Heterokaryon Synthesis and Morphogenesis in *Verticillium*

By J. B. HEALE

*Department of Biology, Queen Elizabeth College, (University of London),
Campden Hill, London, W.8*

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SUMMARY

‘Lucerne’ auxotrophs of *Verticillium albo-atrum* produced by ultraviolet irradiation and derived from the same parental strain formed heterokaryons on minimal agar 4–5 weeks after inoculation. A degree of heterokaryon incompatibility was shown between auxotrophs derived from different isolates of *V. albo-atrum* and one of *V. dahliae*. Conidial methods of heterokaryon synthesis failed but transference of agar inocula from complete medium agar cultures to minimal medium agar was successful. Conidia were uni-nucleate and nuclear migration subsequent to germ-tube fusion was observed. Heterokaryons grew slowly and were unstable, but by using di-auxotrophs a greater stability was achieved and a number were subcultured successfully for more than a year. Spore analysis yielded colonies of both parental types with various ratios. A heterokaryon test between a hyaline and normal dark auxotroph of *V. albo-atrum* indicated that an important system controlling morphogenesis might be linked with a cytoplasmic factor. A hypothesis is proposed to account for the production and degree of stability of the hyaline variants.

INTRODUCTION

The existence of heterokaryons in laboratory strains of many imperfect fungi, including numerous plant pathogens, has often been shown, although as a natural phenomenon occurring in these organisms its significance has not yet been established (Parmeter, Snyder & Reichle, 1963). By using strains of *Verticillium albo-atrum* from hop Hastie (1962) showed that auxotrophs formed unstable heterokaryons and that as a rare event diploid prototrophic colonies could be isolated from concentrated conidial suspensions. Later (1964) Hastie used strains of *V. albo-atrum* from potatoes and tomatoes to show the probable occurrence of a parasexual cycle. Investigations into the verticillium wilt of lucerne (Heale & Isaac, 1963) showed that isolates of *V. albo-atrum* from this host were virulently pathogenic, whereas *V. dahliae* was only slightly pathogenic. Strains of both fungi from other crops never produced symptoms when inoculated into lucerne, suggesting a marked degree of physiological specialization in the lucerne strains of *V. albo-atrum* and, to a lesser extent, of *V. dahliae*. It was for this reason that the first part of the present work was planned in order to investigate heterokaryon synthesis between ‘lucerne’ strain auxotrophs and between these and auxotrophs from ‘tomato’ and ‘potato’ strains of the fungus.

Apart from its possible significance in leading, with parasexualism, to the recombination of characters controlling virulence and pathogenicity, the ability of

imperfect fungi to form heterokaryons affords a convenient system in which to study morphogenesis. With *Verticillium albo-atrum*, development in culture frequently proceeds along two divergent pathways due to the production of variants or 'saltants'. Wild-type isolates of *V. albo-atrum* are usually characterized by the formation of resting mycelium produced from hyphae which swell, undergo septation and develop darkly pigmented walls. This pigment has been described as a melanin (Brandt, 1962). However, there is no evidence that it is a nitrogen-containing melanin (in the limited sense of Thomas, 1955) and it seems more likely to be a product of catechol oxidation and polymerization (Heale & Isaac, 1964). Laboratory strains of *Verticillium* invariably produce a hyaline variant which fails to develop resting structures or dark pigment, a phenomenon which has been frequently observed (Pethybridge, 1916; Rudolph, 1931; Beyma Thoe Kingma, 1939; Nelson, 1950; Robinson, Larson & Walker, 1957). Heale & Isaac (1965) showed that the incidence with which such a variant arose was linked with the age of the parent culture and site of inoculum removal; and they suggested that the operative factor might be carried wholly or partly in the conidia.

After a test involving heterokaryon synthesis between a hyaline and normal auxotroph derived from the hop strain of *Verticillium albo-atrum*, Hastie (1962) suggested that cytoplasmic factors played a dominant role in the expression and perhaps inheritance of the mycelial colour. In the present work the possibility of cytoplasmic inheritance of dark resting mycelium was further examined by using a heterokaryon test and auxotrophs derived from the lucerne strain of this fungus. In addition, a single auxotroph of *V. dahliae* characterized by darkly pigmented microsclerotia (distinguished from the resting mycelium of *V. albo-atrum* essentially by the budding process involved in their formation) was used in an attempt to synthesize, and to follow morphological development in, heterokaryons with lucerne, potato and tomato auxotrophs of *V. albo-atrum*.

METHODS

General procedures. Wild-type strains of *Verticillium albo-atrum* Reinke & Berthold were isolated from lucerne and tomato by incubating at 23° small slices of surface-sterilized stem tissue on moistened sterile filter paper. A single strain of *V. dahliae* Klebahn was obtained from lucerne. Single spore isolates were prepared and the strains were grown at 23° in the dark on potato dextrose agar (PDA). Subsequently, except where ability to produce dark resting structures was being checked, a minimal medium agar (MM) was used; it had the following composition (g./l.): sucrose (Analar), 15.0; NaNO₃, 2.0; MgSO₄·7H₂O, 0.5; KCl, 0.5; KH₂PO₄, 1.0; FeSO₄·7H₂O, 0.01; 'Davies' New Zealand agar, 20. Complete medium (CM) was used with MM for the isolation and growth of auxotrophs and contained the constituents of MM plus casein hydrolysate, yeast extract, peptone and malt extract at 1 g./l., and Japanese agar at 2 %.

Production of auxotrophs. Auxotrophs were isolated after ultraviolet (u.v.) irradiation of a water suspension of conidia in an open Petri dish at a distance of 20 cm. from a 2537 Å 'Hanovia' bactericidal tube, model 11 (with ozone reducing filter). Initial tests showed that although the suspension was shaken at an even rate, there was some variation in the irradiation/survival curves obtained. In sub-

sequent work, the suspensions were irradiated at 30 sec. intervals from 1 to 4 min.; plates showing approximately 5 % survival were selected for their yield of mutants. Several methods of total isolation were employed, including master plate and multi-pin replicator, filter paper and velvet pad techniques, the latter being especially well suited for conidial transference in this organism. Di-auxotrophs were similarly produced by further u.v.-irradiation of selected mutants. A list of auxotrophs is shown in Table 1. Three di-auxotrophs originating from a potato strain of *Verticillium albo-atrum* were kindly provided by Dr A. C. Hastie (Botany Department, Queen's College, Dundee), namely 15 (VP 2/7/1) requiring adenine + nicotinic acid, 16 (VP 2/12/7) inositol + tryptophan and 17 (VP 2/13/16) uracil + nicotinic acid.

Synthesis of heterokaryons. Three methods of heterokaryon synthesis were attempted:

(a) Mixed conidial suspensions were spread on MM agar and incubated at 23° for up to 6 weeks.

(b) Mixed conidial suspensions were incubated in test tubes of liquid CM medium for 3 days at 23° until visible growth occurred, followed by streaking on to MM agar and incubation as above.

(c) Blocks of agar inoculum containing growing hyphae, conidia and some CM agar, were transferred to MM agar, mixed and incubated for a similar period.

To investigate the initial steps in heterokaryon formation, conidial suspensions were plated on to MM agar and examined after staining with Giemsa or Feulgen; an account of the nuclear condition in *Verticillium albo-atrum* and the methods used will be published elsewhere.

Conidial analysis. Heterokaryotic growths were detected visually and transferred to fresh MM agar. Conidial suspensions were later made with 6-week cultures and were shaken vigorously to ensure complete separation of conidia. From a dilution series on CM agar, either a CM master plate was prepared for multi-pin replication or a sterile velvet pad was used in direct transfer to the appropriate supplemented MM agar.

RESULTS

Auxotrophs

As a result of u.v.-irradiation, germination was markedly delayed in surviving conidia, particularly at the higher dosages. The total yield of auxotrophs was of the order of 1/200 at 5 % survival. Two methionine mutants were lost, presumably due to a high reversion rate; however, strain 2.6, which was utilized later, was more stable with regard to its methionine requirement, probably because of the decreased selection pressure in a di-auxotroph. Strain 1.7 was interesting in that after u.v.-irradiation of the adenine parent, a simultaneous requirement for *p*-amino-benzoic acid (pABA) and pantothenic acid was established. This was difficult to interpret, since pABA is thought to be involved in pantothenate synthesis and one would expect growth on the latter alone in the absence of pABA on the basis of an early block. The specific block in auxotroph 2 was established by adding separately the individual compounds involved in choline biosynthesis; auxotroph 2 appears to be incapable of the initial methylation of ethanolamine. Strain 2.6 characteristically formed dark hyphae but yielded a stable hyaline variant [2.6*] which was used in heterokaryon tests between dark and hyaline auxotrophs.

An orange-coloured mutant (designated M 5) was shown to form a large number of carotenoids absent from the wild type (Valadon & Heale, 1964). In addition, several distinctive morphological strains were produced, including dwarf and wrinkled types, usually with decreased conidiation, and also strains with either loss of or a markedly accelerated synthesis of dark pigment. These mutants have not yet been investigated further.

Table 1. *Auxotrophic strains of Verticillium induced by u.v. irradiation*

Strains 1-11 inclusive were derived from wild-type *V. albo-atrum*; 13 was from an isolate of *V. dahliae*.

Auxotroph	Parent strain	Host	Requirement
1	A	Lucerne	Adenine
2	A	Lucerne	Choline
4	A	Lucerne	Arginine
1.2	A	Lucerne	Adenine + arginine
1.3	A	Lucerne	Adenine + glycine
1.7	A	Lucerne	Adenine + pABA + pantothenic acid
1.8	A	Lucerne	Adenine + leucine
2.4	A	Lucerne	Choline + arginine
2.6	A	Lucerne	Choline + methionine
11	B	Tomato	Leucine
13	C	Lucerne	Methionine

Heterokaryon formation

Attempts to synthesize heterokaryons using either fresh conidial, or incubated conidial, suspensions of a large number of paired auxotrophs failed.

Using the block technique the following combinations resulted in heterokaryon formation: 1/2, 1/4, 2/4, 1.3/2.4, 1.7/2.4, 1.7/2.6, 1.8/2.4, 1.8/2.6, 2.6*/1.2, 2.6*/1.3 and 2.6*/1.7. All of these were paired lucerne auxotrophs.

With the same technique, the following combinations failed to produce heterokaryons: 1.3/2.6, 11/1.8, 11/2.4, 11/2.6, 11/13, 11/15, 11/16, 11/17, 13/1.8, 13/2.4, 13/2.6, 13/15, 13/16, 13/17, 15/1.3, 15/1.7, 15/1.8, 15/2.6, 15/16, 15/17, 16/2.4, 16/2.6, 16/17. Of these only the 1.3/2.6 combination consisted of paired lucerne auxotrophs.

Heterokaryons were distinguished from auxotrophic and prototrophic growth, due to carry-over and reversion, respectively, by their characteristic morphology and slow growth rate. They were not visible until at least three and in some cases 4-5 weeks after inoculation. After subculture to fresh MM agar, they again grew slowly, usually reaching 30-44 mm. diameter colonies in 6 weeks at 23° as compared with wild-type or supplemented auxotrophic growth of 30-40 mm. colonies in 12 days under similar conditions. The mycelial mats were considerably thicker and usually formed more pale-yellow/greenish pigment (before the production of darkly pigmented resting mycelium) than wild type. In general dark pigmentation was more marked than in auxotrophs growing separately on supplemented MM agar. A further distinction concerned the growth form: heterokaryons showed a greater tendency to zonation and also developed marked irregularities at the edge of the culture.

Heterokaryons derived from single-requirement auxotrophs were not stable on

MM agar and they frequently broke down 2–3 weeks after inoculation on fresh MM agar. In the case of heterokaryon 2/4, 25 hyphal-tip isolations taken from the fine growth developing from the edge of the heterokaryon were found to have an arginine requirement; this indicated that sufficient arginine leakage occurred from the heterokaryon into the surrounding MM agar to allow limited auxotrophic growth to take place. In all cases the original heterokaryons continued to spread across the plate although they were unstable; some of the auxotrophic hyphae produced as a result of breakdown were slowly enveloped, but heterokaryotic growth terminated when the agar medium eventually dried out.

Di-auxotroph heterokaryons were more stable but showed a tendency to break down about 4–5 weeks after inoculation. Despite this instability, several heterokaryons were cultured on MM agar for more than a year (subcultured at 6-week intervals, away from the growing edge of the colony).

The investigation of the nuclear condition of conidia showed them to be regularly uni-nucleate (Pl. 1, fig. 1). Fusion between germinating conidia appeared uncommon, but it was sometimes seen and in one instance nuclear migration through a germ-tube bridge occurred (Pl. 1, fig. 2).

Conidial analysis

Table 2 gives conidial analysis data for six heterokaryons.

Parental types were recovered in each case. The ratio varied from 1:1 to 1:49 and generally the di-auxotrophic heterokaryons were the more unbalanced. A few prototrophic colonies developed when conidia from heterokaryons 1/4, 2/4 and 1/2 were used and these were regarded as revertants. No prototrophic colonies were observed from the di-auxotroph heterokaryons, but only dilute conidial suspensions were used and too few colonies were tested to detect parasexualism.

Table 2. *Verticillium*: percentage recovery of parental auxotrophs from six heterokaryons

Heterokaryon	Requirements of parent auxotroph	Recovery (%)	Requirements of parent auxotroph	Recovery (%)
1/4	Adenine	50	Arginine	50
2/4	Choline	88	Arginine	12
1/2	Choline	36	Adenine	64
1.7/2.6	Choline + methionine	4	Adenine + pABA + pantothenic acid	96
1.3/2.4	Arginine + choline	2	Adenine + glycine	98
2.4/1.7	Arginine + choline	2	Adenine + pABA + pantothenic acid	98

In the test using hyaline and normal auxotrophs all three heterokaryons synthesized, namely 2.6*/1.2, 2.6*/1.3 and 2.6*/1.7 were similar in morphology and growth to those already described. Tests of conidial ratios indicated that whereas these were unbalanced for heterokaryons 2.6*/1.3 (adenine + glycine types predominating) and heterokaryon 2.6*/1.7 (choline + methionine predominating), for heterokaryon 2.6*/1.2 parental types were recovered in a 1:2 ratio; this heterokaryon was selected for further analysis.

Sampling showed that of 98 colonies derived from heterokaryon 2.6*/1.2, 68 required arginine+adenine (1.2) and 30 grew only when supplemented with choline+methionine (2.6). All colonies produced some dark resting mycelium within 2-3 weeks on supplemented MM or on CM agar. At no time before this test did the 2.6* hyaline parent produce any resting mycelium even when incubated up to 6 weeks under these conditions. During subsequent work a small dark area of resting mycelium suddenly appeared in a 5-week culture of this strain on CM agar. Subculture from this plate gave a modified type of 2.6 strain which produced small areas of dark resting mycelium and increased amounts of fluffy white aerial hyphae. Transference to prune lactose agar gave cultures of a similar appearance to that on CM agar. The behaviour of this and other morphological strains carrying biochemical markers is under investigation.

DISCUSSION

The total yield of auxotrophs (0.5 %) was near the expected value, taking into account failures to detect mutants with requirements not present in CM medium and inhibition effects etc. Together with the evidence from stained conidia, these results corroborate the findings of Waggoner (1956), Caroselli (1957) and Hastie (1962) that the conidia of various strains of *Verticillium albo-atrum* are predominantly uni-nucleate. The marked delaying effect of u.v.-irradiation on the growth of surviving conidia is a general phenomenon and is assumed to reflect the operation of repair mechanisms following u.v.-radiation damage.

The colour mutant m5 was found to contain phytoene, β -carotene, γ -carotene, neo-lycopene A, lycopene, neurosporaxanthin and four unidentified pigments (Valadon & Heale, 1964) and it was suggested that their formation resulted from a de-repression leading to the production of a specific precursor in the pathway of pigment synthesis. The results of further work, involving u.v.-irradiation of mutant m 5 to produce a range of colour mutants which were then analysed for pigments, were published by Valadon & Heale (1965).

It appears from the present work that lucerne auxotrophs (derived from the same parental strain) will form heterokaryons on MM agar provided that the inoculum is in the form of well-established mycelial growth accompanied by some medium from previous CM agar cultures. Auxotrophic growth is at first predominant under these conditions and heterokaryons are not visible until 4 or 5 weeks after inoculation. This does not imply that heterokaryotic relationships do not become established at an early stage. In fact the evidence from staining germinating conidia shows that fusion of germ-tubes and subsequent migration of nuclei can occur within 6 hr of inoculation. It is interesting in this context to recall the first description of *Verticillium albo-atrum* by Reinke & Berthold (1879), for it included an observation of anastomosing germ-tubes. The present results indicate that something militates against the successful development of a heterokaryon until auxotrophic growth has diminished. In particular, the slow growth rate of the heterokaryon gives it a competitive disadvantage compared with active auxotrophic growth. Furthermore, even when auxotrophic development has more or less terminated, there may still be inhibition of heterokaryotic growth. This was indicated on several occasions by the production of heterokaryons after plating on fresh MM agar of mixed lucerne

auxotrophic (including heterokaryotic initials?) hyphae which had not shown obvious heterokaryotic growth during the previous 6 weeks of incubation.

Since Hastie (1962) successfully used mixed conidial suspensions of the hop strain of *Verticillium albo-atrum* on MM agar to produce heterokaryons, the reasons for their failure to develop when lucerne auxotrophs were used are not clear. Under these conditions, auxotrophic growth is severely limited (particularly in the case of di-auxotrophs) and one would expect selection pressure for prototrophic (heterokaryotic) growth to be intense. A negative result was also obtained when mixed conidial suspensions were incubated for 3 days in liquid medium CM (when growth was just visible) before plating on MM agar. It may be suggested that the success of the block technique as compared with the two conidial methods was primarily due to the small amount of CM medium transferred with the inoculum, which was sufficient to promote active auxotrophic growth on MM agar. This would lead to multiple hyphal fusions, thereby facilitating heterokaryon synthesis, and at a later stage (3–4 weeks), when auxotrophic growth declined, to visible heterokaryon development.

Only a few auxotrophs were tested in the present work, and since all the heterokaryons synthesized were derived from a single lucerne strain, it is not possible to conclude that there were significant differences in heterokaryon compatibility between particular host strains of *Verticillium albo-atrum*. It is possible that the relative ease with which the lucerne auxotrophs formed heterokaryons simply reflected the similar genetical constitution of these mutants. However, the results do suggest a heterokaryon incompatibility between auxotrophs derived from different isolates; further work is needed to establish whether this is linked with pathogenic or physiological specialization.

The results obtained from heterokaryon analysis with hyaline and normal darkly pigmented auxotrophs suggest that the inheritance of the dark pigment was cytoplasmically controlled. This is in agreement with the suggestions of Hastie (1962) who worked with hop isolates of the fungus. It is of interest to consider the mechanism involved in the production of the hyaline variant. One way in which it might arise is by the failure of a self-replicating cytoplasmic factor to migrate into the young conidium. In a heterokaryon the pigment-synthesizing system would be reconstituted by the normal cytoplasm of the dark parent and, assuming that migration failure is a comparatively rare event, the great majority of conidia from a heterokaryon of this sort would be normal. Brandt & Roth (1965) showed by a dilution technique that a hyaline variant was far less variable than the black parent and that even when u.v.-irradiated it did not produce any black colonies. This supports the suggestion that stable hyalinity is due to the irreversible loss of a cytoplasmic factor which *ipso facto* removes a source of morphological variation. For this hypothesis to be acceptable it must embrace all the known facts of pigment production in *Verticillium*. In my experience, there are two types of hyaline strains of *Verticillium albo-atrum*. The first is stable, in that dark resting mycelium is probably never formed even under a range of environmental conditions. This is important since light, temperature, nutrient concentrations and age of inoculum have been shown to control the formation of dark resting structures (Heale & Isaac, 1965; Kaiser, 1964; Brandt, 1964). Strains of this type are usually associated with extensive laboratory subculture. The second type, exemplified in the present work

by the 2.6* hyaline strain, apparently retains the latent ability to produce dark pigment. This might be regarded as a reversible change in the cytoplasmic factor rather than an irreversible loss.

Apart from the non-function of an enzyme system which mediates dark-pigment synthesis, the pathways of normal morphogenesis are profoundly altered by the change to hyalinity. In *Verticillium albo-atrum*, resting mycelium (formed by swelling, septation and the development of thickened cell walls in initially slender hyphae) does not develop, considerably more aerial hyphae are produced and spore production is drastically decreased. These widely divergent yet simultaneous changes indicate that an important system controlling morphogenesis in this fungus may be linked to a cytoplasmic factor.

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EXPLANATION OF PLATE

Fig. 1. Conidia of a lucerne strain of *Verticillium albo-atrum* stained with Giemsa after acid hydrolysis for 14 min. at 60°. Note the dense single resting nucleus in each cell.

Fig. 2. Germinating conidia stained with Feulgen after acid hydrolysis for 9 min. at 60°. Note the fusion tube and the migration of an elongate nucleus.