: amino acids, purines, pyrimidines, 20 μg/ml; vitamins, 1 μg/ml. All cultures were grown at 24 °C.

(iii) *Heterokaryons and selection of prototrophic heterozygous diploid conidia*

Prototrophic heterozygous diploid conidia were recovered either by plating large numbers of spores from heterokaryons on MM plates (recovery at a frequency of about 1 in 8×10^6 spores) or from prototrophic sectors formed by mycelial 'pellets' that had been incubated on MM for 4–6 weeks at 21 °C (Typas & Heale, 1976*b*).

(iv) *Analysis of heterozygous diploids*

A modified procedure from that described by Pontecorvo (1959) and Hastie (1962, 1964) was adopted to obtain segregants of independent origin. Selected heterozygous diploids were grown on MM for 3–5 days, until they conidiated, and their spores were then inoculated on CM on which auxotrophic segregants were not at a pronounced selective disadvantage. In order to ensure that any segregants formed on MM would be transferred, and that subsequently both alleles of each originally heterozygous locus would be represented in the inoculum transferred to CM, a large number of conidia from each selected heterozygous diploid was inoculated at one location.

A series of 20–30 segregating cultures was established in this way for the analysis of each heterozygous diploid. Each segregating culture contained both haploid and diploid genotypes; conidial measurements were used to distinguish ploidy levels (Typas & Heale, 1977).

Table 1. *Heterozygous diploids of* Verticillium *spp. analysed via the parasexual cycle with their genotypes*

Key number		Genotypes*
1	*V. albo-atrum*	$\dfrac{pab \; ino \; + \; +}{+ \;\;\; + \;\; arg\text{-}10 \; cho\text{-}4}$
2	,,	$\dfrac{trp\text{-}7 \; nic\text{-}5 \; +}{+ \;\;\;\; + \;\;\; arg\text{-}11}$
3	,,	$\dfrac{nic\text{-}5 \; met\text{-}9 \; + \; +}{+ \;\;\;\; + \;\; pab\text{-}2 \; ino\text{-}8}$
4	,,	$\dfrac{ane\text{-}1 \; ade\text{-}2 \; + \;\;\; + \;\;\; Hyl^+}{+ \;\;\;\; + \;\; met\text{-}3 \; nic\text{-}3 \; Hyl}$
5	,,	$\dfrac{arg\text{-}3 \; cho\text{-}1 \; +}{+ \;\;\;\; + \;\;\; urc\text{-}5}$
6	,,	$\dfrac{ade\text{-}10 \; +}{+ \;\;\; arg\text{-}12}$
7	*V. dahliae*	$\dfrac{arg\text{-}16 \; + \; +}{+ \;\;\; asp \; ino}$
8	,,	$\dfrac{arg\text{-}6 \; + \;\;\; + \;\;\; Hyl^+}{+ \;\; ade\text{-}8 \; gua\text{-}1 \; Hyl}$
9	,,	$\dfrac{arg\text{-}7 \; pro\text{-}1 \; +}{+ \;\;\;\; + \;\;\; leu\text{-}4}$
10	,,	$\dfrac{met\text{-}4 \; +}{+ \;\;\; arg\text{-}19}$
11	*V. albo-atrum*	$\dfrac{met \; nic \; trp \; + \;\;\; +}{+ \;\;\; + \;\;\; + \;\; arg\text{-}9 \; Sot\text{-}1}$
12	,,	$\dfrac{pyr \; phe \; amm \; + \;\;\; +}{+ \;\;\; + \;\;\; + \;\; urc\text{-}1 \; Sot\text{-}4*}$
13	,,	$\dfrac{met \; nic \; trp \; + \;\;\; +}{+ \;\;\; + \;\;\; + \;\; urc\text{-}1 \; Sot\text{-}4*}$
14	,,	$\dfrac{arg\text{-}9 \; Sot\text{-}1 \; + \;\;\; Hyl^+}{+ \;\;\;\; + \;\; ade\text{-}3 \; Hyl}$
15	,,	$\dfrac{ino\text{-}4 \; Sot\text{-}6 \; + \;\;\; + \;\;\; Hyl^+}{+ \;\;\;\; + \;\; arg\text{-}3 \; cho\text{-}1 \; Hyl}$
16	,,	$\dfrac{nic\text{-}5 \; met\text{-}9 \; + \;\;\; +}{+ \;\;\;\; + \;\; urc\text{-}1 \; Sot\text{-}4*}$
17	,,	$\dfrac{ade\text{-}4 \; arg\text{-}4 \; + \;\;\; +}{+ \;\;\;\; + \;\; met\text{-}3 \; nic\text{-}3}$
18	*V. dahliae*	$\dfrac{ade\text{-}5 \; bio\text{-}1 \; +}{+ \;\;\;\; + \;\;\; arg\text{-}16}$
19	,,	$\dfrac{ade\text{-}5 \; bio\text{-}1 \; + \;\;\; + \;\;\; Hyl^+}{+ \;\;\;\; + \;\; met\text{-}7 \; ane\text{-}4 \; Hyl}$

Table 1. (*cont.*)

Keynumber		Genotypes*
20	*V. dahliae*	$\dfrac{arg\text{-}6}{+}\ \dfrac{+}{met\text{-}7}\ \dfrac{+}{ane\text{-}4}\ \dfrac{Hyl^+}{Hyl}$
21	,,	$\dfrac{nic}{+}\ \dfrac{lys}{+}\ \dfrac{+}{arg\text{-}7}\ \dfrac{+}{pro\text{-}1}\ \dfrac{Hyl^+}{Hyl}$
22	,,	$\dfrac{leu\text{-}4}{+}\ \dfrac{+}{ade\text{-}8}\ \dfrac{+}{gua\text{-}1}$ ..
23	*V. albo-atrum*	$\dfrac{pyr}{+}\ \dfrac{phe}{+}\ \dfrac{amm}{+}\ \dfrac{+}{met\text{-}3}\ \dfrac{+}{nic\text{-}3}\ \dfrac{Hyl^+}{Hyl}$

* The genotypes (4, 8, 14, 15, 19, 20, 21, 23) which carry the *Hyl* marker in the 'hetero-zygous' condition only are specified. Symbols concerning pigmentation phenotypes are as follows: *Hyl*+, wild-type phenotype producing normal amounts of dark melanin pigmentation and resting structures as when first isolated from the diseased host: *Hyl*, stable hyaline strains, not producing dark pigment or resting structures, obtained from *Hyl*+ by u.v.-irradiation or acriflavine treatment; *Sot*, 'sooty' mutants kindly provided by Dr A. C. Hastie, Dundee University. They are distinct from wild-type *Hyl*+ *Sot*+ in that their black pigmentation extends to the growing edge of the culture within 4–5 days whereas wild-type isolates form dark resting mycelium only after 10–14 days growth at 24 °C; *Sot*-4*, hyaline strain which was derived from the 'sooty' strain by u.v.-irradiation.

(*a*) *Haploids*

The haploid segregants (products of haploidization) were obtained by plating conidial suspensions from 10- to 14-day-old segregating cultures on CM. To ensure that only haploids of independent origin were considered, and to avoid selecting products of clonal multiplication, only one haploid of any specific genotype from each segregating culture was used. Their genotypes were determined by replica plating on appropriately supplemented MM media and the results were analysed in terms of linkage groups.

(*b*) *Second-order diploids* (*mitotic cross-over products*)

In cases where linkage between two or more markers was indicated in the earlier haploid analyses, analyses of independent diploid segregants (i.e. second-order diploids) was also carried out. This produced evidence related to gene order changes resulting from mitotic crossing over. The genotypes of independent second-order isolates were determined by random conidial analysis of third-order isolates derived from the independent second-order diploids.

3. RESULTS

Among the original segregants (first-order isolates) from the heterozygous diploid *Verticillium* strains, haploids (3–6 μm in length) could be clearly distinguished from diploids (6–11 μm in length); the range of variation in size being less marked within individual haploids and diploids. While haploids tended to show segregation of one or other allele at each, and every locus originally hetero-

10-2

zygous, first order diploids usually showed segregation only for one or a few linked markers, remaining heterozygous for the others; the latter could therefore be detected by their capacity to produce further segregant genotypes (i.e. second-order segregants) as shown by Pontecorvo *et al.* (1953) in *A. nidulans*. An example of the classification of 62 independent haploids amongst the second-order isolates derived from 18 cultures of the prototrophic diploid no. 21

$$\left(\frac{nic}{+} \; \frac{lys}{+} \; \frac{+}{arg\text{-}7} \; \frac{+}{pro\text{-}1} \; \frac{Hyl^+}{Hyl}\right),$$

which segregated for all markers involved, is given in Table 2. None of the auxotrophic allele ratios for the haploids listed in Table 2 differed appreciably from a 1:1 approximation. When recombination between all possible pairs of different

Table 2. *Segregation and recombination of markers* among 62 independent haploids* (*second order isolates*) *derived from diploid no. 21* $\left(\frac{nic}{+} \; \frac{lys}{+} \; \frac{+}{arg\text{-}7} \; \frac{+}{pro\text{-}1} \; \frac{Hyl^+}{Hyl}\right)$ *cultures of V. dahliae*

Segregation

	Markers				
	nic	lys	arg-7	pro-1	Hyl
Mutant phenotype	30	29	31	30	14†
Wild-type phenotype	32	33	31	32	48

Recombination

	Genotypes			Recombination frequency (%)
nic lys 14	nic + 16	+ lys 15	+ + 17	50·0
nic arg-7 1	nic + 29	+ arg-7 30	+ + 2	4·8
nic pro-1 1	nic + 29	+ pro-1 29	+ + 3	6·5
nic Hyl 6	nic + 24	+ Hyl 8	+ + 24	48·4
lys arg-7 13	lys + 16	+ arg-7 18	+ + 15	45·2
lys pro-1 16	lys + 13	+ pro-1 14	+ + 19	56·6
lys Hyl 4	lys + 25	+ Hyl 10	+ + 23	43·5
arg-7 pro-1 29	arg-7 + 2	+ pro-1 1	+ + 30	4·8
arg-7 Hyl 7	arg-7 + 24	+ Hyl 7	+ + 24	50·0
pro-1 Hyl 9	pro-1 + 21	+ Hyl 5	+ + 27	41·9

* *Arg* was epistatic to *pro* and the *arg pro* types were classified as growing on arginine alone.

† In the relevant eight separate diploids analysed the figure for *Hyl* (mutant phenotype) averaged c. 4 %; this ratio for 21 was the highest encountered.

markers was considered, only the proportion of recombinants for *nic* and *arg*-7 (4·8 %), *nic* and *pro*-1 (6·5 %) and *arg*-7 and *pro*-1 (4·8 %) indicated linkage.

The summarized results of independent haploid analyses for all the different heterozygous diploids (listed in Table 1) which segregated for all markers involved are presented in Table 3. Between 55 and 80 independent haploids and 15–30 segregating heterozygous diploids were examined and classified for genetic analyses

Table 3. *Analysis of independent haploid segregants (second-order isolates) from prototrophic diploid cultures of* Verticillium *spp. which segregated for all markers involved*

Hetero-zygous diploid*	Segregation ratio of loci; all *c.* 1:1, except:	Recombination frequency *c.* 50 % for all loci, except:	Linkage
1	—	3·4 % for *pab, ino*	*pab*—*ino*
2	—	3·0 % for *nic*-5, *arg*-11	*nic*-5—*arg*-11
3	—	4·9 % for *pab*-2, *ino*-8	*pab*-2—*ino*-8
4	*Hyl/Hyl*+ 1/77	—	No linkage
5	*urc*-5/+ 26/48	(see text for *urc*-5/+)	No linkage
6	—	—	No linkage
7	—	—	No linkage
8	*Hyl/Hyl*+ all *Hyl*+	—	No linkage
9	—	4·6 % for *arg*-7, *pro*-1	*arg*-7—*pro*-1
10	—	—	No linkage
11	—	4·5 % for *arg*-9, *Sot*-1	*arg*-9—*Sot*-1
12	—	—	No linkage
13	—	93·8 % for *urc*-1, *trp*	*trp*—*urc*-1
14	*Hyl/Hyl*+ all *Hyl*+	5·0 % for *arg*-9, *Sot*-1	*arg*-9—*Sot*-1
15	*Hyl/Hyl*+ 1/78	—	No linkage
16	—	—	No linkage
17	—	3·6 % for *arg*-4, *nic*-3	*arg*-4—*nic*-3
18	—	—	No linkage
19	*Hyl/Hyl*+ 1/74	5·4 % for *bio*-1, *met*-7	*bio*-1—*met*-7
20	*Hyl/Hyl*+ all *Hyl*+	—	No linkage
21	*Hyl/Hyl*+ 14/62	4·8 % for *nic, arg*-7	*pro*-1—*arg*-7—*nic*
		6·5 % for *nic, pro*-1	
22	—	8·6 % for *ade*-4, *leu*-4	*ade*-8—*leu*-4
23	*Hyl/Hyl*+ 6/64	4·7 % for *pyr, nic*-3	*nic*-3—*pyr*
		7·8 % for *amm, met*-3	*met*-3—*amm*

* The diploid genotypes were as in Table 1.

of each diploid strain listed in Table 1. Considering the independent haploids, the segregation ratios of virtually all markers did not depart appreciably from an approximate 1:1 expectation. The only striking exception was that of the *Hyl* marker, as only a small proportion of haploids had the *Hyl* phenotype. In the case of heterozygous diploid 5, the *urc*-5 marker segregated at a ratio of 26/48, probably due to the very poor sporulation of the *urc*-5 strain. In the case of *Hyl* strains, on the other hand, close examination showed that their reduced sporulation was insufficient in itself to fully account for the failure of this marker to segregate normally. In contrast with the *Hyl* marker, the *Sot* locus segregated normally in all diploids tested.

In the analysis of the independent second order diploids it was assumed that all of them were diploids rather than aneuploids, and that they had arisen by mitotic crossing over. An example of a classification of a large number of independent diploid genotypes arising from 18 heterozygous diploid no. 21 cultures

$$\left(\frac{nic\ lys\ +\ +\ Hyl^+}{+\ +\ arg\text{-}7\ pro\text{-}1\ Hyl}\right),$$

with respect to all possible markers is presented in Table 4. Mitotic, like meiotic crossing over, occurs in the four-strand stage. The exchanges are reciprocal and symmetrical, and only two of the four strands take part at any one exchange (Käfer, 1961). When a mitotic crossover occurs in a heterozygous diploid nucleus, all the heterozygous markers which lie distal to the point of exchange become homozygous for half of the possible mitotic products, while all those between the

Table 4. *Classification of 25 independent diploid genotypes derived from 18 diploid no. 21* $\dfrac{(nic\ lys\ +\ +\ Hyl^+)}{+\ +\ arg\text{-}7\ pro\text{-}1\ Hyl}$ *cultures of* V. dahliae *with respect to all possible pairs of markers*

	$\dfrac{nic}{nic}$	$\dfrac{nic}{+}$	$\dfrac{+}{+}$	$\dfrac{lys}{lys}$	$\dfrac{lys}{+}$	$\dfrac{+}{+}$	$\dfrac{arg\text{-}7}{arg\text{-}7}$	$\dfrac{arg\text{-}7}{+}$	$\dfrac{+}{+}$	$\dfrac{pro\text{-}1}{pro\text{-}1}$	$\dfrac{pro\text{-}1}{+}$	$\dfrac{+}{+}$
$\dfrac{Hyl}{Hyl}$	3	3	0	0	4	2	2	2	2	2	2	2
$\dfrac{Hyl}{Hyl^+}$	3	3	2	4	3	1	2	4	2	2	4	2
$\dfrac{Hyl^+}{Hyl^+}$	2	9	0	1	8	2	3	6	2	2	6	3
$\dfrac{pro\text{-}1}{pro\text{-}1}$	2	3	1	1	4	1	0	0	6	.	.	.
$\dfrac{pro\text{-}1}{pro\text{-}1^+}$	2	9	1	3	7	2	0	12	0	.	.	.
$\dfrac{pro\text{-}1^+}{pro\text{-}1^+}$	4	3	0	1	4	2	7	0	0	.	.	.
$\dfrac{arg\text{-}7}{arg\text{-}7}$	4	3	0	1	4	2	.	.	.	.	.	.
$\dfrac{arg\text{-}7}{arg\text{-}7^+}$	2	9	1	3	7	2	.	.	.	.	.	.
$\dfrac{arg\text{-}7^+}{arg\text{-}7^+}$	2	3	1	1	4	1	.	.	.	.	.	.
$\dfrac{lys}{lys}$	2	3	0	.	.	.	.	.	.	.	.	.
$\dfrac{lys}{lys^+}$	5	8	2	.	.	.	.	.	.	.	.	.
$\dfrac{lys^+}{lys^+}$	1	4	0	.	.	.	.	.	.	.	.	.

Hyl/Hyl^+ and Hyl^+/Hyl^+ estimation was subjective, based on whether or not the third-order isolates segregated for this marker.

centromere and the site of crossover remain heterozygous. Thus the sequence of the markers, and to some extent their relative positions, could be determined by the homozygous-heterozygous relationships of the loci of the independent diploid isolates (Käfer, 1958; Holliday, 1961 *a, b*).

When two groups of linked loci segregated simultaneously (i.e. *arg*-7 and *pro*-1 in Table 4) they are assumed to represent markers in arms of homologous chromosomes. The data for such a pair of markers sited on the same or homologous

Table 5. *Analysis of independent diploid segregants amongst the second-order isolates from prototrophic diploid cultures of* Verticillium *spp. which segregated for all markers involved, and derived gene order*

Hetero-zygous diploid*	Linked markers		Same chromo-some arm (I)	Different arms of the same chromo-some (II)	Gene order (from the homozygous/heterozygous relationship) and relative centromere position
1	*pab*	*ino*	+	.	Centromere *pab* *ino*
2	*nic*-5	*arg*-11	.	+	*nic*-5 centromere *arg*-11
3	*pab*-2	*ino*-8	+	.	Centromere *pab*-2 *ino*-8
4	.		.	.	. . .
5	.		.	.	. . .
6	.		.	.	. . .
6	.		.	.	. . .
7	.		.	.	. . .
8	.		.	.	. . .
9	*arg*-7	*pro*-1	+	.	Centromere *pro*-1 *arg*-7
10	.		.	.	. . .
11	*arg*-9	*Sot*-1	+	.	Centromere *Sot*-1 *arg*-9
12	.		.	.	. . .
13	*urc*-1	*trp*	.	+	*urc*-1 centromere *trp*
14	*arg*-9	*Sot*-1	+	.	Centromere *Sot*-1 *arg*-9
15	.		.	.	. . .
16	.		.	.	. . .
17	*arg*-4	*nic*-3	.	+	*nic*-3 centromere *arg*-4
18	.		.	.	. . .
19	*bio*-1	*met*-7	.	+	*bio*-1 centromere *met*-7
20	.		.	.	. . .
21	*nic,*	*pro*-1, *arg*-7	+	+	*nic* centromere *pro*-1 *arg*-7
22	*leu*-4	*ade*-8	.	+	*leu*-4 centromere *ade*-8
23	*pyr*	*nic*-3	+	.	Centromere *nic*-3 *pyr*
	amm	*met*-3	+	.	Centromere *amm*(?) *met*-3(?)

* The diploid genotypes were as in Table 1.

chromosome arm are distributed along one of the diagonals of the appropriate section in the table. The marker *nic* which in the haploid analysis showed linkage with both *arg*-7 and *pro*-1 did not segregate simultaneously with any of these markers; this is accounted for if the loci are on opposite sides of their centromere.

Table 5 presents the summarized results of classification of the independent

diploid genotypes arising from all heterozygous diploids listed in Table 1, with respect to all possible pairs of markers. In the analysis of heterozygous diploid 13

$$\frac{(met \; trp \; nic \; + \quad + \;)}{(\; + \quad + \quad + \quad urc\text{-}1 \; Sot\text{-}4)},$$

because 93·8 % of the haploids recovered had the *trp* and *urc*-1 markers in 'coupling' (see Table 3), it was assumed that a phase change in the nuclear lineage, from 'repulsion' to 'coupling', must have occurred from which a single conidium was taken and purified as diploid 13. In all cases the *Hyl* marker failed to show simultaneous segregation with any of the biochemical markers, thus verifying the absence of linkage indicated earlier by haploid analysis.
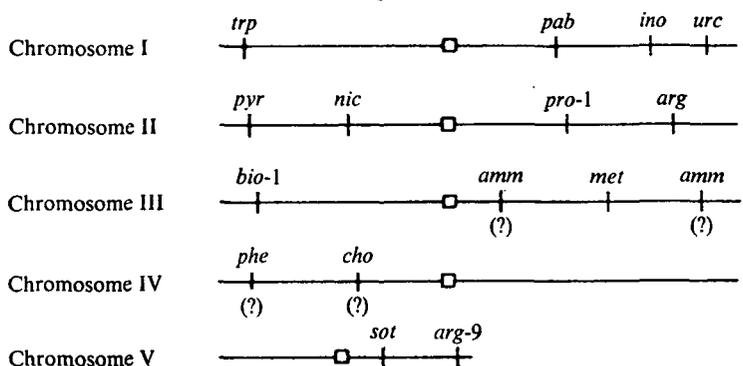
4. DISCUSSION

The application of extensive genetic analyses of heterozygous diploids carrying auxotrophic and morphological marker genes in the present work led to a tentative linkage map for *Verticillium albo-atrum* and *V. dahliae* (Fig. 1). Although haploidization and mitotic crossing over are two independently occurring phenomena, their relatively high frequency in *Verticillium* could result in their both occurring simultaneously, and therefore cause a discrepancy in the results as they affect estimates of gene order. This is one of the factors which makes the task of mapping genetic markers in *Verticillium* far more difficult than in *Aspergillus*.

In the present study using haploid analysis a number of marker genes have been detected in 5 linkage groups in *V. albo-atrum* and 4 in *V. dahliae*. The difference between the haploid (n) chromosome number inferred from genetic data here for *V. albo-atrum* and the reported number for the fungus ($n = 4$; Hastie, 1967; Heale, Gafoor & Rajasingham, 1968) demands some explanation. Brushaber, Wilson & Aist (1967) and Heale *et al.* (1968) reported independently on nuclear division in *Verticillium* and in particular observed the tendency of the chromosomes to link forming ring and filamentous structures. If it is assumed that changes in the phase of linked markers (coupling $\rightleftharpoons$ repulsion) and that formation of homozygotes can be detected, it seems more likely that the chromosomes are joined through their centromeres rather than end to end. Heale *et al.* (1968) also observed preparations in which apparently either three or five chromosomes or chromosome pairs were counted. Loane (1967) in his work with *Penicillium expansum* suggested that the haploid count could be interpreted at $n = 4$ plus 1 DNA containing centriole which stains as a chromosome, whereas Fjield and Strømnaes (1966) concluded that there were 4 linkage groups in this fungus. It could therefore be postulated that the haploid chromosome number in *V. albo-atrum* and *V. dahliae* is 5, and that the fifth (very small) chromosome occasionally fails to segregate and remains linked to another chromosome through their centromeres.

Whereas *Sot* and the auxotrophic markers investigated segregated as one would expect for nuclear genes, *Hyl* showed a very low segregation frequency in various diploids, e.g. 1/77, 0/64, 0/60, 1/78, 1/74, 0/54, 14/62 and 6/64. This, along with

data from heterokaryon analysis and acriflavine treatment (Typas & Heale, 1976 a, b), we interpret as strong evidence for cytoplasmic inheritance, i.e. the Hyl character is almost completely lost from the cytoplasm of the heterozygous diploid conidium produced by the heterokaryon and replaced by the wild-type Hyl^+ 'factor' from one of the two original auxotrophic parents. If the Hyl factor was a nuclear gene, it would have been expected to show linkage with one or more of the many bio-chemical markers used here, but it did not. The Sot marker, however, did show linkage to arg-9 in group v.

Fig. 1. A 'tentative' map of *Verticillium* spp.* marker genes† used in this investigation.

	trp			*pab*	*ino* *urc*
Chromosome I					
	pyr	*nic*		*pro*-1	*arg*
Chromosome II					
	bio-1			*amm* *met*	*amm*
Chromosome III				(?)	(?)
	phe	*cho*			
Chromosome IV					
	(?)	(?)			
		sot *arg*-9			
Chromosome V					

Group I	Group II	Group III	Group IV	Group V
trp	*pyr*	*amm*	*phe*	*arg*-9
urc-1	*pro*-1	*met*-3	*cho*-1 (?)	*Sot*-1
urc-5 (?)	*arg*-4	*met*-7	*cho*-3 (?)	*Sot*-4
trp-7 (?)	*arg*-7	*met*-9 (?)		*Sot*-6
pab	*arg*-11	*bio*-1		
pab-2	*arg*-6 (?)			
ino	*arg*-12 (?)			
ino-8	*nic*			
leu-4	*nic*-3			
ade-8	*nic*-5 (?)			
ade-2 (?)				

* No differences were found in marker locations for *V. albo-atrum* and *V. dahliae*.
† *Hyl* marker could not be assigned to any linkage group; (?) refers to an inconclusive classification.

To try to explain the considerable variation in dark pigmentation of the fungus in the light of the foregoing observations, the authors propose the following: The overwhelming majority of stable completely hyaline variants of *V. albo-atrum* or *V. dahliae* in our experience do not produce an otherwise typical form of resting structure (either resting mycelium or microsclerotia respectively) with hyaline cell walls. It is therefore the absence of the typical form of resting structure that usually causes hyalinity, and not the absence of dark melanin pigment *per se*. However, Bell *et al.* (1976) have reported recently on albino variants of *V. dahliae* which produce hyaline microsclerotia. The only such hyaline mutant analysed

genetically in detail was *alm*-1, which behaved as though it was due to a single nuclear gene lesion. A number of non-allelic genes (some cytoplasmic and some nuclear in origin) may therefore control the development of darkly pigmented resting structures. The presence or absence of resting structures *per se* is at least partially controlled by a major extrachromosomal factor (Hyl^+) which is self-replicating and transmitted via the cytoplasm of the conidia.

Hyaline variants therefore appear to comprise at least three types: (1) *completely hyaline stable strains lacking resting structures*, originating from dark parental cultures at a spontaneous rate of approximately 1 in 10^5–10^6; (2) *partially hyaline strains* (showing variation both regarding the time required for dark resting structures to form, and also in the amount of such resting bodies ultimately produced) are more frequent, occurring spontaneously at approximately 1 in 10^3; and (3) *completely hyaline stable strains with hyaline resting structures*. These apparently occur very rarely and have not yet been observed for *V. albo-atrum*. The explanation of the origin of the first type of variant is within the range of mutation, and its apparent stability is suggestive of an almost irreversible change in the factor. Mutation in the dark Hyl^+ type to produce a hyaline variant (Hyl) appears to be of a pleiotropic type; hyalinity is usually associated with faster growth rate, extensive fluffy aerial mycelium, fewer conidia and conidiophores and changes in respiration (Pilkington & Heale, 1969). The rate of origin of partially hyaline variants however, could not be explained on a similar basis. In the third type of hyaline variant we can assume that the cytoplasmic (Hyl^+) factor is present in the cell, but a nuclear gene mutation results in the blocking of pigment formation and consequently leads to production of hyaline resting structures. The occurrence of 'sooty', *alm*-1 and a range of partially hyaline variants is probably explained by the operation of several nuclear genes which control the amount of pigment *per se* and the time required for the pigment to form.

It is therefore envisaged that the phenotypic expression of such 'dark pigment' nuclear genes depends upon (i.e. is hypostatic to) the cytoplasmically determined (Hyl^+) presence or absence of resting structures. When the wild-type Hyl^+ cytoplasmic factor is present, 'sooty', normal, partially hyaline and possibly albino (with hyaline resting structures) phenotypes can be expressed.

REFERENCES

BELL, A. A., PUHALLA, J. E., TOLMSOFF, W. J. & STIPANOVIC, R. D. (1976). Use of mutants to establish (+)-scytalone as an intermediate in melanin biosynthesis by *Verticillium dahliae*. *Canadian Journal of Microbiology* **22**, 787–799.

BRUSHABER, J. A., WILSON, D. L. & AIST, J. R. (1967). Asexual behaviour of some plant pathogenic fungi. *Phytopathology* **57**, 43–46.

BUXTON, E. W. (1956). Heterokaryosis and parasexual recombination in pathogenic strains of *Fusarium oxysporum*. *Journal of General Microbiology* **15**, 133–139.

DEMEREC, M., ADELBERG, E. A., CLARK, A. J. & HARTMAN, P. E. (1966). A proposal for a uniform nomenclature in bacterial genetics. *Genetics* **54**, 61–76.

FJIELD, A. & STRØMNAES, Ø. (1966). The parasexual cycle and linkage groups in *Penicillium expansum*. *Hereditas* **54**, 389–403.

FORDYCE, C. (1962). Studies of the mechanisms of variation of *Verticillium albo-atrum* Rke and Berth. Ph.D. thesis, Purdue University.

HASTIE, A. C. (1962). Genetic recombination in the hop wilt fungus *Verticillium albo-atrum*. *Journal of General Microbiology* **27**, 373–382.

HASTIE, A. C. (1964). The parasexual cycle in *Verticillium albo-atrum*. *Genetical Research* **5**, 305–315.

HASTIE, A. C. (1967). Mitotic recombination in conidiophores of *Verticillium albo-atrum*. *Nature*, **214**, 249–252.

HASTIE, A. C. (1968). Phialide analyses of mitotic recombination in *Verticillium*. *Molecular and General Genetics* **102**, 232–240.

HASTIE, A. C. (1970). The genetics of asexual phytopathogenic fungi with special reference to *Verticillium*. *Root Diseases and Soil-Borne Pathogens* (ed. T. A. Toussoun, R. V. Bega and P. E. Nelson), pp. 56–62. Berkeley: University of California Press.

HEALE, J. B. (1966). Heterokaryon synthesis in *Verticillium*. *Journal of General Microbiology* **45**, 419–427.

HEALE, J. B., GAFOOR, A. & RAJASINGHAM, K. C. (1968). Nuclear division in conidia and hyphae of *Verticillium albo-atrum*. *Canadian Journal of Genetics and Cytology* **10**, 321–340.

HOLLIDAY, R. (1961*a*). The genetics of *Ustilago maydis*. *Genetical Research* **2**, 204–230.

HOLLIDAY, R. (1961*b*). Induced mitotic crossing over in *Ustilago maydis*. *Genetical Research* **2**, 231–248.

KÄFER, E. (1958). An 8-chromosome map of *Aspergillus nidulans*. *Advances in Genetics* **9**, 105–145.

KÄFER, E. (1961). The process of spontaneous recombination in vegetative nuclei of *Aspergillus nidulans*. *Genetics* **46**, 1581–1609.

LOANE, M. M. (1967). The nuclear division in *Penicillium expansum*. *Canadian Journal of Genetics and Cytology* **9**, 342–351.

PILKINGTON, S. & HEALE, J. B. (1969). Respiration and morphogenesis in *Verticillium*. *Transactions of the British Mycological Society* **43**, 467–470.

PONTECORVO, G. (1954). Mitotic recombination in the genetic system of filamentous fungi. *Caryologia, Supplement* **6**, 192–200.

PONTECORVO, G. (1956*a*). Allelism. *Cold Spring Harbor Symposium on Quantitative Biology* **21**, 171–174

PONTECORVO, G. (1956*b*). The parasexual cycle in fungi. *Annual Review of Microbiology* **1**, 393–400.

PONTECORVO, G. (1959). *Trends in Genetic Analysis*, pp. 1–145. New York: Columbia University Press.

PONTECORVO, G. & ROPER, J. A. (1952). Genetic analysis without sexual reproduction by means of polyploidy in *Aspergillus nidulans*. *Journal of General Microbiology* **6**, vii (abstr.).

PONTECORVO, G., ROPER, J. A. & FORBES, E. (1953). Genetic recombination without sexual reproduction in *Aspergillus nidulans*. *Journal of General Microbiology* **8**, 198–210.

PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advances in Genetics* **5**, 141–238.

PONTECORVO, G. & SERMONTI, G. (1954). Parasexual recombination in *Penicillium chrysogenum*. *Journal of General Microbiology* **11**, 94–104.

PONTECORVO, G., TARR GLOOR, E. & FORBES, E. (1954). Analysis of mitotic recombination in *Aspergillus nidulans*. *Journal of Genetics* **52**, 226–237.

ROBINSON, D. B., LARSON, R. H. & WALKER, J. C. (1957). *Verticillium* wilt of potato in relation to symptoms, epidimiology and variability of the pathogen. *Wisconsin Agricultural Experimental Station Research Bulletin* **202**, 1–49.

STRØMNAES, Ø., GARBER, E. D. & BERAHA, L. (1964). Genetics of phytopathogenic fungi. IX. Heterocaryosis and the parasexual cycle in *Penicillium italicum* and *Penicillium digitatum*. *Canadian Journal of Botany* **42**, 423–427.

TINLINE, R. D. & MACNEILL, G. B. (1969). Parasexuality in pathogenic fungi. *Annual Review of Phytopathology* **7**, 147–170.

Tuveson, R. W. & Coy, D. O. (1961). Heterocaryosis and somatic recombination in *Cephalosporium mycophilum. Mycologia* **53**, 244–253.

Typas, M. A. (1976). Genetical aspects of the mechanisms of variation and differentiation in species of *Verticillium*. Ph.D. thesis, University of London.

Typas, M. A. & Heale, J. B. (1976*a*). Acriflavine-induced hyaline variants in *Verticillium albo-atrum* and *V. dahliae. Transactions of the British Mycological Society* **66**, 15–25.

Typas, M. A. & Heale, J. B. (1976*b*). Heterokaryosis and the role of cytoplasmic inheritance in dark resting structure formation in *Verticillium* spp. *Molecular and General Genetics* **146**, 17–26.

Typas, M. A. & Heale, J. B. (1977). Analysis of ploidy levels in strains of *Verticillium* using a Coulter Counter. *Journal of General Microbiology* **101**, 177–180.