

Spore size revisited: analysis of spore populations using an automated particle sizer

I. H. CHAPELA

Biotechnology, Preclinical Research, Sandoz Pharma Ltd.
4002 Basel, Switzerland

CHAPELA, I.H. (1991). Spore size revisited: analysis of spore populations using an automated particle sizer. – SYDOWIA 43: 1–14.

An automated, electroning sizing device, the Coulter counter, was used to analyse the distribution of spore sizes in representatives of the fungal genera *Hypoxyylon*, *Colletotrichum*, *Hormonema* and *Microsphaeropsis*. This proved a fast, reproducible and reliable method that provided a frequency distribution of spore sizes in populations of up to 10^5 spores within a couple of minutes. These profiles were qualitatively more informative than other morphometric determinations based on smaller sample sizes. Frequency distributions of large samples provided evidence of differentiation, and possible specialization, in spore populations of *Colletotrichum* and *Hypoxyylon*, in that all samples from these genera (one and five species, respectively) presented a bimodal size distribution. Differentiation of species and ecological types within species was also made possible through comparisons of spore size frequency distribution profiles.

Keywords: *Hypoxyylon*, *Colletotrichum*, *Hormonema*, Coulter counter, image analysis, spore measurement.

Spore size, a relatively constant character for a given species, has naturally been used as diagnostic in mycology ever since the discovery of the microscope. Spore size is simple to assess under the microscope for small samples yet allows one to distinguish otherwise very similar fungi in nature or in culture. A problem arises, however, when variability in spore size is acknowledged and attempts are made at measuring that variability under the microscope. In those cases, a range of spore sizes is usually the best available expression of spore variability. Establishing spore size ranges is time-consuming and the results are of questionable statistical reliability. Size ranges provide at best an estimate of the extremes in a frequency distribution, completely neglecting the properties of the actual distribution which would describe the composition of a spore population. Nevertheless, a detailed expression of spore size variability can provide crucial ecological and taxonomic information. For example, GOURBIÈRE & MORELET (1979) showed that a biometric characterization of spores according to their length and width could be used to distinguish species in the genus *Hormonema* which are otherwise undistinguishable. Similarly, PETRINI & al. (1987, 1989), have recently

shown that host-specific groups can be distinguished at specific and sub-specific levels on the basis of spore size even for taxa usually assumed to display homogeneous spore size distributions.

The present is an attempt to address the shortcomings of expressing spore size distribution only from microscopic observations. Using three practical examples, I evaluate the potential of an automated particle counter and sizer for use in fungal ecology and taxonomy.

Materials and methods

Spore size ranges were estimated microscopically (1000 X) from a sample of approximately 200 spores and using an automated particle sizer and counter, the Coulter counter (Coulter Electronics, Ltd., Luton, England). The Coulter counter measures the alteration of a constant-intensity electric current across a small aperture caused by non-conducting particles crossing it. The increase in resistance as a particle travels through the current-dense orifice is accompanied by a voltage pulse which is measured, amplified and counted with electronic scalars. The height of the voltage pulse is proportional to the volume of the particle according to the relationship

$$\Delta V = \frac{v \cdot \beta \cdot i \cdot f_E}{r^4}$$

where ΔV is the amplitude of the voltage pulse; v is the particle volume; β is the electrolyte resistance; i is the orifice current; f_E is a particle-shape factor, relevant in the case of very elongated particles such as filiform, sigmoid or radiate spores; and r is the orifice radius. A discussion of the principles and application of the Coulter counter technique was provided by KACHEL (1979; Fig. 1). An additional electronic device, the Coulter Channelizer 256 was used to register separately pulses of different amplitude. The Channelizer accumulates the number of pulses (counts) registered by the Coulter counter on each of 256 channels corresponding to as many particle size classes. The cumulative count for every channel is displayed and printed by the Channelizer as a size frequency-distribution. Figures presented here are hand-drawn copies of printouts.

Fungal spores were suspended in buffered isotonic electrolyte (Dulbecco's phosphate-buffered saline solution without Ca or Mg, Gibco) at a final concentration of 10^4 – 10^5 spores ml⁻¹ and maintained under agitation during sizing. For measurement, spores were drawn, one at a time, through a 100 μ m orifice in the wall of a glass probe which was filled with the same electrolyte and contained one of the electrodes in its inner compartment. The other electrode was

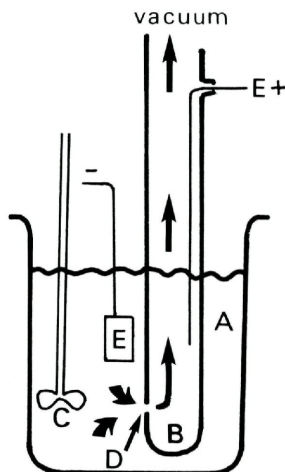


Fig. 1. - Diagrammatic representation of electronic particle sizer. A, spores suspended in electrolyte; B, glass probe; C, stirrer; D, orifice through which spores are drawn; E, electrodes.

placed in the vessel containing the spore suspension, so that an electrical current was established across the orifice (Fig. 1). This orifice current was maintained at $400 \mu\text{A}$. The amplification of the voltage signal produced by spores crossing the orifice was altered by changing the amplitude and gain settings of the amplifier, thus allowing for measurement of spore sizes within three ranges: 1 – 25 fl (femtoliter, i.e. 10^{-15} l), 10 – 400 fl and 100 – 2500 fl (equivalent sphere diameters, 1.24 – 3.63 μm , 2.67 – 9.14 μm and 5.76 – 16.84 μm , respectively). For each size range, two latex bead standards (spherical particles of known volume obtained from Coulter Electronics Ltd.) were measured. The scale of printouts for the corresponding setting was then calibrated according to the latex standards. For comparison with microscopic measurements, the modal volume (highest peak) was read directly on the frequency-distribution curves. Spores were assumed to be ellipsoid for transformations between linear and volumetric measurements. When using the Coulter counter, a minimum of 3×10^4 spores were measured for each sample, usually within 1 – 2 minutes.

An independent control was provided by the estimation of size frequency-distribution using a computerized image analysis system (Leica AG, Glattbrugg, Switzerland). From digitalized microscopic

images of lactic acid-mounted spore preparations of *Colletotrichum orbiculare* (BERK. & MONT.) V. ARX and *Hypoxyylon fragiforme* (SCOP.) KICKX, the image analysis system calculated, using the PARTAN C software, the diameter of circles with the same area as each spore in the microscopic field. This diameter was used to calculate the equivalent sphere volume which was displayed as a frequency-distribution plot for a total of 500 spores. Lactic acid was used as a mountant to avoid spore germination between sampling and measurement.

Three groups of fungi were selected for the analysis of spore size frequency-distributions. First, a single conidiospore isolate of the plant pathogen *C. orbiculare* was used to ascertain the reproducibility of size determinations in the particle counter, as well as the constancy of a given frequency-distribution profile for repeated measurements on conidia produced from a genetically homogenous source under diverse conditions. Subcultures of this isolate were grown on 2% malt agar on Petri plates for four to sixty days or on 2% malt broth in shake flasks (200 rpm) at 21 C in the dark for one week. Spores were collected by flooding culture plates with 5 ml sterile tap water and gently swirling, or by filtering broths from liquid cultures through ca. 1 cm loose fiber glass in a funnel and immediately measured.

The second group comprised *H. cohaerens* (PERS.) FR., *H. rubiginosum* (PERS.) FR. and *H. fragiforme* from *Fagus sylvatica* L.; *H. fuscum* (PERS.) FR. from *Corylus avellana* L. and *H. mammatum* (WAHLENB.) MILLER from *Populus tremula* L. selected as a taxonomically well-defined group where spore size ranges, while being an important taxonomic character, commonly overlap from one species to the other. Stromata were collected in the field and placed in moist chambers covered by a glass slide suspended 2–5 mm above the stromatal surface. Ascospores discharged onto the slide over a 24 h period were either measured immediately or stored on the slides at room temperature in the dark until measured, up to 15 months after discharge.

In the third group, isolates were selected from fungi living in tissues of taxonomically distant but spatially contiguous plant species (the mistletoe *Viscum album* L. and one of its hosts, the fir *Abies alba* MILLER; I. CHAPELA, in preparation). In this case, it was crucial to distinguish between culturally similar isolates that occurred on both host plant in order to establish the degree of host specificity in two endophytic communities. Of interest in this respect were four isolates each of the hyphomycete *Hormonema* sp. and the coelomycete *Microsphaeropsis* cf. *olivacea* which occurred on both mistletoe and its fir host. Fungi in this third group were grown on 2% malt agar on Petri plates at 24 C in the dark. Conidia were collected by flooding plates with sterile tap water and measured immediately.

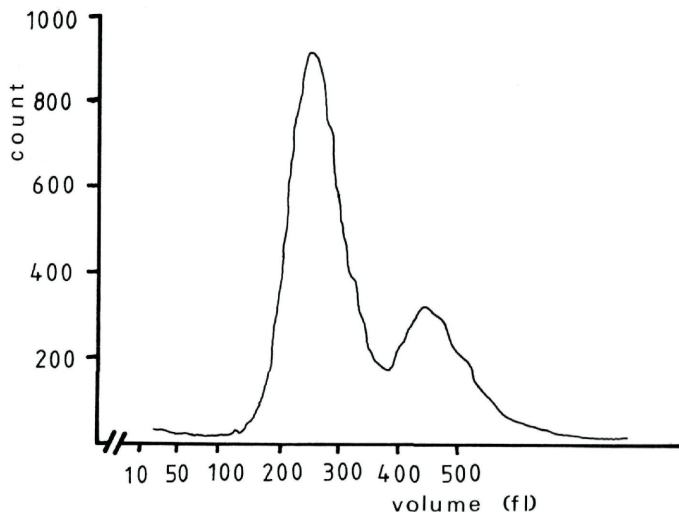


Fig. 2. – Frequency-distribution curve of a conidial population of *Colletotrichum orbiculare* obtained with an automated electronic particle sizer. Bimodal nature of the distribution is evidenced by the presence of two distinct frequency peaks.

Results

Sizing of a genetically homogeneous spore population

The use of a single-spore isolate of *C. orbiculare* allowed for the analysis of size frequency-distribution profiles with a minimum of genetically-determined variability. By careful sampling to avoid mycelial particles, background noise due to debris was minimized and the profiles obtained represented therefore a close picture of the actual conidial size-distribution (Fig. 2).

The conidial population of this fungus always presented a bimodal distribution, with peaks at about 300 and 450 fl. Peak heights corresponding to each of the two subpopulations of conidia maintained always an approximately 3:1 relationship between the number of conidia in the smaller and larger sub-groups. For 15 independent counts, the number of larger spores related to that of smaller ones was extracted from the frequency-distribution curves. An average of 75% of the total count comprised small- and 25% large spores (range 66–82% and 18–34%, respectively; standard deviation:

5.7). Due to the close proximity of the two peaks (equivalent sphere diameter: 8.31 and 9.51 μm , respectively), the existence of the two conidial subpopulations could not be observed directly under the microscope. Since the Coulter counter measures overall volume and not any particular dimension of the particles crossing the sampling orifice, the bimodal distribution observed was not an artifact, as would be expected if length and width were measured separately for elongated spores.

The volumes registered by the automatic particle counter are commensurate with the corresponding microscopic measurements as

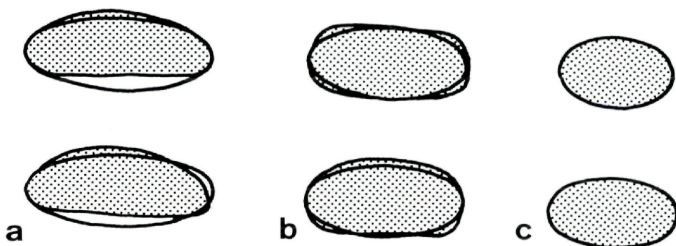


Fig. 3. – Spore profiles of *Hypoxylon fragiforme* (a), *Colletotrichum orbiculare* (b) and *Hormonema* sp. (c) obtained from enlarged photomicrographs (shadowed), superimposed on the profiles of ideal ellipsoids with corresponding length/width dimensions (not shadowed). Spore size is smaller than, larger than, and equal to the ideal ellipsoid in *H. fragiforme*, *C. orbiculare* and *Hormonema* sp., respectively. These differences might account for small discrepancies between electronically- and microscopically measured spore sizes (cf. Tab. 1).

well as with those reported in the literature, although discrepancies were observed for spores departing significantly from the assumed ideal ellipsoidal shape (Tab. 1). Taking into account the fact that small variations in linear measurements can have a large influence on volume estimates (linear discrepancies are cubed in the calculation of volumes), the discrepancies between Coulter counter measurements and those derived from microscopic observations are relatively small (Fig. 3).

For the analysis of various subcultures of the single-spore isolate of *C. orbiculare*, no change larger than an equivalent sphere diameter of 0.5 μm (standard deviation for the volume: 10.64) was ever observed in the position of either of the two main peaks in any of these observations.

Tab. 1. – Comparative summary of spore sizing provided by an electronic particle sizer and from calculated volumes derived from a computerized image analysis system (IAS), from direct microscopic measurements of the same samples and from measurements for the corresponding species as published in the following references: ¹SUTTON (1980); ²HERMANIDES-NIJHOF (1979); ³DENNIS (1981); ⁴ELLIS & ELLIS (1985). o.s. unreliable out-of-scale measurement; n.m. not measured; – not applicable.

Fungus	Particle sizer modal volume (fl)		Microscopic measurements		
	peak 1	peak 2	IAS modal volume (fl)	size range (fl)	
				direct	literature
<i>Colletotrichum orbiculare</i>	300	450	263	100–301	148–282 ¹
<i>Hormonema</i> , <i>Viscum</i> isolate	143	–	n.m.	92–1770	198–586 ²
<i>Abies</i> isolate	838–998	–	n.m.	92–1770	198–586 ²
<i>Hypoxylon cohaerens</i>	104	165	n.m.	144–359	75–130 ³
<i>Hypoxylon fuscum</i>	240	o.s.	n.m.	359–503	157–384 ³
<i>Hypoxylon fragiforme</i>	143	226	315	359–570	144–384 ³
<i>Hypoxylon mammatum</i>	1317	1839	n.m.	1885–4123	377–3694 ⁴
<i>Hypoxylon rubiginosum</i>	166	250	n.m.	157–502	75–226 ³
<i>Microsphaeropsis</i> cf. <i>olivacea</i>	15–20	–	n.m.	10–45	13–105 ¹

Closely related species with overlapping spore size ranges and use of herbarium material

Species of *Hypoxyylon* with distinct ascospore sizes also produced totally distinct peaks in their frequency-distribution profiles. This is clearly seen when comparing *H. mammatum* with either *H. fragiforme*, *H. rubiginosum*, *H. fuscum* or *H. cohaerens* (Fig. 4). By contrast, and as expected from direct microscopic observation, frequency peaks corresponding to the latter four species occurred close to each other, and the frequency-distribution curves from ascospores of *H. fragiforme* and *H. rubiginosum* were practically indistinguishable.

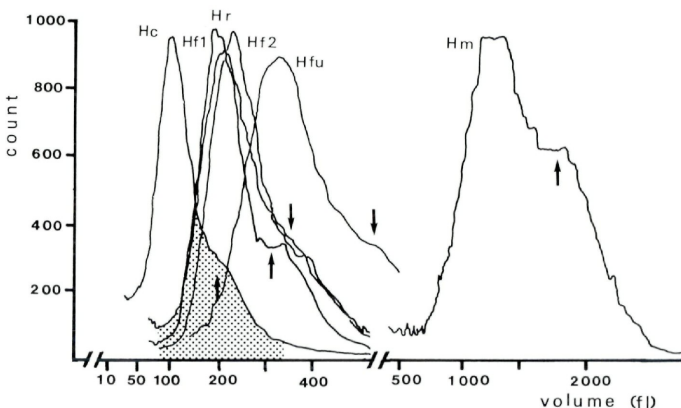


Fig. 4. – Frequency-distribution curve of ascospore populations of *Hypoxyylon* spp. obtained with an automated electronic particle sizer. Hc, *H. cohaerens*; Hf1, Hf2, *H. fragiforme*, showing the two most dissimilar curves obtained in this study; Hfu, *H. fuscum*; Hm, *H. mammatum*; Hr, *H. rubiginosum*. A bimodal distribution of spore size is evidenced by a shoulder in the curves (arrowed). The area of overlap between curves for *H. cohaerens* and *H. rubiginosum* is shadowed.

shable. Nevertheless, displaying the full distribution profiles for these fungi allowed to differentiate the ascospores of *H. fragiforme* and *H. rubiginosum* from those of *H. cohaerens* on the one hand and *H. fuscum* on the other (Fig. 4).

The reproducibility of the method and its applicability to spore material stored under herbarium conditions could also be estimated in this case for a group of genetically diverse samples from each fungus. As illustrated in Fig. 4, the variability of size frequency-distribution profiles was minimal for a given fungal species: the largest divergence found was between freshly discharged *H. fragi-*

forme ascospores and a sample of the same species stored for 15 months. The difference between the respective frequency-distribution profiles is minimal (Fig. 4) and corresponds to a difference of less than 0.5 μm in linear dimensions.

All five *Hypoxyylon* spp. studied presented a bimodal size frequency-distribution reminiscent of that also observed in *C. orbiculare*. The second frequency peak in the case of *Hypoxyylon* spp. was relatively close to the first one appearing only as a shoulder in their frequency-distribution curves (arrows, Fig. 4). Herbarium material stored for 15 months had a practically unchanged frequency-distribution profile as compared with freshly discharged ascospores.

Culturally indistinguishable sporulating isolates from different habitats

Frequency distribution curves of *Microsphaeropsis* isolates obtained from fir or mistletoe overlapped over more than 50% of their areas, making it impossible to consider the two isolate groups as distinct populations on the basis of spore size (Fig. 5a). Similarly, frequency profiles for the three *Hormonema* isolates from fir overlapped over more than 80% of their subjacent area, as would be expected of samples from a single population. *Hormonema* fir isolates, however, were clearly distinguishable from the only available isolate from mistletoe, demonstrating that the spores of isolates from each host belonged to distinct populations (Fig. 5b). By contrast, all four *Hormonema* isolates were indistinguishable on the basis of traditional microscopic and cultural criteria, fitting the description of the *Hormonema* state of *Rhizosphaera* spp. (GOURBIÈRE & MORELET, 1978; E.J. HERMANIDES-NIJHOF, pers. comm.; cf. Tab. 1).

Comparison with a computerized image analysis

Direct microscopic measurement of 500 spores each of *C. orbiculare* and *H. fragiforme* using a computerized image analysis system were compatible with results obtained using the electronic particle sizer (Tab. 1 and Fig. 6). However, the resolution obtained with the image analysis was much poorer than that provided by the Coulter counter, mostly due to the comparatively reduced size of the sample examined with the former method (500 spores measured). Furthermore, none of the fine features of distributions obtained with the electronic sizer, such as the bimodal distribution of spore size in *C. orbiculare* and *Hypoxyylon* spp., were distinguishable using the computerized image analysis (cf. Figs. 2, 4 & 6).

Discussion

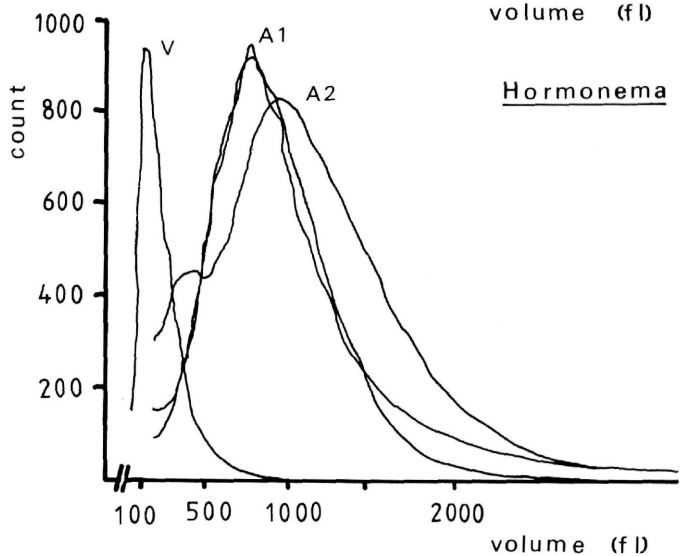
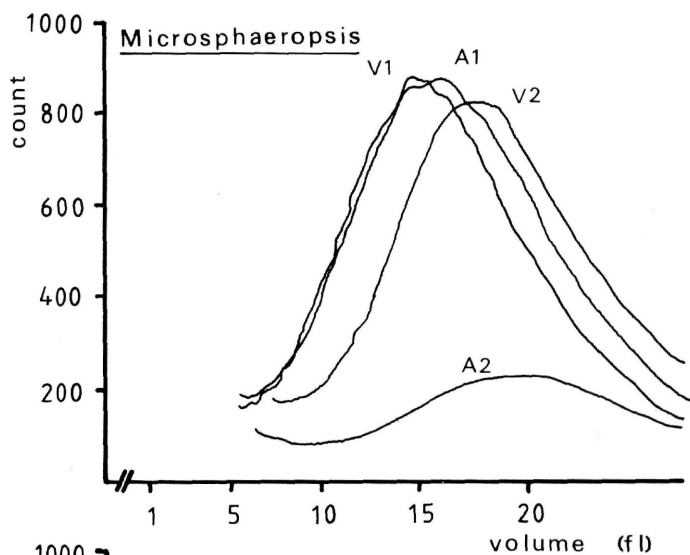
Statistical analyses involved in many taxonomic and ecological studies require a minimum amount of size determinations which are tedious, if not impossible, to obtain from direct microscopic measurements. This limitation is largely circumvented by the use of an automated sizing and counting device which can increase the number of measurements 10- to 1000-fold (obtained by the image analysis and electronic counting approach, respectively).

The electronic counting and sizing method used here was reproducible and highly accurate, as shown by the very close correspondence of measurements on spores of a genetically homogeneous culture of *C. orbiculare* after growth on either liquid or solid media.

Displaying size frequency-distributions and not only size ranges produced further evidence that conidia of *C. orbiculare* do not conform a homogeneous population. Two spore populations, completely indistinguishable from each other under the microscope, were clearly recognized on the basis of their size for the isolate studied. *C. orbiculare* thus seems to share with several other deuteromycetes the ability to produce at least two distinct types of conidiospores, although this observation could be best confirmed by analysis of isolates from various precedences. As in other taxa, such as *Phoma* and *Fusarium* where spore subpopulations (traditionally termed micro- and macroconidia) are readily distinguishable by their shape, gross size or colour, it is reasonable to assume that each subpopulation plays a different role in the life history of these fungi (CARMICHAEL, 1981). The question is also opened as to the existence of such distinct spore subpopulations in other fungal groups.

The application of an automated sizer was also shown to be useful in traditional taxonomic studies, since it provided an immediate picture of spore size distributions which could then be used directly to compare various taxa. Within the genus *Hypoxylon*, various species such as *H. cohaerens*, *H. fragiforme*, *H. fuscum* and *H. rubiginosum* are reported in the literature to have overlapping spore size ranges, forming a continuum where it is difficult to judge their actual delimitation (Tab. 1). However, when frequency-distribution profiles are considered, it can be seen that spore size can help to discriminate between *H. cohaerens* and any of *H. fragiforme*, *H. fuscum* or *H. rubiginosum* (Fig. 4). The degree of overlap between frequency-distribution curves of the various species shows that spore size is more unreliable to distinguish between *H. fuscum* and the group formed by *H. fragiforme* and *H. rubiginosum*, while of no

Fig. 5. — Frequency-distribution curves obtained with an automated electronic particle sizer for various isolates of *Microsphaeropsis* sp. (above) and *Hormonema* sp. (below) isolated from *Viscum album* (V isolates) or from *Abies alba* (A isolates).



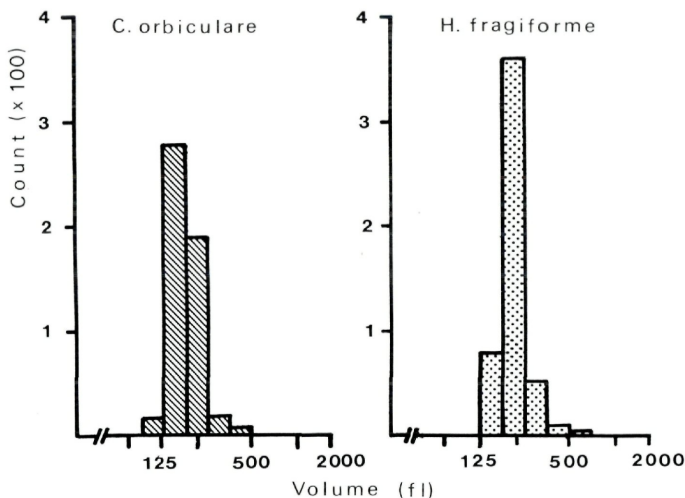


Fig. 6. – Frequency-distribution of spore sizes calculated using a computerized image analysis system for *Colletotrichum orbiculare* and *Hypoxyylon fragiforme*. By this method no bimodal distribution can be seen.

diagnostic value at all when the last two fungi are compared. A similar taxonomic evaluation using a computerized image analysis was recently published by HAGEN & HAGEN (1990) using four species of *Fusarium*.

A bimodal population was also clearly discerned in each of the five *Hypoxyylon* species studied, pointing to the possible differentiation of spore subpopulations which might play different ecological roles. This substructuring of the spore population could not be observed either directly under the microscope nor even at the level of resolution provided by 500 spore measurements using a computerized image analysis (cf. Figs. 2, 4 & 6).

Fungal variability in the field poses a major challenge in ecology, where the delimitation of functional individuals, populations and communities forms the basis of most studies (COOKE & RAYNER, 1983). The methodological problems resulting from variability are exacerbated many times by the limited number of individuals available. Under these circumstances, detailed analysis of a few individuals becomes of crucial importance. The automated sizing method described here was also useful in determining the homogeneity of a few field isolates obtained from an actual ecological study. Thus,

while *Microsphaeropsis* isolates from both fir and mistletoe could be considered to belong to a single population on the basis of their spore size, corresponding *Hormonema* isolates were clearly distinct according to the host from which they were obtained. While no definitive taxonomic implications can be derived from such a reduced number of isolates, the speed with which size frequency-distribution profiles can be obtained (typically $10^4 - 10^5$ spores measured in 1 - 2 minutes), makes this an ideal technique to be performed prior to more elaborate biochemical tests normally used to study field isolates.

The three examples used here provide evidence that an automated sizer can be successfully used to approach ecological and taxonomical problems which until now have been addressed in only a few cases and with great effort. The electronic sizer provides fast, reproducible and accurate measurements of large numbers of spores. Other techniques now widely available in research and academic institutions which could yield similar results are provided by fluorocytometric devices, laser diffraction sizers and by the computerized analysis of video images obtained directly from the microscope (GROGAN & COLLINS, 1990; HAGEN & HAGEN, 1990; NEWTON & KENDRICK, 1990). Despite the widespread routine use of these techniques in many biological areas, other fields such as mycology and botany could still profit from their widespread application in taxonomical and ecological research.

Acknowledgments

Critical reading of the manuscript and suggestions by M. DREYFUSS (Sandoz Ltd, Basel), L.E. PETRINI (Zürich, Switzerland) and an anonymous reviewer are gratefully acknowledged. I am also grateful to M. DREYFUSS and J. HARR (Sandoz Ltd) for their continuous help. The technical support of B. FEHÉR and the computerized image analysis by G. MARRER (Leica AG, Zürich, Switzerland) are also appreciated.

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Jahr/Year: 1991

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Autor(en)/Author(s): Chapela I. H.

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