Relationship between DNA Content and Spore Volume in Sixteen Isolates of *Verticillium lecanii* and Two New Diploids of *V. dahliae* (= *V. dahliae* var. *longisporum* Stark)

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Sixteen wild-type isolates of *Verticillium lecanii* from different insects and non-insect hosts, soils and locations exhibited a wide range in spore volumes, from $2.18 \mu\text{m}^3$ to $18.25 \mu\text{m}^3$, as recorded by Coulter counter analysis. A cold hydrolysis technique for Feulgen DNA microdensitometry indicated that all the isolates were haploid. Two wild-type strains of *V. dahliae*, from rape and sugarbeet from Sweden, were shown to be stable diploids by the criteria of conidial volume and relative DNA content. After treatment with *p*-fluorophenylalanine, both *V. dahliae* strains produced haploid segregants with half the conidial volume and relative DNA content of the original parents. Both diploid strains are regarded as new records for *V. dahliae* var. *longisporum* (Stark).

INTRODUCTION

Verticillium lecanii (Zimm.) Viegas and *V. dahliae* Kleb. are widespread deuteromycete fungi. *V. lecanii* includes isolates with the ability to parasitize insects (Burgess, 1981), although some isolates hyperparasitize fungi (Hall, 1980) and others are saprophytic. Due to its entomopathogenic nature, certain isolates of *V. lecanii* have been developed as commercial microbial insecticides by Microbial Resources Ltd (formerly Tate and Lyle Ltd), against glasshouse aphids and whiteflies (Hall, 1981). *V. dahliae* is a vascular wilt plant pathogen (Isaac, 1949), attacking a wide range of crops. Both species reproduce asexually via the production of verticillately borne, single-celled, uninucleate conidia (Domsch *et al.*, 1980).

Heterokaryosis and the parasexual cycle in imperfect plant pathogenic fungi are frequently proposed as mechanisms involved in the origin of new pathogenic races (Day, 1960; Tinline & MacNeill, 1969). Buxton (1956) found that several haploid segregants from a heterozygous diploid, formed between auxotrophic strains of *Fusarium oxysporum* f. sp. *pisi*, were recombinant for both auxotrophic markers and pathogenicity. As early as 1879, Reinke & Berthold reported anastomosing germ-tubes of plant pathogenic species of *Verticillium*. Schreiber & Green (1966) observed anastomosis between hyphae from germinating conidia of *V. albo-atrum* (= *V. dahliae sensu* Isaac, 1949); later Singh (1972) observed hyphal anastomosis and nuclear migration in *V. lecanii*. These reports indicated the possibility of heterokaryon formation in the wild by both species studied here. The parasexual cycle has been demonstrated in the laboratory for both *V. dahliae* (Hastie, 1970; Typas & Heale, 1976) and *V. lecanii* (Jackson & Heale, 1983). Evidence for the parasexual cycle in the wild is still limited although large-spored, wild-type, diploids have been reported in various fungi, e.g. in *Aspergillus niger* and *A. nidulans* (Nga *et al.*, 1975; Upshall, 1981), in *V. albo-atrum*, *V. dahliae*, *V. tricorpus* (Hastie & Heale, 1984) and in *V. dahliae* var.

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Table 1. *Original host and geographical location of Verticillium isolates*

The CBS isolates of *V. lecanii* were kindly supplied by Dr W. Gams, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Isolates C3 and C42 of *V. lecanii* were kindly supplied by Dr R. A. Hall, Glasshouse Crops Research Institute, Littlehampton, UK. *V. dahliae* isolates SOS and SBS were kindly supplied by Dr A. A. Bell and Dr J. Puhalla, National Cotton Pathology Research Laboratory, College Station, Texas, USA. Isolate CMI 21176 was obtained from the Commonwealth Mycological Institute, Kew, UK. *V. dahliae* var. *longisporum* is our laboratory strain.

Isolate	Original hos.	Geographical location
<i>V. lecanii</i>		
C3	<i>Macrosiphoniella sanborni</i> (aphid)	UK
C42	<i>Myzus persicae</i> (aphid)	India
CBS 318.70K	<i>Nematus pavidus</i> (sawfly-nymph)	Unknown
CBS 318.70D	<i>Pristiphora abietina</i> (sawfly)	Czechoslovakia
CBS 170.76	<i>Carpocapsa pomonella</i> (codling moth)	Poland
CBS 384.35	<i>Lecanium coryli</i> (scale)	Czechoslovakia
CBS 318.70L	<i>Diprion pini</i>	E. Germany
CBS 140.60	Wooden floor	Netherlands
CBS 446.54	Soil	Zaire
CBS 132.56	Soil	Zaire
CBS 413.70A	<i>Tuber maculatum</i> (truffle)	Netherlands
CBS 123.42	<i>Citbotium schiedei</i> (fern)	Netherlands
CBS 531.72	<i>Salvinia rotundifolia</i> (aquatic plant)	Poland
CBS 455.70A	<i>Saissetia olea</i>	Morocco
CBS 455.70B	Unknown	USSR
CMI 21176	Unknown	Unknown
<i>V. dahliae</i> var. <i>longisporum</i>	Horse-radish	Germany
<i>V. dahliae</i> SOS	Rape	Sweden
<i>V. dahliae</i> SBS	Sugarbeet	Sweden

longisporum (Stark, 1961; Ingram, 1968). Further, Typas & Heale (1976) observed segregation for acriflavine resistance in a large-spored *V. albo-atrum* strain from tomato, which they regarded as evidence for a heterozygous diploid occurring in the wild.

A doubling in spore size is often regarded as evidence for a doubling in the nuclear DNA content, e.g. for *Puccinia graminis* (Williams & Mengden, 1975), *Aspergillus nidulans* (Clutterbuck, 1969), *V. nigrescens*, *V. nubilum*, *V. albo-atrum* (Tolmsoff, 1972) and *V. dahliae* (Typas & Heale, 1977). In work on genetic recombination with *V. dahliae* and *V. albo-atrum*, Typas (1983) used conidial length data to differentiate between diploids, prototrophic haploid recombinants and aneuploids. Feulgen DNA microdensitometry has previously been used for DNA estimations of *V. dahliae* and *V. albo-atrum* conidia (Typas & Heale, 1980), but the hot (60 °C) hydrolysis method used did not always produce consistent results.

An earlier paper (Jackson *et al.*, 1985) described factors associated with virulence in 18 different isolates of *V. lecanii*, ranging from highly pathogenic to saprophytic types. In this paper, we report DNA microdensitometry determinations using a cold hydrolysis procedure on 16 isolates exhibiting a considerable range in spore size. In view of a report by Puhalla & Hummel (1983) that two large-spored isolates of *V. dahliae* from rape and sugarbeet failed to mutate after UV treatment, these two isolates and an established stable diploid strain of *V. dahliae* (*V. dahliae* var. *longisporum*) were also included in the investigation.

METHODS

Fungal isolates. Sixteen wild-type prototrophic isolates of *V. lecanii* and three of *V. dahliae* were used (Table 1). Single-spore isolations were made from each and stock cultures maintained on complete agar slopes in the dark at 4 °C.

Media. Complete medium (CM) was as described previously (Jackson *et al.*, 1985). Autoclaving was at 120 °C for 20 min. To prepare haploidization medium, CM was cooled to 40 °C and a filter-sterilized solution of *p*-fluorophenylalanine added prior to pouring (final concentration: 0.0116%, w/v).

Conidial volume. Conidia were harvested with 10 ml sterile distilled water from semi-synchronous 7-d-old spread-plate cultures, incubated at 22–24 °C in the dark. Conidial suspension ($0.2 \text{ ml}, 1 \times 10^6 - 1 \times 10^7 \text{ ml}^{-1}$) was

Fig. 1. Effect of 'cold' hydrolysis (5 M-HCl, 26 °C) (□) and 'hot' hydrolysis (1 M-HCl, 60 °C) (○) on the intensity of Feulgen staining of nuclei of conidia of *V. lecanii*. The bars indicate SD ($n = 50$).

added to 18 ml Isoton solution (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 and 1 g NaN_3 in 1 litre of water) in a plastic cuvette. Volume measurements were made with a Coulter counter (Typas & Heale, 1977) model ZB1, fitted with a probe with a 30 μm diameter orifice, connected to a Coulter Channelyzer C100 (Coulter Electronics Ltd) and interfaced to a computer programmed to calculate mean values and standard error. Accuracy was checked by using latex granules of known volume.

Feulgen staining. The methods of Heale *et al.* (1968) and Typas & Heale (1980) proved unsatisfactory for optimum staining of *V. lecanii* conidia. The following technique was developed by varying fixation, hydrolysis and staining procedures.

A conidial suspension of one test isolate was dried onto one end of a coverslip (22 $\times$ 50 mm), spores from a control strain being applied to the opposite end. The coverslips had been previously coated with a gelatin-based (Jensen, 1962) adhesive (5 g gelatin and 0.5 g chromic potassium sulphate dissolved in 1000 ml distilled water at 30–38 °C).

Fixation was done for 5 min in freshly prepared Helly's solution (5 g mercuric chloride and 3 g potassium dichromate in 100 ml distilled water; just before use, 1 ml formaldehyde was added). After washing in distilled water, the conidia were hydrolysed at 26 °C in 5 M-HCl for 45 min; the coverslips were transferred to freshly made Schiff's solution (Fox, 1969, slightly modified), prepared by adding 1.5 g basic fuchsin (Gurr) and 3.8 g sodium metabisulphite to 200 ml 0.15 M-HCl. The solution was stirred for 3 h at room temperature, decolorized with 2 g activated charcoal and filtered through Whatman no. 1 filter paper. The conidia were stained for 80 min, then washed in sulphur dioxide water (0.5 M-potassium metabisulphite/1 M-HCl, 2:1, v/v), dehydrated through an alcohol series and mounted in DPX (BDH), thinned with xylene.

DNA content of conidia. A Vickers M86 scanning microdensitometer was used to record integrated stain densities per nucleus (Typas & Heale, 1980), compared with an internal control of known ploidy, e.g. *V. dahliae* var. *longisporum*, a stable diploid (Ingram, 1968), or *V. lecanii* isolate C3 (subsequently shown to be haploid). A $\times 100$ oil immersion objective was used, and the microdensitometer settings were: slit width 41.5 units, wavelength 560 nm, orifice size A2 and screening area settings of X1, Y1. Three readings were taken per conidium to calculate the mean. Fifty spores were screened for each treatment, zero levels being checked after every five readings.

RESULTS

Preliminary experiments involving Feulgen staining suggested that 'hot' hydrolysis (60 °C in 1 M-HCl for 10–15 min) similar to that used by Typas & Heale (1980) for *V. albo-atrum* and *V. dahliae* was unsuitable, and hydrolysis with 5 M-HCl at 26 °C for 45 min was preferable. The reason for this was indicated by the calibration curves of hydrolysis time against amount of Feulgen stain per conidial nucleus, recorded on the scanning microdensitometer (Fig. 1). For both types of hydrolysis, the optimal values were about the same for the two methods, but the 'hot' hydrolysis optimum occurred after only 12 min and persisted for 1 min at the most.

Table 2. *Relationship between conidial volume and ploidy level inferred from Feulgen DNA microdensitometry of sixteen isolates of V. lecanii*

Isolate	Conidial volume ($\mu\text{m}^3 \pm \text{SE}$)	DNA content* (arbitrary units $\pm \text{SE}$)	Ploidy
1. C3 (control)	4.06 ± 0.22	1.22 ± 0.02	haploid
2. 123.42 (Control)	2.18 ± 0.14	0.99 ± 0.04	haploid
3. 531.72 (Control)	3.06 ± 0.20	1.19 ± 0.03	haploid
4. 446.54 (Control)	3.48 ± 0.17	1.32 ± 0.04	haploid
5. 140.60 (Control)	3.91 ± 0.28	1.31 ± 0.39	haploid
6. 413.70A (Control)	4.35 ± 0.29	0.90 ± 0.08	haploid
7. CMI (Control)	4.38 ± 0.29	1.21 ± 0.05	haploid
8. 455.70B (Control)	4.83 ± 0.22	0.87 ± 0.02	haploid
9. 170.76 (Control)	5.45 ± 0.38	1.05 ± 0.07	haploid
10. 455.70A (Control)	5.45 ± 0.39	1.08 ± 0.17	haploid
11. 318.70L (Control)	5.47 ± 0.39	1.05 ± 0.03	haploid
12. 384.35 (Control)	5.48 ± 0.37	1.12 ± 0.04	haploid
13. 318.70D (Control)	5.81 ± 0.36	1.08 ± 0.07	haploid
14. 318.70K (Control)	6.21 ± 0.40	1.12 ± 0.02	haploid
15. 132.56 (Control)	9.58 ± 0.52	1.15 ± 0.01	haploid
16. C42 (Control)	18.25 ± 1.30	1.00 ± 0.36	haploid
<i>V. dahliae</i> var. <i>longisporum</i>	16.48 ± 0.61	1.03 ± 0.07	haploid
		2.69 ± 0.07	diploid

* The Feulgen DNA microdensitometry values are mean values per conidium from a total of 50 conidia per isolate. For each isolate tested, isolate C3 was used as an internal control to reduce experimental error.

Hydrolysis at 26 °C in 5 M-HCl resulted in a much broader peak of approximately 10 min for optimal staining, beginning after about 35 min. Slight variability between tests was therefore less of a problem when 'cold' hydrolysis was used. Freshly made Schiff's reagent produced more intense staining than did commercial preparations.

Conidial volumes for 16 different wild-type isolates of *V. lecanii* (Table 2) indicated considerable variability between isolates ranging from $2.18 \pm 0.14 \mu\text{m}^3$ (isolate 123.42) to $18.25 \pm 1.3 \mu\text{m}^3$ (isolate C42). Feulgen staining revealed that the conidia were regularly uninucleate, and despite the differences in size, DNA microdensitometry values indicated that all were haploid (Table 2). When the 'standard' diploid (*V. dahliae* var. *longisporum*) isolate was compared, the values obtained for DNA content were approximately double those for *V. lecanii* isolates (2.69 ± 0.07 units compared with 1.22 ± 0.02 for *V. lecanii*), confirming the haploid state of the latter.

The conidial volumes of *V. dahliae* isolates SOS and SBS were $15.04 \pm 0.57 \mu\text{m}^3$ and $15.30 \pm 0.69 \mu\text{m}^3$, respectively, similar to the stable diploid *V. dahliae* var. *longisporum* ($16.48 \pm 0.61 \mu\text{m}^3$). DNA microdensitometry indicated that all three isolates had approximately the same amount of DNA per conidial nucleus (Table 3) and all conidia were uninucleate. When these two test isolates were grown on media containing the haploidizing agent *p*-fluorophenylalanine, sectoring was observed, *V. dahliae* SOS producing fewer sectors than *V. dahliae* SBS. Single-spore isolations from 10 sectors of *V. dahliae* SOS and 15 sectors of *V. dahliae*

Table 3. *Conidial volume and DNA content of natural diploids and induced haploids of V. dahliae*

Isolate	Conidial volume ($\mu\text{m}^3 \pm \text{SE}$)	DNA content* (arbitrary units $\pm \text{SE}$)	Ploidy
<i>V. dahliae</i> var. <i>longisporum</i>	16.48 $\pm$ 0.61	3.56 $\pm$ 0.07	diploid
<i>V. dahliae</i> SOS	15.04 $\pm$ 0.57	3.49 $\pm$ 0.07	diploid
<i>V. dahliae</i> var. <i>longisporum</i>	16.48 $\pm$ 0.61	2.75 $\pm$ 0.04	diploid
<i>V. dahliae</i> SBS	15.30 $\pm$ 0.69	2.81 $\pm$ 0.06	diploid
<i>V. dahliae</i> SBS	15.30 $\pm$ 0.69	2.55 $\pm$ 0.17	diploid
<i>V. dahliae</i> SBS-1	6.44 $\pm$ 0.56	1.20 $\pm$ 0.08	haploid
<i>V. dahliae</i> SBS	15.30 $\pm$ 0.69	5.08 $\pm$ 0.13	diploid
<i>V. dahliae</i> SBS-2	7.87 $\pm$ 0.34	2.29 $\pm$ 0.10	haploid
<i>V. dahliae</i> SOS	15.04 $\pm$ 0.57	4.57 $\pm$ 0.08	diploid
<i>V. dahliae</i> SOS-1	8.13 $\pm$ 0.40	2.24 $\pm$ 0.06	haploid

* The Feulgen DNA microdensitometry values are mean values per conidium from a total of 50 conidia per isolate.

SBS were made. One colony from *V. dahliae* SOS (SOS-1) and two from *V. dahliae* SBS (SBS-1 and SBS-2) resulting from these sectors produced relatively small conidia, volume 6–8 μm^3 (Table 3). The nuclear DNA content of the small-spored strains was half the value of the parent isolate from which they were derived (Table 3), thus indicating a reduction to haploidy.

DISCUSSION

Once the method was established for Feulgen staining, there was still variation in the intensity of the Feulgen reaction. Thus it was essential to have an internal control on the same coverslip as the conidia to be tested, in agreement with the observations of Dhillon *et al.* (1977). It was also necessary to standardize culture age and environmental conditions, both of which affect spore size (Pelletier & Aube, 1970). Leslie & Parbery (1972) showed that *V. lecanii* conidia produced on malt agar had mean lengths of 4.9 μm , and the addition of 0.158 M-fluoride to the medium resulted in conidial lengths of 6.1 μm . There are numerous reports on factors that influence spore size (Smith, 1965). Even where spore size is a reliable indication of ploidy, spontaneous haploidization of diploids produced in the laboratory (Hastie, 1964; Das & Ilzuck, 1978) can result in the confusion of haploid recombinants with diploids (Messias & Azevedo, 1980; Bergeron & Al-Aidroos, 1982).

The measurement of conidial volume as performed here, using a Coulter counter (Typas & Heale, 1977), allowed rapid analysis of a large population of spores. For *V. lecanii*, there was a wide variation in mean values between isolates, viz. 2.18 μm^3 to 18.25 μm^3 . Results for DNA values indicated that despite such differences, the isolates were all haploid. The microdensitometer readings were about half those obtained for *V. dahliae* var. *longisporum*, a natural stable diploid (Typas & Heale, 1980). In additional studies not reported here, auxotrophic and morphological mutants of *V. lecanii* have been obtained with expected frequencies from wild-type parental isolates, following treatment with *N*-methyl-*N'*-nitro-nitrosoguanidine or UV irradiation. Cytological studies with Feulgen DNA microdensitometry confirmed that these wild-type isolates of *V. lecanii* were haploid and invariably uninucleate.

Tolmsoff (1983), using an extraction procedure, estimated values of 0.076 pg DNA per nucleus for *V. dahliae* var. *longisporum*, which agree well with values (approximately half this level) obtained using microdensitometry and a known standard (carp hepatic nuclei) for ungerminated conidia from haploid strains of *V. dahliae* (0.025–0.030 pg) (Typas & Heale, 1980). By comparison with the microdensitometry data obtained in the present work, we infer that the conidia of *V. lecanii* contain approximately 0.038 pg DNA per nucleus.

The two new naturally occurring diploids of the plant pathogenic species *V. dahliae* studied here, SOS and SBS, in contrast with most other isolates of *V. dahliae*, were large-spored and did

not yield auxotrophic mutants when treated with UV light by Puhalla & Hummel (1983). Our results for haploidization, conidium size and relative DNA content per nucleus have established that isolates SOS and SBS are both stable diploids. Until now, the only other proven natural diploid of *V. dahliae* was *V. dahliae* var. *longisporum*, originally isolated by Stark (1961). On the basis of conidium size, the ability of *p*-fluorophenylalanine to induce small-spored (haploid) forms, the induction of auxotrophic mutants only from the small-spored forms and not from the large-spored form, and the synthesis of types indistinguishable from the large-spored form using haploid auxotrophs, Ingram (1968) concluded that *V. dahliae* var. *longisporum* was a true diploid. Further studies comparing conidium size (Typas & Heale, 1977) and relative DNA content between *V. dahliae* var. *longisporum* and haploid isolates of *V. dahliae* have confirmed this conclusion. There have been other reports of naturally occurring diploids in *V. dahliae* and *V. tricornis* (Tolmsoff, 1973; Kasyanenko & Portenko, 1978), but these require additional genetic evidence and DNA determinations before they can be fully accepted. Wild somatic diploids have also been described for other 'normally' haploid plant pathogenic fungi (Caten & Day, 1977) including *Puccinia graminis* f. sp. *tritici* (Williams & Mengden, 1975) and *Aspergillus niger* (Nga *et al.*, 1975).

Many deuteromycete fungi can be induced to form diploids under laboratory conditions via the parasexual cycle, using auxotrophic haploid parents (Fincham *et al.*, 1979), species of *Verticillium* being unexceptional in this respect (Hastie, 1970; Hastie & Heale, 1984). These heterozygous diploids are mainly unstable, although exceptions do occur (Heale *et al.*, 1981). In contrast, *V. dahliae* var. *longisporum* and the two *V. dahliae* isolates investigated here appeared extremely stable. These are assumed to be homozygous diploids arising as a result of total mitotic nondisjunction or from somatic nuclear fusion in a homokaryotic cell. Further evidence for homozygosity was the observation that all haploid segregants appearing after treatment with *p*-fluorophenylalanine were similar in morphology to the parent isolate. In fact, Tolmsoff (1983) suggested that production of aneuploids from homozygous diploids is a significant source of genetic variability.

Unequivocal demonstration of the parasexual cycle as a major cause of variability in *Verticillium* species in the wild is difficult, presumably because of its rare and transient nature in most cases, and some workers dispute its relevance. However, when MacNeill & Chilton (1981) inoculated tomato plants with two distinct prototrophic isolates of *Fusarium oxysporum* f. sp. *lycopersici*, many recombinants were recovered. Similarly, Clarkson & Heale (1985) infected *Antirrhinum* plants with two haploid complementary diauxotrophic isolates of *V. albo-atrum* from hop and were able to reisolate a stable, pathogenic, prototrophic diploid. Even where diploidy is rare and transient, there may be profound evolutionary potential in terms of genetic recombination for many deuteromycete fungi.

We suggest that the two new strains of *V. dahliae* described here be assigned to *V. dahliae* var. *longisporum*, a form previously isolated only from horse-radish in Germany.

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