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Heterokaryosis and the Role of Cytoplasmic Inheritance in Dark Resting Structure Formation in *Verticillium spp*

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Summary. Auxotrophic and morphological mutants of *Verticillium albo-atrum* (producing darkly pigmented resting mycelium) and *V. dahliae* (forming dark microsclerotia) were isolated after treatment of conidia (haploid and uninucleate) with ultraviolet light. Hyphal tip and conidial analysis revealed that complementation between pairs of auxotrophs on minimal medium was due to a mosaic of homokaryotic and heterokaryotic regions with some hyphal tips growing syntrophically. A degree of incompatibility was observed in a few intraspecific, but in most of the interspecific, heterokaryon tests. Heterozygous diploid conidia (6–11 μ in length compared with 3–6 μ for haploids) were recovered at a frequency of 1 in 8×10^6 by plating spores at high density on MM. Young diploid colonies segregated to give haploid and diploid sectors, some of which were recombinant types (parasexual cycle). Heterokaryons between complementary auxotrophs which were wild-type for dark pigmentation (hyl^+) resembled wild-type and only darkly pigmented colonies were recovered by conidial analysis. Heterokaryons between hyl^+ and hyaline (hyl) auxotrophs again resembled hyl^+ morphology and usually only hyl^+ colonies of *both* auxotrophic genotypes were recovered. Conidia from heterokaryons formed by stable hyl auxotrophs produced only hyl colonies of both auxotrophic genotypes. The important role played by cytoplasmic factors in the inheritance of darkly-pigmented resting structures in *Verticillium* was strongly confirmed by the present work.

Introduction

Most species of *Verticillium* lack a sexual phase and are therefore classified in the Fungi Imperfecti. They are characterized by verticillate conidiophores bearing groups of conidia in mucilage, and the development of darkly-pigmented resting structures (resting mycelium for *V. albo-atrum* and microsclerotia for *V. dahliae*) from the mycelium which is regularly septate.

Heterokaryosis is considered to be an important component of genetic systems in fungi (Pontecorvo, 1946; Raper, 1955; Buxton, 1960; Hastie, 1962), although its full significance in wild populations is unknown. When independent strains of the same species are mixed, heterokaryon formation may be prevented by incompatibility systems (Caten and Jinks, 1966; Esser and Kuenen, 1967; Fincham and Day, 1971). Apart from Heale's (1966) and Schnathorst's (1973) observations of a degree of heterokaryon incompatibility between auxotrophs derived from different isolates of *Verticillium*, little is known about the mechanisms involved in this fungus. When two independently-isolated auxotrophs inoculated together on minimal medium grow, they frequently complement (Pontecorvo, 1959), because the individual defect in each is compensated for by the corresponding wild-type allele in the other nuclear type. Hastie (1962) showed that auxotrophs derived from hop strains of *V. albo-atrum* form unstable heterokaryons, and that as a rare event, diploid prototrophic colonies can be isolated. The instability of such heterokaryons was observed by Hastie (1962) and Heale (1966), while on the contrary Puhalla and Mayfield (1974) claim that once established, the heterokaryons on MM are very stable.

Morphological variation affecting the development of darkly-pigmented resting structures in *Verticillium spp* has been recorded frequently (Rudolph, 1931; Beyma Thoe Kingma, 1939; Nelson, 1950; Caroselli, 1957; Pegg, 1957; Van den Ende, 1958; Heale and Isaac, 1965). Different mechanisms have been proposed to explain such variability including mutation (Pethybridge, 1916), heterokaryosis and the dual phenomenon (Hansen, 1938), genetic recombination

(Robinson, Larsen and Walker, 1957), the parasexual cycle (Hastie, 1964), cytoplasmic inheritance (Hastie, 1962; Heale, 1966) and more recently, changes in ploidy (Tolmsoff, 1972). Evidence from heterokaryons and heterozygous diploids led Hastie (1962) to suggest that cytoplasmic factors might play a dominant role in the expression and perhaps in the inheritance of the mycelial colour, and later Heale (1966) performed preliminary heterokaryon tests, the results of which substantiated this view. Subsequently, Pilkington and Heale (1969) reported on some respiratory differences between the dark wild-type (hyl^+) strains of *V. albo-atrum* which are characterized by deposition of non-indolic melanin in the walls of the resting structures (Gafoor and Heale, 1971), and the hyaline variants (hyl) which lack the dark pigment. The effects of acriflavine in increasing the spontaneous rate of production of stable hyaline and partially hyaline variants have already been reported (Typas and Heale, 1976). In the present work extensive heterokaryon tests have been employed which contribute substantial evidence in support of cytoplasmic inheritance, and in addition aspects of "incompatibility", complementation, and mechanisms of heterokaryon formation in this fungus are discussed. Additionally, analysis of heterozygous diploids is presented as substantiating evidence for the parasexual cycle.

Methods and Materials

Strains

Table 1 details the host and location from which the strains of *V. albo-atrum* and *V. dahliae* were isolated. All wild-type (hyl^+) strains were purified by single spore isolation. 'Sooty' mutants of *V. albo-atrum* kindly provided by Dr. A.C. Hastie, Dundee University, were also used in some of the heterokaryon tests as a modified type of dark strain ($hyl^+ \cdot sot$).

Table 1. Strains of *Verticillium* and their origin

Species	Strain number	Host plant	Location
V. albo-atrum	1	potato	Swansea, Wales
V. albo-atrum	2	tomato	London, England
V. albo-atrum	3	lucerne	Norfolk, England
V. albo-atrum	4	chrysanthemum	Devonshire, England
V. albo-atrum	5	antirrhinum	Swansea, Wales
V. dahliae	6	pear	Australia
V. dahliae	7	cotton	U.S.A.
V. dahliae	8	sunflower	Swansea, Wales
V. dahliae	9	peppermint	U.S.A.
V. dahliae	10	potato	Swansea, Wales

Media

Stock cultures of all strains were maintained on freshly made potato dextrose agar (PDA) and on modified prune extract agar medium (PE) described by Talboys (1960). A Czapek-Dox agar containing (g/l); 'Analar' sucrose 15.0; $NaNO_3$ 2.0; $MgSO_4$ 7 H_2O 0.5; KCL 0.5; KH_2PO_4 1.0; $FeSO_4$ 7 H_2O 0.01; Japanese 'Davies' agar 20 g; distilled water up to 1 l, was employed as a minimal medium (MM). The complete medium (CM) contained minimal medium plus casein hydrolysate, yeast extract, peptone and malt extract at 2 g/l and Japanese 'Davies' agar 2%. Strains were grown continuously in the dark at 23°C except when heterokaryons were 'forced' and then transferred to 21°C after two days.

Production of Hyaline Variants and Auxotrophs

All wild-type strains (hyl^+) of both species normally produce darkly pigmented resting structures i.e. black torulose mycelium in the case of *V. albo-atrum* and black microsclerotia in *V. dahliae* within 2–3 weeks. Hyaline variants arose in low frequencies (0.1%) from such wild-type strains but most of these usually formed small amounts of resting structures when maintained on PE, PDA or CM for more than 5–6 weeks. These hyaline variants which eventually formed isolated dark resting structures on the above media are referred to as partially hyaline. Stable hyaline variants (hyl) which never reverted to either partially hyaline or darkly-pigmented parental type, were obtained either by acriflavine treatment (Typas and Heale, 1976) or by exposing conidia to ultra-violet irradiation. In preliminary tests a water suspension of conidia from wild-type strains was prepared (which did not show any clumping) in a sterile petri dish which was irradiated (uncovered), with a 2,537 Å "Hanovia" bactericidal tube, model II (with ozone reducing filter) at a distance of 30 cm. Although shaken during irradiation, an uneven distribution of variants/survivals was always observed; subsequently spore suspensions were transferred (400–500 conidia/plate) onto CM and irradiated for the time necessary to give 5% survival (ca. 4–4$^1/_2$ min). The surviving conidia formed distinct colonies after 4–6 days in the dark at 23°C and they were further tested for hyalinity in a range of different media (Brandt, 1962; Heale and Isaac, 1965).

Auxotrophs were induced by reducing the U.V. light distance to 20 cm and by selecting from the 3% survivals. Presumptive auxotrophs, were tested for response to amino acids, vitamins or organic bases (purine-pyrimidine) and were classified accordingly (the gene symbols are as in *Aspergillus nidulans*, Roper, 1968). A number of these auxotrophs were further irradiated to produce strains with two or more requirements.

Stable hyaline auxotrophs were obtained after extensive U.V.-irradiation of the 'sooty' auxotrophs, and they were designated $hyl \cdot sot$ strain. The 'sooty' $hyl^+ \cdot sot$ mutants are distinct from wild-type *V. albo-atrum* hyl^+ strains in that their black pigmentation is produced throughout the entire culture in only 4–5 days, whereas wild-type isolates ($hyl^+ \cdot sot^+$) form dark resting mycelium mainly in the central area, and then only after 10–14 days growth. According to Hastie (1968) the sot marker is linked with arg-9, and therefore has a nuclear pattern of inheritance.

Heterokaryon Synthesis

Four different methods were compared. Except where otherwise stated final incubation was for 2–6 weeks at 21°C. The usual 'reversion' checks were employed.

1. Aliquots (0.25 ml) of mixed auxotroph spore suspensions (10^6 conidia/ml) were spread on MM. Another series of MM plates was inoculated with isolated drops of the mixed suspensions without spreading.

2. Mixed auxotrophic suspensions in liquid CM, in test tubes were firstly incubated for two days at 23° C. Aliquots (0.5 ml) were then streaked or spread on MM.

3. Small CM agar blocks from the edge of young auxotrophs were placed adjacent to one another on MM.

4. Mixed spore suspensions (as in 2) were incubated for 2 days at 23° C. Then ca. 25 small droplets (0.1–0.15 ml) were transferred onto the lower surface of the petri plate and moist sterile filter paper was inserted in the upper half and incubated for 2 days at 23° C. The mycelial pellets resulting from each droplet were transferred to plates containing MM.

Heterokaryon Analysis

Conidia harvested from putative 4–6 week-old heterokaryons were plated on CM and their nutritional requirements were determined by replica plating. To distinguish whether complementation between auxotrophs was due to heterokaryosis rather than to cross-feeding (syntrophism), hyphal tips approximately 0.5 mm and 1.0 mm long were cut from the putative heterokaryons and inoculated on MM. This enabled us to examine their growth requirements as well as the genotypes of resulting conidia. Ryan tubes (45 cm) were used for linear growth measurements.

Selection of Heterozygous Diploids

Conidia of *V. albo-atrum* and *V. dahliae* are uninucleate (Hastie, 1962; Buxton and Hastie, 1962; Heale, Gafoor and Rajasingham, 1968) and therefore when arising from 'forced' heterokaryons they are usually unable to grow on MM. However, conidia containing a heterozygous diploid nucleus, will grow on MM (Roper, 1952; Hastie, 1964). Such heterozygous diploids were selected in the present work by three different techniques. Firstly, large samples of conidial suspensions (10^6–10^8 conidia/ml) from heterokaryons were plated on MM and the resulting colonies were tested for auxotrophy and conidial size. Secondly, prototrophic sectors formed by mycelial 'pellets' (Method 4) that had been incubated on MM for 4–6 weeks at 21° C, were selected and their relatively large conidia were subsequently proved to be diploid. Thirdly, prototrophic sectors on MM formed by heterokaryons that had grown to a diameter of 1–2 cm at 21° C and which were later transferred to 30° C, were selected and when tested were proved to be heterozygous diploids. The temperature of 30° C is one at which haploid strains of both *V. albo-atrum* and *V. dahliae* will not grow normally; (*V. dahliae* may make very slight growth at this temperature).

Microscopic Observations of Nuclear Number and Size of Conidia

Conidia were stained with Feulgen as described by Heale et al. (1968). Ploidy was inferred cytologically by conidial size measurement using a calibrated eye piece micrometer.

Results

Production of Hyaline Variants and Auxotrophs

U.V.-irradiated conidia showed a considerable delay in germination, forming distinct colonies only after 4–5 days incubation. Figure 1 shows the average survival rate and the mutation (auxotrophy) frequency in conidia from 5 isolates of strain 1 and 5 isolates

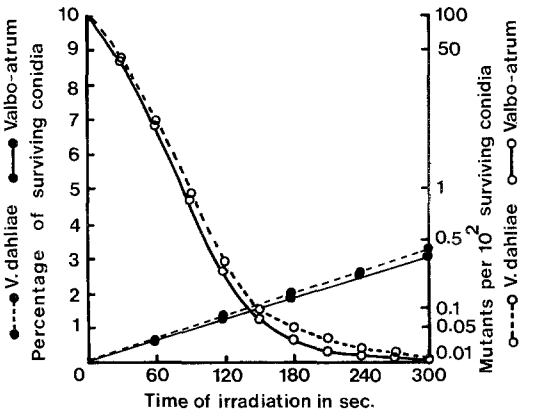

Fig. 1. Mutagenic effect of U.V.-irradiation. Relationship between the time of irradiation and the frequency of forward mutation in conidia of *V. albo-atrum* and *V. dahliae*

of strain 6, after various doses of U.V.-irradiation. All other strains of both species gave similar results and are omitted for clarity. From a total of ca. 30,000 surviving colonies (3% survival level) which were screened, 127 biochemical (auxotrophic) and 280 morphological mutants were isolated. Of these, ca. 14,000 survivors screened from wild-type *V. albo-atrum* strains resulted in 58 auxotrophic (28 amino acid, 17 nucleic acid and 13 vitamin requiring) and 134 morphological mutants; ca. 16,000 survivors screened from *V. dahliae* strains resulted in 69 auxotrophic (30 amino acid, 23 nucleic acid and 16 vitamin requiring) and 146 morphological mutants. For every host strain employed both the darkly pigmented wild-type (hyl^+) and its stable hyaline (hyl) variant – selected after previous U.V. or acriflavine treatment – were irradiated, i.e. 20 strains in all (10 pairs). There were no marked differences in the range and frequency of mutant production of hyl^+ and hyl types amongst these strains. The most frequent types were those requiring arginine, adenine and methionine constituting 66% of the total yield of biochemical mutants. Most of the vitamin-requiring auxotrophs had a slight ability to grow on MM ("leakiness" – due presumably to use of an alternative pathway or to some residual enzyme activity), and included cho-2, nic-3, pab-2, ane-1, pab-3. Darkly-pigmented auxotrophs usually produced resting structures in amounts similar to the wild-type (hyl^+) strain from which they originated, while all stable hyaline (hyl) auxotrophs completely lacked dark resting structures. When the latter were tested for long term stability, they were shown to retain the hyaline trait for up to 2 years.

Heterokaryon Synthesis

In both the first and second methods, after a considerable lag period (2–6 weeks), the mixed inoculum

Table 2. Summary of phenotypes from 108 different heterokaryons and classification (with respect to pigmentation) of colonies arising from conidia taken from heterokaryons of *Verticillium spp* (data as % figures with mean, of at least 700 conidia sampled and examined for each heterokaryon)

Heterokaryon group		Phenotype of heterokaryon	Numbers of different hetero-karyons tested	Auxotrophic type A pigmentation class[a]			Auxotrophic type B pigmentation class[a]		
A	B			I and II	III	IV	I and II	III	IV
hyl^+	$/hyl^+$	wild-type	21	15–67 (40)	–	–	33–85 (60)	–	–
hyl^+.sot	$/hyl^+$.sot$^+$	wild-type	5	44–87 (65)	–	–	13–56 (35)	–	–
hyl^+.sot	$/hyl^+$.sot	wild-type	1	15 (15)	–	–	85 (85)	–	–
hyl^+	/hyl	most (39) wild-type	46	15–91 (65)	0.0–0.5 (0.2)	–	8–84 (33)	0.7–2.5 (1.5)	0.0–1.0 (0.3)
hyl^+.sot	/hyl.sot$^+$	or near wild-type,	3	51–61 (56)	0.1–0.5 (0.3)	–	36–52 (32)	1.8–2.9 (2.5)	0.0–0.3 (0.2)
hyl^+	/hyl.sot$^+$	some (13) partially	4	36–48 (43)	0.0–0.6 (0.3)	–	49–61 (54)	1.7–2.9 (2.3)	0.1–0.6 (0.4)
hyl^+.sot	/hyl.sot$^+$	hyaline	1	50 (50)	0.2 (0.2)	–	48 (48)	2.2 (2.2)	0.2 (0.2)
hyl	/hyl	all hyaline[b]	21	–	–	19–60 (47)	–	–	40–81 (53)
hyl.sot$^+$	/hyl	all hyaline[b]	5	–	–	37–53 (45)	–	–	42–64 (55)
hyl.sot$^+$	/hyl.sot$^+$	all hyaline[b]	1	–	–	19 (19)	–	–	81 (81)

[a] Pigmentation class refers to I, II, III, IV. Class I = wild-type pigmentation, class II = almost wild-type pigmenation, class III = partially hyaline appearance (few dots of pigmentation in centre of colonies), class IV = completely hyaline (no pigment). The sot phenotypes were classified under I and II

[b] The exception of heterokaryon leu-4/ade-8, gua-1 (*V. dahliae*) has been discussed in full details in the text

produced fans of mycelium which when transferred onto fresh MM yielded (after 4–6 weeks), conidia of both parental types, clearly indicating that they derived from heterokaryotic cultures. Approximately 60% of the MM plates in the first method and 73% in the second, resulted in heterokaryotic cultures. Using the "agar block inocula" (Method 3) and the "mycelial pellet" (Method 4) techniques approximately 90–95% successful formation of heterokaryons was observed and since they were also convenient they were used extensively throughout the present work. Irrespective of the techniques used, however, there were some pairs of auxotrophs which invariably failed to produce heterokaryons, and others which formed heterokaryons with great difficulty. A large number of heterokaryons (obtained from 108 different pairs of complementing di- or tri-auxotrophs, 57 of *V. albo-atrum* and 51 of *V. dahliae*) were tested and their morphology with respect to pigmentation is given in Table 2.

Heterokaryotic growth was not visible usually until at least 2 weeks, and sometimes 3–5 weeks, after incubation. After subculturing onto fresh MM the heterokaryons always grew at a slower rate than wild-type cultures. The optimum temperature for growth of wild-type strains of *V. albo-atrum* and *V. dahliae* is 23°C; a slightly lower temperature of 21°C appeared to be optimum for heterokaryon formation in our studies. Typical growth rate (at 21°C) data (in mm/day) from Ryan tube experiments containing MM was as follows: For *V. albo-atrum* heterokaryons ino-4, sot-6/arg-4, ade-4 (hyl/hyl) and ane-1, ade-2/met-3, nic-3 (hyl^+/hyl) 2.8 and 2.7 respectively, for wild-type (strain 1) 3.0 and for hyl^+ and hyl auxotrophs (strain 1) 2.9 and 3.1 respectively; for *V. dahliae* heterokaryons nic, lys/arg-7, pro-1 (hyl^+/hyl) and ade-5, bio-1/arg-6 3.3, for wild-type (strain 6) 3.5 and for hyl^+ (strain 10) and hyl (strain 9) auxotrophs 3.5 and 3.7 respectively.

Heterokaryons formed between two darkly pigmented auxotrophs (hyl^+/hyl^+) invariably resembled wild-type, but the colonies were smaller with irregular edges. In general, dark pigmentation in heterokaryons was more marked than in 'parental' auxotrophs growing separately. Heterokaryons of the hyl^+/hyl type usually showed wild-type phenotype – classed as (I) – or near wild-type (II) with reduced amounts of dark pigmentation, but sometimes resembled the appearance of partially hyaline mutants (III). Heterokaryons between hyaline auxotrophs (hyl/hyl) never produced dark pigmentation (with the exception of a heterokaryon leu-4/ade-8, gua-1 which will be discussed later).

Almost all the compatible paired auxotrophs showed a 90–100% success rate for all attempts. Seven pairs however, gave only a partial success rate (15–25%) and a few: heterokaryons ade-5, bio-1/ser-1 (*V. dahliae* pear, Australia/*V. dahliae* peppermint, U.S.A.); nic, lys/ser-1 (*V. dahliae* potato, Swansea Wales/*V. dahliae* peppermint, U.S.A.), ser-1/met-7, ane-4 (*V. dahliae* peppermint, U.S.A./*V. dahliae* potato, Swansea Wales) completely failed to form heterokaryons. Heterokaryons derived from mono-auxotrophs were less stable than those derived from di-

auxotrophs. The former 'broke down' 2–3 weeks after inoculation onto fresh MM, whereas the latter did so less frequently even after 4–6 weeks incubation. Heterokaryons between vitamin-requiring auxotrophs broke down rapidly to their homokaryotic components (as in *Aspergillus nidulans* Pontecorvo, 1953). Although heterokaryons appeared in general unstable, by careful subculturing from their older central regions onto fresh MM plates we were able to maintain several cultures for more than a year.

As a part of this investigation we also studied interspecific heterokaryon synthesis between different hyl^+ auxotrophic strains of *V. albo-atrum* and *V. dahliae*. The success rate here was approximately 10–20%, similar to that encountered for some of the relatively incompatible intraspecific combinations already dealt with. Where heterokaryotic growth did occur, dark pigmentation was observed as in the intraspecific hyl^+/hyl^+ heterokaryons. Microscopic examination of the interspecific heterokaryons failed to reveal distinct examples of hybrid resting structures; instead areas of the culture appeared to consist of either fairly typical microsclerotia of *V. dahliae* or the resting mycelium of *V. albo-atrum*.

Heterokaryon Analysis

Mycelial agar block transfers taken from the centre of the heterokaryons invariably grew on MM without delay. An average of 110 hyphal tips (ca. 50 of each of 0.5 and 1.0 mm long) from each of 24 different heterokaryons (6 of hyl^+/hyl^+ group, 11 of hyl^+/hyl and 7 of hyl/hyl) were examined. Most (95–100%) of the 0.5 mm long hyphal tips from the advancing edge of heterokaryons however failed to grow on MM. After 1–2 weeks extra incubation however, in about 18% of cases some very slow growth commenced, and conidial analysis resulted in the re-isolation of both auxotrophic components.

When 1 mm long hyphal tips were transferred onto fresh MM however, prototrophic growth was almost always (90–100%) obtained. Conidial analysis of these colonies resulted in isolation of both auxotrophic components. When cellophane was interposed between the two agar block inocula (when heterokaryon formation was attempted) no growth was observed.

Puhalla and Mayfield (1974) and Tuveson and Garber (1961) observed that the nuclear ratios of a heterokaryon could be drastically shifted by altering the growth medium. We obtained very similar results from our experiments. Heterokaryons where one of the auxotrophic strains required either a vitamin, arginine, methionine or adenine often 'broke down' and this component then dominated the whole culture. In these cases almost all of the 1 or 5 mm long hyphal tips contained this major component alone. There were 9 complementing combinations which formed predominantly syntrophic colonies, e.g. *V. albo-atrum* heterokaryon met, nic, trp/arg-3, cho-1 (hyl^+/hyl), and *V. dahliae* heterokaryon nic, lys/arg-7, pro-1 (hyl^+/hyl). In the case of *V. albo-atrum* heterokaryons arg-9, sot-1/ade-1, ile ($hyl^+ \cdot sot/hyl$); and *V. dahliae* heterokaryons ade-5, bio-1/arg-6 and pab-3, gly-1/asp, ino (hyl^+/hyl^+); ade-5, bio-1/met-7, ane-4 and asp, ino/ser-1 (hyl^+/hyl); and leu-4/met-7, ane-4 (hyl/hyl), most of the hyphal tip isolations from the edge of the heterokaryons were found to have an arginine, adenine, inositol, biotin, inositol, or aneurine requirement respectively.

To attempt to minimize complications arising from the apparent 'dominance' of one auxotrophic requirement over the other, areas from the centre of the heterokaryons (being almost 100% heterokaryotic) were chosen for further conidial analysis studies. The central areas were cut out, washed with sterile distilled water to produce a conidial suspension which was plated onto CM and later, replica plated in the usual way. Results for conidial analysis from central heterokaryon areas showed recovery of parental types which was very near to a 1:1 ratio (1:1 to 1:2), whereas similar data for conidia harvested over the entire surface of heterokaryons resulted in 1:1 to 1:9 recovery rates. In both cases while the auxotrophic markers were recovered in a normal nuclear pattern, the extent of darkly pigmented resting structures produced varied greatly. The few prototrophic colonies occasionally arising from the 5 heterokaryons i.e., *V. albo-atrum* ane-1, ade-2/pab-2, ino-8 (hyl^+/hyl^+); met, nic, trp/arg-3, cho-1 (hyl^+/hyl); ade-4, arg-4/met-3, nic-3 (hyl/hyl); *V. dahliae* nic, lys/arg-7, pro-1 (hyl^+/hyl) and arg-7, pro-1/ade-8, gua-1 (hyl/hyl), were further examined and found to be haploid revertants (with the exception of two heterokaryons which proved to contain heterozygous diploids, see next section).

In the hyl^+/hyl^+ groups, the heterokaryons resembled the wild-type hyl^+ in appearance and almost all colonies resulting from conidial analysis produced normal or almost normal amounts of dark pigmentation. In tests of groups hyl^+/hyl the heterokaryons were again mainly wild-type hyl^+ (I) or near wild-type hyl^+ (II), but some were partially hyaline (III). While the auxotrophic markers were recovered during conidial analysis in a ratio of 1:1 to 1:7 as expected considering their nuclear pattern of inheritance, the factor(s) controlling pigment formation was present in virtually all the spores tested except in the case of about 1/250 (and here combined with hyl parent auxotrophic marker) of conidia harvested from hyl^+/hyl heterokaryons. The difference in partial hyalinity

percentages from hyl^+/hyl heterokaryons and the presence of hyl^+ in the great majority of spores from such heterokaryons might be attributable to diffusion in the medium, translocation through the septa of the hyphae or to a truly genetical self-duplicating ability. To distinguish between these possibilities, subsequent subculturing of the colonies on CM was followed by close examination for long periods. The colonies remained darkly-pigmented throughout the three next generations, without any major alterations in morphology.

Conidial analysis of heterokaryons between 2 darkly-pigmented 'sooty' ($hyl^+ \cdot$ sot/$hyl^+ \cdot$ sot) auxotrophs showed that both the nutritional and the 'sooty' characters were inherited in a nuclear pattern and only 'sooty' auxotrophic parental types of one or the other kind were observed. Conidial analysis of darkly-pigmented 'sooty' and stable hyaline auxotroph heterokaryons ($hyl^+ \cdot$ sot/hyl·sot^+) resulted in mainly darkly-pigmented colonies in the first generation. In the case of auxotroph 1 genotype these were of the $hyl^+ \cdot$ sot type, but in the case of auxotroph 2 these were of the $hyl^+ \cdot sot^+$ type. Again, while both nutritional and 'sooty' markers were recovered in a nuclear pattern, colonies with a parental stable hyaline auxotrophic genotype produced hyl^+ type dark pigmentation in the subsequent generations. Conidial analysis of heterokaryons between 'sooty' strain $hyl^+ \cdot$ sot and hyaline strain hyl·sot ($hyl^+ \cdot$ sot/hyl·sot) auxotrophs gave similar results; all darkly-pigmented first generation colonies with strain hyl·sot auxotrophic genotype were of the 'sooty' pigmented type i.e. $hyl^+ \cdot$ sot.

Tests between the normal stable hyaline auxotrophs (hyl/hyl) with the exception of leu-4/ade-8, gua-1 led to the recovery of only parental types for both auxotrophic and morphological characteristics. In general, all heterokaryons of hyl/hyl type showed very sparse sporulation, producing 10–100 times less spores than the heterokaryons of hyl^+/hyl^+ or hyl^+/hyl type incubated for a similar period. The hyl/hyl *V. dahliae* heterokaryon leu-4/ade-8, gua-1 invariably produced an almost hyaline culture, which nevertheless had a small area of dark pigmentation near its centre. When conidial analysis of this heterokaryon was carried out, 72% of the 'daughter' colonies resembled the parental phenotypic (IV) appearance and auxotrophic genotypes, 15% had a partially-hyaline phenotype (III), and 13% were of the darkly-pigmented (I, II) phenotypes. Isolation and further examination of the latter showed that the pigment character was stable for the next three generations. Finally, heterokaryons between hyaline 'sooty' strain hyl auxotrophs (hyl·sot/hyl·sot) resulted in progeny resembling hyaline parental appearance and auxotrophic genotype at a 1:1 to 1:4 ratio. Predictably, the 'sooty' character was not expressed in the absence of resting structures determined by the hyl locus. When the same hyaline strain hyl·sot 'sooty' auxotrophs were paired with darkly pigmented hyl^+ sot^+ auxotrophs ($hyl^+ \cdot sot^+$/hyl·sot) the 'sooty' character showed linkage to the auxotroph 2 genotype (i.e. arg-9) as expected.

Selection of Heterozygous Diploids

When large samples of conidia (10^6–10^8/ml) from heterokaryons were plated on MM a number of colonies growing prototrophically were recorded. Isolation and microscopic examination of their morphology showed that their conidial volume was approximately twice that of the parental auxotrophs. The recovery rate of prototrophic conidia from heterokaryon spore analysis was approximately 1 in 8×10^6 (0.6–1.6 for six different hyl^+/hyl^+ heterokaryons, 0.0–10.4 for eleven different hyl^+/hyl heterokaryons, and 0.0–15.1 for seven different hyl/hyl heterokaryons based on a 8×10^6 conidial sampling in each case). Heterokaryons met, nic, trp/arg-3, cho-1 (hyl^+/hyl^+ of *V. albo-atrum*) and arg-7, pro-1/ade-8, gua-1 (hyl/hyl of *V. dahliae*) showed a much higher recovery rate of prototrophically growing diploids, approximately 5 in 8×10^6. Most of the prototrophic colonies isolated by this method produced longer conidia (see below) than those derived from the haploid parental auxotrophs. After 1–3 weeks incubation the prototrophic colonies all segregated into sectors having both parental and non-parental auxotrophic genotypes. Spore size measurements indicated that each sector produced either haploid or diploid (and possibly aneuploid) conidia. Conidial analysis showed that all conidia within each sector had the same genotype (parental or recombinant). The morphology of haploid and diploid sectors having parental genotypes (shown by conidial analysis) resembled the auxotrophic parents, but was significantly different when new genotypes had arisen, e.g. wild-type recombinants. Prototrophic sectors from mycelial pellets were isolated and their relatively large conidia suggested they were also diploid. Again, colonies developing from such conidia were very unstable. Similar results were obtained from prototrophic sectors of heterokaryons which were transferred from 21° C to 30° C. Conidial analysis of 10–14 days-old heterozygous diploid colonies arising from *V. dahliae* heterokaryons ade-5, bio-1/arg-6 (hyl^+/hyl) and arg-7, pro-1/ade-8, gua-1 (hyl/hyl) and *V. albo-atrum* heterokaryons met, nic, trp/arg-3, cho-1 (hyl^+/hyl) resulted in 47.2%, 41.5% and 45.4% recombinant types respectively (both hap-

loids and diploids) from 536, 614 and 582 conidia tested in each case.

Microscopic Observations of Nuclear Number and Size

When freshly harvested conidia of darkly pigmented auxotrophic parents were subjected to Feulgen staining, approximately 99% of the 400 conidia tested for each strain were uninucleate. Almost all the conidia which contained more than one nucleus were binucleate and bicellular, and very rarely trinucleate conidia were observed. From a large number of conidia tested in each case none had more than three nuclei. Approximately 97–98% of the conidia produced by the hyaline (hyl) auxotrophs were uninucleate. Again most of the multinucleate conidia were binucleate and bicellular, and very rarely trinucleate.

Conidia arising from prototrophic colonies recovered from plating of heterokaryon conidia on MM, were relatively longer (6–11 μ) than those from parental auxotrophs (3–6 μ) and had a volume almost twice (1.8×) that of parental conidia. Spore suspensions of these colonies were stained with Feulgen and 98–99% of the 6,000 conidia examined were uninucleate. When samples of these conidial suspensions were plated on CM and further examined for prototrophy, an estimate of more than 35% prototrophic conidia was recovered in each case. This strongly indicates that almost all the prototrophic conidia were uninucleate. Spore size measurements for conidia arising from segregating heterozygous diploids showed that some of the sectors contained haploid, some diploid and some presumably aneuploid conidia (4–10 μ). Again, when stained with Feulgen virtually all these conidia were found to be uninucleate.

Discussion

The results from the dose-mutation curve are in agreement with Buxton and Hastie's (1962) observations for *Verticillium*. The very slight but consistently different U.V.–sensitivity observed for *V. albo-atrum* and *V. dahliae*, with the latter being somewhat less sensitive (Fig. 1) is similar to the difference recorded by Puhalla (1973). Biochemical mutants were relatively easily produced at a frequency of 0.40% for *V. albo-atrum* and 0.42% for *V. dahliae* from the 3% U.V.–irradiated survivors.

The high frequency of induced biochemical mutation and the stability of the auxotrophs in culture, coupled with the fact that auxotrophs were also recovered after a parasexual cycle, strengthen the assumption that conidia of *Verticillium* are uninucleate and haploid, and that auxotrophy is the result of a single nuclear gene mutation. Further, evidence from cytological observations of a number of wild-type and auxotrophic strains, where no obvious differences in conidial size and number of nuclei were obtained, supports the above assumption.

From the present work, it seems perfectly clear that in *Verticillium* complementation between different auxotrophs is a common phenomenon. Apparent complementation could however arise through the operation of one or more of several mechanisms i.e. back-mutation, formation of heterozygous diploid nuclei, syntrophism (cross-feeding) or true heterokaryosis. The almost invariable recovery of only parental types from random conidial analysis of the complementing combinations excludes the possibility of back-mutation. A fusion between dissimilar nuclei would result in the formation of a heterozygous diploid nucleus, but this was demonstrated to occur only at very low frequencies (approximately 1 in 10^7 conidia) among conidia from heterokaryons. This excludes the possibility of diploidy being a determining factor for complementation in the fungus. Most heterokaryon test combinations involving vitamin auxotrophs ('leaky') appeared to complement due to syntrophism, for a large proportion of the hyphal tips and the conidia examined in these combinations were homokaryotic for vitamin deficiency. Also, the general failure of 0.5 mm long hyphal tips in growths from all auxotrophic paired combinations to develop on MM is indicative of a homokaryotic state in these regions of the 'heterokaryon' as reported by Clutterbuck and Roper (1966) in *Aspergillus nidulans*. However, growth in most of the test auxotroph combinations was unequivocally heterokaryotic because the majority of 1 mm long hyphal tips grew on MM and both nuclear components of the parental types were recovered by random conidial analysis.

Previous investigations with *Fusarium* (Tuveson and Garber, 1959, 1961; Coy and Tuveson, 1961; Buxton, 1954), *Cephalosporium mycophilum* (Tuveson and Coy, 1961) *Ascochyta imperfecta* (Sanderson and Srb, 1965), and *Penicillium cyclopium* (Jinks, 1952a, b) have shown that certain heterokaryons yielded conidia with nuclei carrying markers characteristic of only one of the component strains. These observations are in contrast with the situation in *Neurospora* (Pittenger and Atwood, 1956) in which it was found that the nuclear proportions are relatively constant during growth and any slight changes that do occur are not adaptive. In *Verticillium*, growth rates were variable for different heterokaryons and appeared to be related to the ratio of the two component nuclei, as reported by Warr and Roper (1965) in *Aspergillus nidulans*. However, heterokaryons here always grew

slower on MM than the wild-type under similar conditions. There are various mechanisms that could explain the observed changes in nuclear ratios. Different nuclei may divide at different rates in a common cytoplasm or, by chance, hyphal tips may acquire altered nuclear ratios, such tips being best adapted to the immediate situation and having a selective advantage over tips with other nuclear ratios. To distinguish between these hypotheses is not, at present, possible. Although Pontecorvo (1946) claimed that nuclei did not divide synchronously in Fungi Imperfecti, subsequent investigators strongly suggest that they do so divide both in homokaryons and heterokaryons (Rees and Jinks, 1952). In *Verticillium*, our cytological studies and those of MacGarvie and Isaac (1966) suggest that all hyphal cells, except a small number, are uninucleate and yet there is now substantial genetical evidence of heterokaryosis from a number of sources. Although the septum possesses a pore, in extensive microscopical examination of hyphae during micromanipulation experiments, nuclear migration apparently failed to occur between cells (M. Typas unpublished data). If this is indeed the case, it seems likely that the formation and maintainance of heterokaryons depends upon a few multinucleate cells becoming heterokaryotic via anastomoses. Since the nuclei do not appear to migrate in significant numbers, the mycelium may develop as a mosaic of genetically-distinct uninucleate cells surrounding the anastomosed areas. This would easily explain why the region of the hyphae between the anastomosed cells and the edge of the colony remains homokaryotic and auxotrophic. It would also explain the production of distinct areas of typical resting mycelium and microsclerotia observed in the interspecific heterokaryons. Recent investigations using *V. dahliae* only (Puhalla and Mayfield, 1974) led to the following conclusions regarding heterokaryons of this species: (1) most hyphal cells are uninucleate except for a small, but important, number of binucleate anastomosed cells formed between hyphae; (2) there is no migration of nuclei from cell to cell; and (3) the tip cells of the colony edge remain homokaryotic. Our genetic data is in accord with these cytological observations both for *V. dahliae* and *V. albo-atrum*.

The stimulus which initiates hyphal fusion does not seem to be completely species specific. Intraspecific and intrageneric fusions have been reported to occur between *V. albo-atrum* × *V. dahliae* (Fordyce and Green, 1964; Hastie, 1973; Schnathorst, 1973), and *V. dahliae* × *V. nubilum* and *V. albo-atrum* × *V. nubilum* (Schnathorst, 1973). On the other hand, hyphal fusions appear to form more readily between hyphae of the same species than between different species as confirmed in the present work. The failure of certain intraspecific auxotrophic combinations to form heterokaryons as well as the formation of heterokaryons with great difficulty by others, is suggestive of some degree of incompatibility in the fungus. This incompatibility could involve one or more of the following: (1) a different pool of genetic material, due to the varying geographic origin of the combining auxotrophs (2) Raper's (1952) suggestion that each of the hyphal tips may produce small quantities of a labile substance controlling fusion which diffuses outwards, setting up in each case a steep diffusion gradient around their sources of origin. (3) a 'heterokaryon incompatibility' may be envisaged as found in *Neurospora* (Garnjobst and Wilson, 1956; Wilson, Garnjobst and Tatum, 1961). Here actual development is determined by two pairs of alleles C/c and D/d (cytoplasmically controlled) which do not prevent hyphal fusions but if incompatible the fusion cell is rapidly sealed off and dies by autolysis.

In heterokaryons, cytoplasmic differences can be detected by having a marker gene in each component nucleus which can be followed in any segregation; any dissociation or reassociation of features originally associated with either homokaryotic nucleus is evidence of a cytoplasmic difference. The results obtained from heterokaryon analysis with darkly-pigmented auxotrophs (hyl^+) and hyaline auxotrophs (hyl) strongly suggest that the inheritance of the dark pigmentation is, at least in part, cytoplasmically controlled. However, observations on colonies developing from spores taken from hyl^+/hyl heterokaryons also showed that: (1) 1/250 spores carried the hyl factor associated with the original hyl auxotrophic genotype, and (2) a larger percentage of spores showed partial hyalinity associated with the original hyl auxotrophic genotype; these facts may be interpreted as evidence of some nuclear control. Dark pigmentation is certainly not inherited as a single gene difference otherwise it would have segregated with a similar ratio to the nutritional markers used in the heterokaryon tests and in the heterozygous diploid strain analysis. The existence of 'sooty', partially hyaline and complete hyaline stable mutants favours the hypothesis that more than two genes are involved in the control of pigment formation (Types and Heale, 1976). Evidence for this hypothesis comes from the results of random conidial analysis of various heterokaryons between darkly pigmented ($hyl^+ \cdot sot$ and $hyl^+ \cdot sot^+$) and hyaline auxotrophs ($hyl \cdot sot$ and $hyl \cdot sot^+$). In particular, most of the colonies arising from the selective plating of conidia from the $hyl^+ \cdot sot/hyl \cdot sot^+$ heterokaryons, produced pigmentation and segregated at ca. 1:1 ratio for both the nutritional markers and the 'sooty'/normal character, whereas in the $hyl^+ \cdot sot/hyl \cdot sot$ heterokaryon both types of conidia

carried only the 'sooty' character. This suggests that the two marker genes used here for dark resting structures are independent of one another; one of them (hyl) is linked to a cytoplasmic factor while the other (sot) is nuclear. Further, from complementation observed between two hyaline auxotrophs (hyl/hyl, leu-4/ade-8, gua-1) which resulted in a number of pigmented colonies (when conidial analysis was made), we conclude that the cytoplasmic factors controlling dark resting structures/hyalinity in these two auxotrophs carry mutations of the hyl type but in different functional units (cistrons) if not in different loci.

To try to explain the considerable variation in dark pigmentation in the fungus, in the light of the foregoing observations we propose the following: A stable hyaline variant of *V. albo-atrum* or *V. dahliae* apparently never produces 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 invariably causes hyalinity, and not the absence of pigment per se. The presence or absence of resting structures in *Verticillium* cultures is at least partially controlled by one or more major extrachromosomal factors (hyl) which is self-replicating and transmitted via the cytoplasm of the conidia. Hyaline variants however are clearly of two types: completely hyaline stable strains originate from dark parental cultures at a spontaneous rate of approximately 1 in 10^5–10^6; partially hyaline strains showing a range of strongly reduced pigmentation are more frequent, occurring spontaneously at approximately 1 in 10^3. Both rates are greatly increased during ageing. The frequency of the origin of the stable hyaline type of variant is within the range of mutation, and its stability is suggestive of an almost irreversible change (perhaps a major deletion or loss) in the factor. The rate of origin of the partially hyaline variants however, cannot be explained on this basis, and it is difficult to conceive of a mechanism which does not involve differential multiplication rates and/or migration of the cytoplasmic factor controlling hyl.

The occurrence of a wide range of partially hyaline variants may also be explained by the operation of more than one gene (i.e. sot) which are nuclear in origin. The phenotypic expression of nuclear genes would depend upon the cytoplasmically determined presence or absence of resting structures. Only when the wild-type cytoplasmic factor (hyl^+) is present in the cell are 'sooty', normal and partially hyaline variants detected. Gene mutation in the dark pigmented hyl^+ type which produces a hyaline variant (hyl) appears to be of a pleiotropic type for hyalinity is constantly associated with faster growth rate, extensive fluffy aerial mycelium, and fewer conidia and conidiophores. Alternative explanations for the origin of such hyaline variants such as genetic recombination (Robinson et al., 1957) or changes in ploidy (as reported by Tolmsoff, 1972) remain unsubstantiated at the present time.

Precise and elegant genetic analysis in the fungus has already been carried out by Hastie and the parasexual cycle has also been demonstrated (Hastie, 1962, 1964, 1967, 1968). The heterozygous diploids isolated from heterokaryons in the present work showed haploid and diploid segregants differing from the parental genotypes. This clearly indicates the occurrence of mitotic crossing-over and haploidization in the diploid lineage, and therefore contributes further evidence of the occurrence of the parasexual cycle in *Verticillium*. The mechanisms which control pigment formation are under further investigation by genetical analysis through the parasexual cycle, and they should help to elucidate the system operating here.

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