Analysis of Ploidy Levels in Strains of *Verticillium* using a Coulter Counter

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INTRODUCTION

Verticillium albo-atrum and *V. dahliae* are predominantly haploid and are classified as Fungi Imperfecti. Both species form verticillate conidiophores and develop darkly-pigmented resting structures; resting mycelium is produced by *V. albo-atrum* and microsclerotia by *V. dahliae* (Isaac, 1967). Single spore analysis indicates that black parental (Hyl⁺) cultures of both species frequently yield small numbers of hyaline (white) colonies which include both partially hyaline (Hyl⁺.p) and completely hyaline (Hyl) forms. There is substantial evidence that the inheritance of darkly-pigmented resting structures in these fungi is under cytoplasmic control (Heale, 1966; Typas & Heale, 1976*a, b*).

Cytological investigations, including those of Robinson, Larson & Walker (1957), Heale, Gafoor & Rajasingham (1968) and Pelletier & Aubé (1970), strongly indicate a uninucleate state for conidia of *Verticillium*. The percentage of binucleate spores was usually about 1 % or less, although Roth & Brandt (1964) reported a frequency as high as 15 % for binucleate and multinucleate conidia in one hyaline strain. Hastie (1964, 1968) produced unstable heterozygous diploids through heterokaryons and demonstrated the occurrence of the parasexual cycle in this fungus.

In this paper we report on the use of the Coulter counter (Kubitschek, 1969) for reliably estimating the ploidy levels of large populations of conidia of *V. albo-atrum* and *V. dahliae*.

METHODS

Strains. The 12 strains of *Verticillium* used are listed in Table 1. Auxotrophs were produced by ultraviolet irradiation (Typas & Heale, 1976*b*).

Media. Czapek-Dox agar served as minimal medium (MM) (Typas & Heale, 1976*b*). Complete medium (CM) contained the constituents of MM with the addition of casein hydrolysate, yeast extract, peptone and malt extract (each at 2 g l⁻¹). Solid media contained 2 % (w/v) 'Japanese' agar. All cultures were grown at 24 °C.

Heterokaryons and prototrophic heterozygous diploid conidia. Large numbers of spores sampled from heterokaryons yielded prototrophic heterozygous diploid conidia at a frequency of about 1 in 8 × 10⁶ spores (Typas & Heale, 1976*b*). Conidial volumes for spores from such prototrophic colonies on MM were analysed over a time course.

Ploidy levels. Ploidy levels were determined by examining 12000 to 16000 spores from each strain with a Coulter counter, model 2B, connected with a Coulter Channelyzer C-100 (Coulter Electronics, Dunstable, Bedfordshire). Both the 30 µm and 50 µm orifices were used and 1 ml of conidial suspension (adjusted to 1 × 10⁶ conidia ml⁻¹) was diluted in 9 ml of Isoton (isotonic solution; Coulter Electronics) before screening the sample.

Conidia were also stained with Feulgen (Heale *et al.*, 1968) and examined microscopically.

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Table 1. *Volumes of conidia from darkly-pigmented (Hyl⁺), partially hyaline (Hyl⁺.p) and completely hyaline (Hyl) prototrophic (wild-type) strains of V. albo-atrum and V. dahliae*

Each volume result (Coulter counter data) gives the mean, with standard error, of five measurements, each corresponding to 12 000 to 16 000 conidia.

Species	Host plant	Location	Mean conidial volume (μm^3)		
			Hyl ⁺	Hyl	Hyl ⁺ .p
<i>V. albo-atrum</i>	Potato	Swansea	17.88 $\pm$ 0.18	19.30 $\pm$ 0.20	17.52 $\pm$ 0.14
<i>V. albo-atrum</i>	Tomato	London	15.84 $\pm$ 0.14	17.58 $\pm$ 0.13	16.49 $\pm$ 0.17
<i>V. albo-atrum</i>	Lucerne	Norfolk	16.37 $\pm$ 0.25	18.00 $\pm$ 0.14	16.44 $\pm$ 0.21
<i>V. albo-atrum</i>	Chrysanthemum	Devonshire	15.86 $\pm$ 0.12	18.61 $\pm$ 0.16	16.04 $\pm$ 0.16
<i>V. albo-atrum</i>	Antirrhinum	Swansea	17.83 $\pm$ 0.13	19.52 $\pm$ 0.09	17.97 $\pm$ 0.16
<i>V. dahliae</i>	Pear	Australia	12.62 $\pm$ 0.12	13.76 $\pm$ 0.09	12.85 $\pm$ 0.15
<i>V. dahliae</i>	Peppermint	U.S.A.	12.15 $\pm$ 0.10	13.15 $\pm$ 0.13	12.40 $\pm$ 0.12
<i>V. dahliae</i>	Sunflower	Swansea	11.56 $\pm$ 0.11	13.34 $\pm$ 0.15	11.62 $\pm$ 0.11
<i>V. dahliae</i>	Cotton	U.S.A.	11.37 $\pm$ 0.13	13.12 $\pm$ 0.15	11.95 $\pm$ 0.17
<i>V. dahliae</i>	Potato	Swansea	11.61 $\pm$ 0.16	12.89 $\pm$ 0.19	10.92 $\pm$ 0.18
<i>V. dahliae</i>	Sprout	Evesham	11.98 $\pm$ 0.10	12.98 $\pm$ 0.18	11.02 $\pm$ 0.12
<i>V. dahliae</i> var. <i>longisporum</i> *	Horseradish	Germany	38.67 $\pm$ 0.19	39.73 $\pm$ 0.22	38.50 $\pm$ 0.21

Hyl⁺, Wild-type phenotype producing normal amounts of dark pigmentation as when first isolated from the diseased host.

Hyl⁺.p, Partially hyaline, producing very small amounts of pigment (as dots around the centre of the culture); obtained from the Hyl⁺ wild-type by ultraviolet irradiation or acriflavine treatment (Typas & Heale, 1976a).

Hyl, Stable hyaline strains, not producing pigment and resting structures; obtained from Hyl⁺ by ultraviolet irradiation or acriflavine treatment.

* *V. dahliae* var. *longisporum* (Stark, 1961) is a naturally-occurring diploid strain (Ingram, 1968) and was kindly supplied by Dr A. C. Hastie.

RESULTS AND DISCUSSION

The volume data (Table 1) indicate that there was no significant difference in spore size between strains belonging to the same pigmentation class of the same species. Comparison of the mean values for all haploid strains of the Hyl⁺ type shows a constant spore size difference between *V. albo-atrum* and *V. dahliae*, with the former being relatively larger, namely $5.16 \pm 0.09 \mu\text{m}$ (length) and $16.75 \pm 0.16 \mu\text{m}^3$ (volume) compared with $4.55 \pm 0.07 \mu\text{m}$ and $11.87 \pm 0.12 \mu\text{m}^3$ respectively for *V. dahliae*. It might therefore be possible to distinguish between the two species on these grounds. However, this is not considered to be a major criterion and it should obviously be accompanied by a series of other observations, i.e. dark pigmentation in the base of the conidiophores, temperature response, appearance of dark resting mycelium for *V. albo-atrum* and microsclerotia for *V. dahliae* (Heale & Isaac, 1965; Schnathorst, 1973; Typas, 1976).

Size distribution curves of conidial samples from the *V. dahliae* var. *longisporum* strain confirmed the difference in ploidy level between this natural diploid strain (Ingram, 1968) and all other wild-type haploid strains of *V. dahliae* tested. A few investigators have also observed conidial lengths which might suggest the natural occurrence of diploids (Chaudhuri, 1923; Van der Meer, 1925).

Mean data for spores from Hyl strains of both species suggested that they were slightly larger (about 4 % in length and 14 % in volume) than those from either Hyl⁺ or Hyl⁺.p pigmented strains, the latter two types being indistinguishable on this basis. This was probably due to the higher frequency (up to 3 %) of bi- and multinucleate conidia recorded for Hyl strains compared with less than 1 % for dark parental types. Tolmsoff's (1973) suggestion that a mixture of ploidy within the same strain accompanied the formation of resting structures therefore remains unsubstantiated.

Fig. 1. The effect of culture age on average frequencies of haploid conidia in segregating heterozygous diploid cultures of *V. dahliae* and *V. albo-atrum*. Data represent the means for eight different heterozygous diploid cultures.

Conidial suspensions from auxotrophic colonies of the Hyl⁺ and Hyl types showed size distributions which were indistinguishable from those obtained from the parental prototrophic Hyl⁺ and Hyl colonies respectively.

Analysis of conidial samples from 1-week-old prototrophic colonies, recovered after plating conidia from heterokaryons at very high density on MM, resulted in size distribution curves and volume data which invariably indicated diploidy, being similar to those obtained from *V. dahliae* var. *longisporum*.

Once spore-size distributions of both haploid and diploid conidial populations were established, it was possible to prepare a series of standard curves from conidial samples with a range of given haploid/diploid ratios, i.e. 100 % haploid 0 % diploid, 90 % haploid 10 % diploid, 80 % haploid 20 % diploid etc. These curves, which were of a bimodal type, enabled us to estimate the haploid/diploid ratio in an unknown sample; the sensitivity was such that a level of diploidy down to 2–3 % was still just detectable.

Changes in spore size, which tended towards a bimodal distribution, were noted in conidial samples taken from prototrophic heterozygous diploid cultures over a growth period of 4 weeks. The proportion of diploid conidia (Fig. 1) fell markedly in the second and third week and by the end of the fourth week almost all the conidia were haploid.

The peak of the curve for conidia from the stable diploid *V. dahliae* var. *longisporum* was relatively smooth in contrast to those obtained from segregating heterozygous diploid cultures which appeared to show very small side peaks on either side of the main one, presumably indicating the presence of various aneuploid states. Investigations on nuclear behaviour in phialides (Hastie, 1967, 1968) have indicated that haploid genotypes are not formed directly from diploids but through intermediate aneuploid stages.

Examination of conidia of wild-type darkly-pigmented (Hyl⁺) haploid strains of both species after Feulgen staining revealed nuclear diameters ranging from about 1.0 to 1.1 μm ; the diameters of nuclei in conidia of *V. dahliae* var. *longisporum* and all prototrophic heterozygous diploid strains were estimated to be in the range 1.8 to 2.0 μm .

Among a large number of fungi, only *Cephalosporium mycophilum* (Tuveson & Coy, 1961) spontaneously yields high proportions of haploid segregants from heterozygous diploids similar to those found for *Verticillium*. The frequency of haploids recovered in our work may be attributed mainly to a high haploidization frequency, but also at least in part to the prolific sporulation observed in the haploid, as opposed to the diploid, mycelium of *Verticillium*.

Spore size, therefore, is a convenient criterion for reliably establishing the level of ploidy. Such an approach should always be verified by cytological checks of nuclear-stained conidia, as well as by genetic analysis. In future experiments, single spore analysis and screening of large conidial samples from wild-type *Verticillium* isolates could lead to estimations of the frequency at which diploids might be found in natural populations. Further, the discovery of large-spored variants which give bimodal spore-size distributions (similar to those obtained here from rapidly segregating heterozygous diploids) would be the simplest way of demonstrating the occurrence of the parasexual cycle under field conditions in this, and possibly other, fungi.

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