Mycologia, 97(2), 2005, pp. 474–484.

Spore dimensions of *Puccinia* species of cereal hosts as determined by image analysis[1]

Y. Anikster
T. Eilam

Department of Botany and Institute for Cereal Crops Improvement, Tel Aviv University, Ramat Aviv, 69978, Israel

W.R. Bushnell[2]

USDA-ARS, Cereal Disease Laboratory, 1551 Lindig Street, University of Minnesota, St. Paul, Minnesota 55108

E. Kosman

Institute for Cereal Crops Improvement, Tel Aviv University, Ramat Aviv, 69978, Israel

Abstract: Digital image analysis was used to measure dimensions of spores produced by *Puccinia coronata, P. graminis, P. hordei, P. recondita, P. striiformis* and *P. triticina.* Included were teliospores, basidiospores, urediniospores and, except for *P. striiformis,* pycniospores and aeciospores. Length, width and projection area of spores were measured with NIH Image or Scion software. By using limits on size, spores were automatically selected and measured, except for teliospores, which required manual elimination of the pedicel and separation of images of adhering spores. Length and width were determined as the major and minor axes of the best fitting ellipse for each spore. This procedure gave values for length and width close to results obtained with an ocular micrometer. Projection area was determined as the number of pixels within spore boundaries multiplied by the area represented by each pixel, giving values that are not feasible to obtain accurately with an ocular micrometer. Of the species studied, spores of *P. recondita* had the largest dimensions, *P. triticina* had the smallest. The rank of the six species based on increasing width, length or projection area was almost the same, using each spore type except pycniospores. Generally, differences of 5% in a given spore dimension between two species were significant. Differences between species were greater with basidiospores and aeciospores than with other spore types. Teliospores were unique in that length and width were negatively correlated, resulting in less variation in area than in length or width. The results indicate that image analysis is useful for measuring spore dimensions, that projection area of spores is a useful added parameter for characterizing rust species and that dimensions of teliospores, basidiospores, aeciospores and urediniospores each are potentially useful for differentiating species.

Key words: aeciospore, basidiospore, coronata, graminis, hordei, pycniospore, recondita, striiformis, teliospore, triticina, urediniospore

INTRODUCTION

The taxonomy of the Uredinales within genera is based largely on morphological characters, together with range of hosts, including alternate hosts of heteroecious rust fungi. Among useful morphological traits are length and width of teliospores. Rust manuals generally give teliospore dimensions, usually including the range most commonly encountered and the maximum range. Dimensions of other spores in the life cycle also are given sometimes, especially for urediniospores, which often are readily available.

For Puccinia species on cereal hosts, spore dimensions and host range both tend to overlap between species, obscuring species boundaries. Distinctive morphological traits such as projections on teliospore caps of *P. coronata* or the extraordinarily long teliospores of *P. elemi* are useful when present. For most species, however, the more accurately spore dimensions can be determined the better will be the delineation of species. Spore dimensions have also been used to define groups within species such as subspecies (Savile 1984).

Since their advent in recent years, software programs for digital image analysis have been used to investigate several aspects of fungus morphology and development. These include dimensions and shape of spores (Mitchell et al 1997, Benyon et al 1998), spore numbers (Jones et al 1992), spore germination (Paul et al 1993, Oh et al 1996), germ tube length (Hilber and Schüepp 1992), length and branching of hyphae (O'Shea and Walsh 1996) and size of mycelial aggregates (Adams and Thomas 1988, Cox and Thomas 1992, Tucker et al 1992). Likewise, image

Accepted for publication 23 Feb 2005.

[1] Mention of a trademark name or proprietary product does not constitute a guarantee by Tel Aviv University, the University of Minnesota or the U.S. Department of Agriculture.

[2] Corresponding author. E-mail: billb@umn.edu

TABLE I. Number and country of origin of isolates of six species of Puccinia

Species	Origin and number of isolates	Total number of isolates	Hosts	
			Telial/uredinial	Pycnial/aecial
Puccinia coronata	Israel 2 USA 2	4	*Avena sterilis* *A. sativa*	*Rhamnus palaestina* *R. cathartica*
P. graminis		8		
f.sp. *secalis*	USA 3		*Elytrigia repens*	*Berberis vulgaris*
f.sp. *tritici*	Hungary 1; Germany 1; USA 3		*Triticum aestivum*	*B. vulgaris*
P. hordei	Israel 1; Germany 1; USA 1	3	*Hordeum vulgare*	*Ornithogalum* spp.
P. recondita		8		
Aegilops longissima type	Israel 2		*Aegilops longissima*	*Anchusa aggregata*
Ae. ovata type	Israel 1		*Ae. ovata*	*Echium glomeratum*
Ae. variabilis type	Israel 2		*Ae. variabilis*	*An. strigosa*
Secale montanum type	Israel 1		*Secale montanum*	*Lycopsis arvensis*
T. turgidum var. *durum* type	Morocco 2		*T. turgidum* var. *durum*	*An. italica*
P. striiformis	Israel 4	4	*T. aestivum*	
P. triticina		8		
T. aestivum type	Israel 2; China 1; USA 1		*T. aestivum*	*Thalictrum speciossissimum*
T. turgidum var. *durum* type	Israel 1; Ethiopia 3		*T. turgidum* var. *durum*	*Th. speciossissimum*

analysis has been used to determine volume of yeast cells (Makarov et al 1998), budding of yeasts (O'Shea and Walsh 1996) and shifts from yeast-like to filamentous growth in dimorphic fungi (McCarthy et al 1998).

For rust fungi, Hernandez et al (2002) used image analysis to measure length and width of urediniospores and teliospores of *P. hemerocallidis*. Johnson et al (1999) compared urediniospores of *P. menthae* isolates from spearmint and peppermint, showing that 15 of 18 characteristics, based mainly on dimension and shape, differed between isolates of the two groups. In an investigation of several members of the *P. recondita* complex from wheat, wild wheat and rye, image analysis was used to determine the length, width and projection area of teliospores (Anikster et al 1997). The results showed that the rust collections fell into two groups differing in teliospore size: teliospores of group I (tentatively termed *P. triticina*) were smaller in both length and width than those in group II (the remaining members of the *P. recondita* complex). The differences in teliospore dimensions were most pronounced when comparison was based on area. Coupled with differences in host range and lack of sexual compatibility, the differences in teliospore dimensions contributed to the conclusion that group I was a species distinct from the rest of the *P. recondita* complex. Likewise spore dimensions obtained digitally were used to assist in the differentiation of groups within the *P. allium* complex (Anikster et al 2004) and to define a new forma specialis of *Puccinia triticina* from *Aegilops speltoides* (Ben Yehuda et al 2004).

Here we extend investigation of spore dimensions to include a total of six Puccinia species of cereal rusts (TABLE I), including *P. triticina* and other members of the *P. recondita* complex as investigated earlier. For each species, isolates from several regions of the world were included to ensure thorough representation. Using image analysis, we measured length, width and projection area of all spore types produced by each species (as shown for *P. recondita* and *P. triticina*, FIG. 1). Our objectives were: (i) to evaluate digital image analysis as an aid to measuring spore dimensions, including use of projection area as a potential descriptor for rust fungus species; (ii) to learn how differences in teliospore dimensions among species relate to dimensions of other spore types in the rust life cycle; (iii) to characterize variation in dimensions in populations of a given spore type within individual species. We found to our surprise that length and width of teliospores are negatively correlated (i.e., variation in length was compensated for by opposing variation in width).

MATERIALS AND METHODS

The number of isolates for the six rust species investigated are provided (TABLE I) with country of origin and host spe-

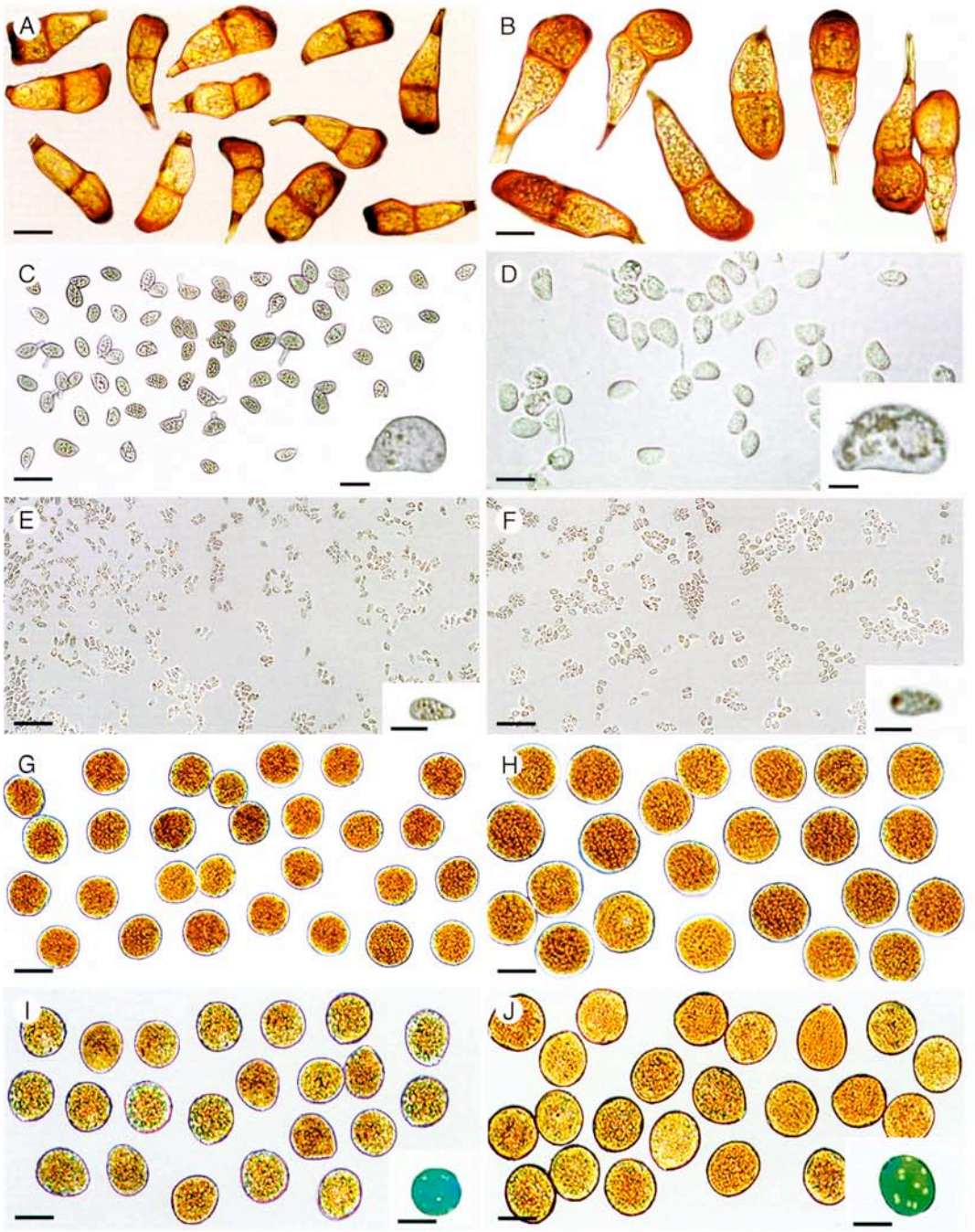

FIG. 1. The five spore types of *Puccinia triticina* (left) and *P. recondita* (right). A, B. Teliospores. C, D. Basidiospores. E, F. Pycniospores. G, H. Aeciospores. I, J. Urediniospores. Unstained specimens, except urediniospores in insets (I, J) were stained with 0.1% aniline blue in lactic acid to show germ pores. Bar = 15 μm, except 7.5 μm for pycniospores (E, F) and 3 μm for insets of basidiospores (C, D) and pycniospores (E, F).

cies. Three to eight isolates were used for each species. Methods for growing and inoculating plants and for harvesting spores were as described by Anikster et al (1997). Spores were prepared for image analysis as follows:

Teliospores. Leaves from telia were brought in from the field and dried in paper bags on the laboratory bench for about a week. The bags then were stored in a refrigerator at 4 C for 2 mo to 10 y. For image analysis, teliospores were scraped from 10–15 telial clusters and mounted in 50% aqueous glycerol under a cover slip on a glass microscope slide. At least five fields with 10–20 spores/field were measured for each isolate.

Basidiospores. Teliospores (in telia) were preconditioned for germination by incubating them 1–6 wk in distilled water at 4 C (Eilam et al 1992). The telia then were placed on a wet filter paper on the lid of a Petri dish so that ba-

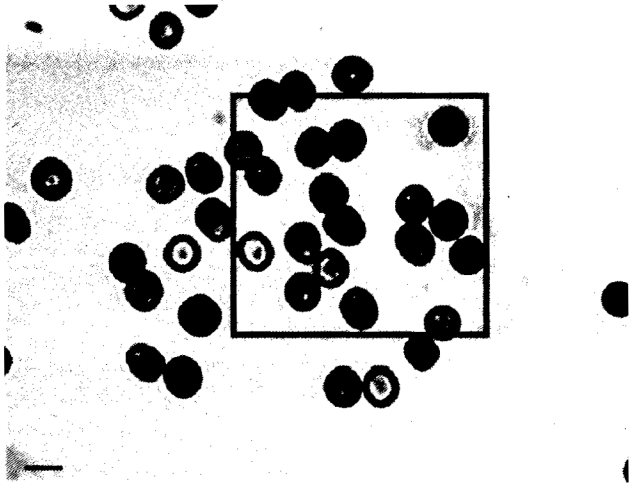

FIG. 2. Example of rectangular field used for image analysis as viewed on the computer screen. The square within the field shows size of subfields of FIG. 3. Shown are urediniospores of *P. recondita*. Digital image derived from black and white video image. Bar = 30 μm.

sidiospores ejected from basidia produced by the germinating teliospores would fall on cover slips on the bottom of the dish. After a sufficient number of basidiospores were ejected, usually after 20 h incubation at 18 C, the cover slips were inverted on a drop of 1% cotton blue in lactophenol on a microscope slide. The slide was heated briefly on an electric hot plate to 40 C; the basidiospores then were viewed under the microscope. For each isolate, spores were measured in 10 or more fields, each with 10–20 spores. (Basidiospores of FIG. 1 were unstained.)

Pycniospores. To harvest pycniospores, pycnia were touched lightly with a cover slip so that nectar (containing pycniospores) adhered as a droplet. The nectar was allowed to dry 10 min until pycniospores were affixed to the cover slip. A droplet of 1% cotton blue in lactophenol was placed on a microscope slide. The cover slip with pycniospores then was inverted and placed so the spores were immersed in the fixative under the cover slip. The slide was heated as described for basidiospores and examined under the microscope. At least four fields, each with about 200 spores, were measured for each isolate. (Pycniospores shown in FIG. 1 were unstained.)

Aeciospores and urediniospores. Freshly harvested aeciospores and urediniospores were mounted under cover slips on slides in lactophenol at room temperature. A minimum of 10 microscope fields, each with about 20 spores, was measured for each isolate.

Image analysis.—Spore images were obtained by differential interference contrast (DIC) microscopy with a Zeiss Axioskop equipped with 10× eyepieces. A 40× oil immersion Plan-Neofluar objective lens (N.A.1.30) was used for pycniospores; a 20× Plan-Neofluar lens (N.A. 0.50) was used for all other spore types. For analysis, microscope fields were selected in which illumination was uniform and spores were not crowded (FIG. 2). Images were captured with a LIS 700 CCD black and white video camera (Applitec, Israel). Gain and offset were adjusted usually with the automatic option of the camera. Exposure time was usually 1/500 s. In initial experiments, video images were digitized with a 50 Hz (PAL) Quick Capture frame grabber (Data Translation, Marlboro, Massachusetts) in a Macintosh 8100. Later a video capture frame grabber (All in Wonder, ATI Technologies, Markham, Canada) was used with a NEC (Pentium 4-3 1 Mgh) Packard Bell PC computer. The result was a rectangular array of 640 × 480 pixels with gray levels in increments of 0–255 steps. The width of the rectangle, as viewed on the computer screen (FIG. 2), was equivalent to one-half the diameter of the original microscope field.

Spore images obtained with the Macintosh were analyzed with NIH Image Software (version 1.60) written by Wayne Rasband, National Institute of Health, Maryland. Images obtained with the PC were analyzed with Scion Image version 4.02 (Scion Corp., Frederick, Maryland). To illustrate procedures, close-up views of a portion of the analyzed rectangle are shown (FIG. 3) for each spore type. Initial digital images (FIG. 3A, D, G, J, M) were processed as follows. (i) Background was usually subtracted with "2D rolling ball" algorithms, especially when the background was uneven. (ii) An automatic threshold setting (typically 70–150) was used to produce binary images (FIG. 3B, E, H, K, N). Thresholding sets the minimum gray level required for viewing an image. The threshold is set by automatic analysis of gray levels of the current selection histogram. For basidiospores, germ tubes were excluded because the narrow, low contrast base of the germ tube (FIG. 3D) was below threshold gray level (FIG. 3E). (iii) Spores with areas 10% larger or 10% smaller than the smallest spore (as measured in a preliminary subsample) were eliminated. This removed miscellaneous debris as well as images of two or more spores touching each other (except for teliospores as described below). Spores touching frame boundaries were rejected automatically. (iv) Length and width of spores were measured as the major and minor axes of the best fitting ellipse. Area was computed in pixels and displayed in μm^2 as the sum of all pixels in an object. Spatial calibration of pixels was done with a stage micrometer. Any white spaces within the boundaries of threshold spores were included automatically in spore area. Dimensions were determined automatically for all suitable spores in the field, except for teliospores, which were measured individually. A white line was drawn across the base of each teliospore to remove the pedicel before measurements (FIG. 3B). Because teliospores tended to stick together, spores in clumps of 2–3 were separated by drawing a white line between them if areas of overlap were judged insignificant (FIG. 3B). Single-celled teliospores (mesospores) were excluded from measurements. Each prepared teliospore image then was measured by touching the image with the "magic wand" tool (of NIH image) and using the best fitting ellipse procedure as used with other spores. Spores that were included in measured samples were numbered automatically (FIG. 3C, F, I, L, O).

Length, width and area measurements were copied to Excel and are reported as the sample mean and the sample

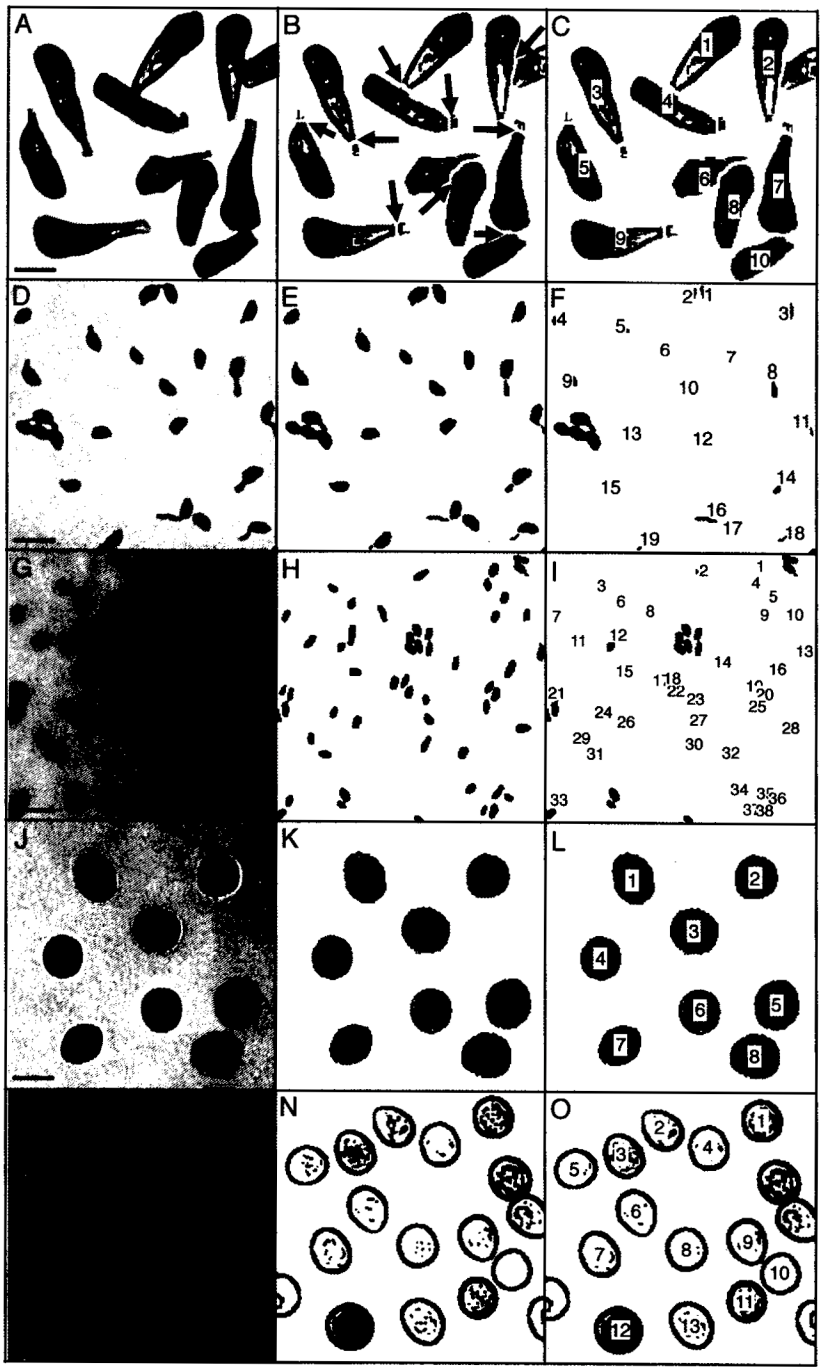

FIG. 3. Preparation of images for analysis for five spore types of *Puccinia recondita*: teliospores (A, B, C), basidiospores (D, E, F), pycniospores (G, H, I), aeciospores (J, K, L), and urediniospores (M, N, O). A, D, G, J, M. Initial digital image. B, E, H, K, N. Binary image after background was subtracted and gray levels below threshold were eliminated using software of NIH Image or Scion. C, F, I, L, O. Spores selected for measurement as numbered. For teliospores only (FIG. 3B), images were modified with white lines to separate pedicels from the main spore body and to separate contiguous spores. Close-up views of only a portion of the rectangular fields used for image analysis, as in FIG. 2. Bar = 20 μm, except 10 μm for G, H and I.

coefficient of variation (CV), which expresses standard deviation as a percentage of the sample mean. In addition, the square root of the spore area and its CV are provided as a way to compare CVs of length and width to CVs as described in Results. Significance of differences in sample means for species was determined by one-way ANOVA using Tukey's test from SPSSa software. The relation between length and width was characterized by the Pearson correlation coefficient. Photographs of spores (FIG. 1) were taken with a Nikon Coolpix 4500 digital camera.

RESULTS

Differences in size and morphology among the five types of spores investigated are shown (FIG. 1) for *P. triticina* and *P. recondita*. For both species, there was an approximate 10-fold difference in both length and width between pycniospores (the smallest of the spore types) and teliospores (the largest) (FIG. 1). Teliospores (FIG. 1A, B) were almost all two-celled, had caps formed by apical wall thickenings and had pedicels that usually were broken off but remained intact in *P. graminis*. The pedicel of *P. triticina* had a pigmented, cone-shaped deposit in the remnant of pedicel that remained attached to the teliospore (FIG. 1A). Basidiospores (FIG. 1C, D) were asymmetrical with one side more highly curved than the other. Basidiospores produced germ tubes by the time the spores were fixed for observation, as described in Materials and Methods. Pycniospores (FIG. 1E, F) were pear-shaped with thin walls. Aeciospores and urediniospores (FIG. 1G, H, I, J) were spheroid, except for urediniospores of *P. graminis* (not shown) which were elongated. Urediniospore germ pores (FIG. 1I, J) usually were scattered but were equatorial in *P. graminis* (not shown). Surfaces of urediniospores had spines (not clearly visible at the magnification of FIG. 1I, J).

Evaluation of image analysis protocols.—Because surfaces of aeciospores, teliospores and urediniospores are hydrophobic, small air bubbles accumulated around each spore if mounted in water, interfering with measurements. To counter this problem, spores were mounted in glycerin (for teliospores) or lactophenol (for the four other spore types). In preliminary trials comparing water to the mounting media used for each spore type, the media had no detectable effect on spore dimensions.

For all five spore types of an isolate of *P. recondita*, values for spore length and width obtained by image analysis were compared to values obtained directly with an ocular micrometer on the microscope and also to values obtained by using a ruler on the computer screen before the thresholding step. For measurements with the ocular micrometer, a 100× objective lens was used. For digital images a 20× objective lens was used except 40× for pycniospores, as in our standard protocol. Larger numbers of spores were present for measurement in individual microscope fields at 40× (used for pycniospores) or 20× (used for the other spore types) than at 100×, aiding rapid analysis of spore populations. Results for the three methods agreed within 7% of values obtained with the ocular micrometer (TABLE II). This indicated that the thresholding step, the use of axes of the best fitting ellipse for length and width and other aspects of the image analysis procedure gave satisfactory results.

TABLE II. Length and width of *Puccinia recondita* spores as measured by three methods: with an ocular micrometer on the microscope, with a ruler on the computer screen (before threshold was applied), and by image analysis (after the threshold step) with Scion software

	Method					
	Ocular micrometer		Ruler		Image analysis	
Spore type	μm	CV	μm	CV	μm	CV
Teliospores						
Length	48.1	11	47.3	9	51.3	9
Width	20.2	12	20.0	11	19.4	10
Basidiospores						
Length	10.3	9	10.9	7	10.5	9
Width	8.0	8	7.6	9	8.1	8
Pycniospores						
Length	4.1	12	4.5	12	4.3	14
Width	2.0	14	2.1	13	1.9	10
Aeciospores						
Length	25.8	9	25.6	4	24.6	5
Width	23.3	6	23.4	4	23.2	6
Urediniospores						
Length	27.4	10	24.1	6	25.6	7
Width	22.7	9	23.2	5	23.1	7

Projection area.—Mean projection areas for each spore type in order of increasing values among the species investigated are provided (TABLE III). The order was almost the same whether based on values for the teliospores, basidiospores, aeciospores or urediniospores but not if based on values for pycniospores. Mean values ranged from 6.71 μm^2 for pycniospores to 719 μm^2 for teliospores. Among species, areas were largest for *P. hordei* and *P. recondita* and smallest for *P. triticina* for all spore types except pycniospores, which, in contrast, were larger for *P. triticina* than *P. recondita*.

Examples of the distribution of area values within spore types are shown in histograms (FIG. 4) for *P. triticina* and *P. recondita*, representing the species with the smallest and largest teliospores. Although the spore populations of the two species overlapped, their means were significantly higher for *P. recondita* than *P. triticina* for all spore types except pycniospores (TABLE III). Generally differences in area of a given spore type between any two species were significant if mean values differed by 5% or more.

Length and width.—The rank of species according to increasing length or width usually paralleled rank by

TABLE III. Dimensions and length-width correlation of five spore types of *Puccinia* species as measured by image analysis. Data are for all spores of all isolates for each species (TABLE I) combined into a single population. Species ranked in order of increasing means for area of each spore type

Spore type/ species	Number of spores	Area, μm²		√Area, μm		Length, μm		Width, μm		Length-width correlation coefficient
		Mean[a]	CV	Mean	CV	Mean	CV	Mean	CV	
Teliospores										
P. triticina	279	583 a	15	24.1 a	7	45.9 a	17	16.3 a	10	−0.49
P. striiformis	110	703 b	15	26.4 b	7	49.4 b	13	18.1 b	10	−0.22
P. coronata	78	713 b	13	26.7 b	7	52.1 c	15	17.8 b	14	−0.51
P. graminis	241	760 c	10	27.5 c	5	49.5 b	13	19.8 c	12	−0.66
P. recondita	253	771 c	13	27.7 c	7	49.1 b	12	20.1 c	12	−0.37
P. hordei	70	784 c	17	27.9 c	9	45.6 a	13	22.1 d	16	−0.34
Mean		719	13.8	26.3	7.0	48.6	13.8	19.0	12.3	
Basidiospores										
P. triticina	730	41.0 a	19	6.37 a	10	8.27 a	11	6.28 a	12	0.30
P. graminis	564	49.9 b	20	7.03 b	10	9.83 b	15	6.46 b	12	0.04
P. coronata	261	59.7 c	19	7.70 c	9	10.3 c	12	7.10 c	11	0.43
P. striiformis	212	60.1 c	23	7.68 c	13	10.6 d	18	7.40 d	12	0.09
P. hordei	610	69.1 d	21	8.27 d	10	12.0 e	13	7.29 d	14	0.19
P. recondita	1042	70.4 d	19	8.35 d	10	11.8 e	15	7.59 e	13	−0.01
Mean		59.0	20.2	7.60	10.3	10.5	14.0	7.03	12.3	
Pycniospores[b]										
P. coronata	1260	5.67 a	26	2.36 a	13	3.52 a	17	2.04 a	16	0.20
P. graminis	675	6.19 b	21	2.47 b	10	3.65 b	16	2.16 b	14	−0.05
P. recondita	1467	6.78 c	27	2.57 c	14	3.99 c	15	2.13 b	19	0.41
P. triticina	1296	7.13 d	22	2.65 d	11	3.93 d	16	2.28 c	15	0.23
P. hordei	1803	7.28 d	30	2.69 e	15	4.12 e	21	2.26 c	15	0.36
Mean		6.71	25.2	2.57	12.6	3.89	17.0	2.41	15.8	
Aeciospores[b]										
P. triticina	770	271 a	16	16.4 a	8	20.1 a	9	17.1 a	10	0.42
P. graminis	155	299 b	21	17.2 b	10	21.3 b	14	17.9 b	9	0.47
P. coronata	406	312 c	21	17.6 c	10	21.3 b	11	18.6 c	11	0.67
P. recondita	693	335 d	19	18.2 d	9	22.1 c	11	19.2 d	11	0.57
P. hordei	199	431 e	19	20.7 e	10	25.3 d	11	21.6 e	11	0.56
Mean		317	19.2	17.7	9.4	21.6	11.2	18.6	10.4	
Urediniospores										
P. coronata	204	370 a	21	19.1 a	11	23.1 a	12	20.2 b	11	0.75
P. triticina	613	375 ab	17	19.3 ab	9	23.2 a	11	20.4 c	9	0.49
P. striiformis	365	380 ab	18	19.4 ab	9	24.4 b	12	19.7 b	10	0.30
P. graminis	440	388 b	17	19.6 b	8	28.3 d	14	17.5 a	12	−0.15
P. hordei	150	421 c	13	20.5 c	7	24.4 b	14	21.9 d	7	0.61
P. recondita	365	489 d	11	22.1 d	6	26.2 c	6	23.7 e	7	0.48
Mean		404	16.2	20.0	8.3	25.0	11.5	20.6	9.3	

[a] Values in a given column followed by the same letter did not differ significantly ($P > 0.05$).

[b] Pycniospores and aeciospores were not available for *P. striiformis*.

measuring area for basidiospores, pycniospores or aeciospores, although the position of species with largest spores, *P. hordei* and *P. recondita*, sometimes switched (TABLE III). The ranking with pycniospores, although different from ranking based on other spore types remained almost the same when ranked by length or width as by area. With urediniospores, the order by length and width also differed from that by area, mainly because *P. graminis* had relatively long (283 μm) and narrow (17.5 μm) urediniospores compared to other species. Further the urediniospores of *P. graminis* had a low length/width corre-

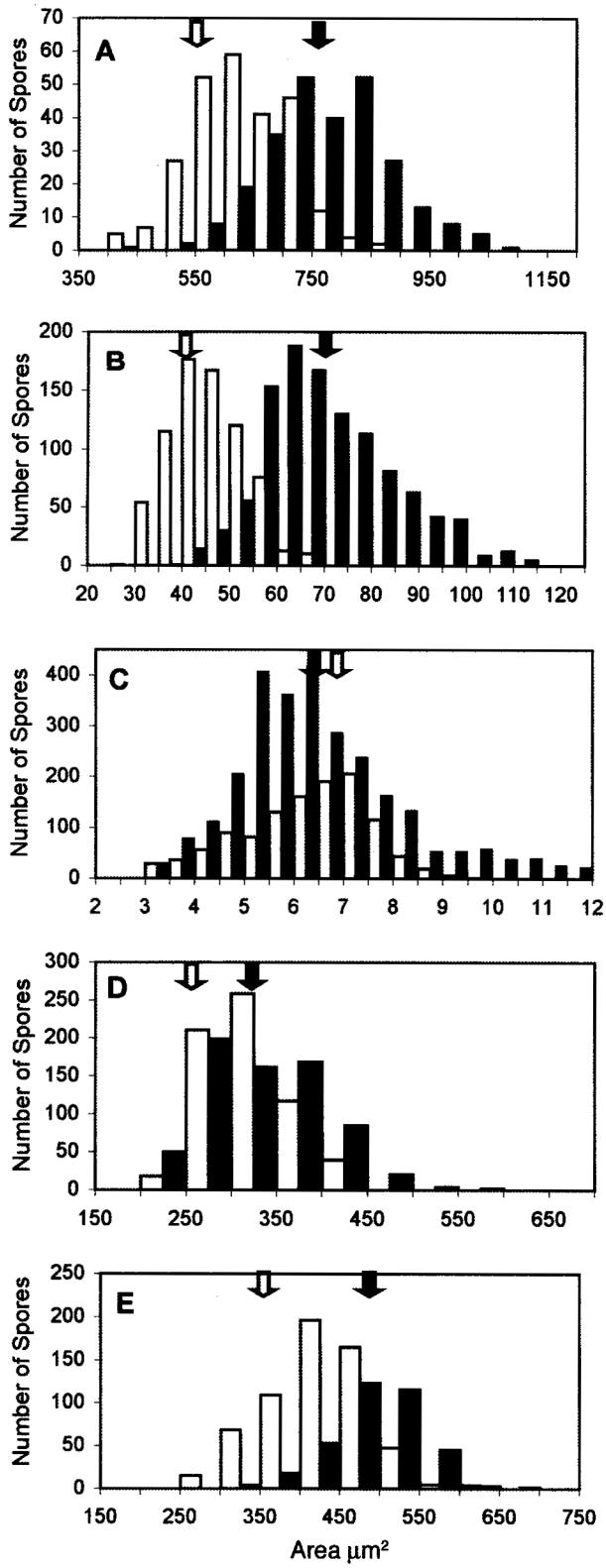

FIG. 4. Histograms of values obtained for cross sectional area for spores of *Puccinia triticina* (open bars) and *P. recondita* (closed bars). A. Teliospores. B. Basidiospores. C. Pycniospores. D. Aeciospores. E. Urediniospores. Open arrow indicates mean value for *P. triticina*; closed arrow indicates mean value for *P. recondita*.

lation coefficient (−0.15) compared to those of other species (0.30–0.75) (TABLE III). Increases in length were not associated with increases in width in *P. graminis* as they tended to be in urediniospores of other species.

For similar reasons, the rank of teliospores by increasing length did not relate to ranking by area (TABLE III). Teliospores of all species had highly negative length/width correlation coefficients. This indicated that teliospore width decreased as length increased. This is illustrated by teliospore length/width plots for *P. graminis* (correlation coefficient, −0.66) and *P. striiformis* (correlation coefficient, −0.25) (FIG. 5). Therefore rank of species by order of increasing length differed from rank by area. For example, teliospores of *P. hordei* ranked with *P. triticina* as having the shortest teliospores, in contrast to being the largest when ranked by area (TABLE III).

Another consequence of the negative length/width correlation coefficient for teliospores was lower variation within spore populations for area than for length or width. To demonstrate this we calculated the CV of the square root of area so that, like the CVs for length and width, the unit of measurement was µm (and not µm²). The mean CV for square root of teliospore area (for all species combined) was 7.0, compared to 13.8 and 12.3 for length and width respectively (TABLE III). Note also that the CV of square root area for all species was less for teliospores (7) than for the other spore types (8.3–12.6), which usually had positive length/width correlation coefficients.

DISCUSSION

With the assistance of digital image analysis, we compared length, width and projection area of the five spore types produced by five full cycled Puccinia species: *P. coronata, P. graminis, P. hordei, P. recondita,* and *P. triticina* plus the spores produced by *P. striiformis,* which lacks known pycnial and aecial stages. The results indicate that relative spore size among species tends to be the same for teliospores, basidiospores, aeciospores and urediniospores. For example, species that had large teliospores compared to those of other species also had relatively large basidiospores, aeciospores and urediniospores. The complex genetic and physiological factors controlling spore size within a given species apparently act in common in all four spore types.

The size of pycniospores, on the other hand, did not relate to size of the other four spore types. The pycniospore is highly specialized in that it functions in the environment of pycnial nectar in which it fuses to receptive hyphae (i.e., it does not require protec-

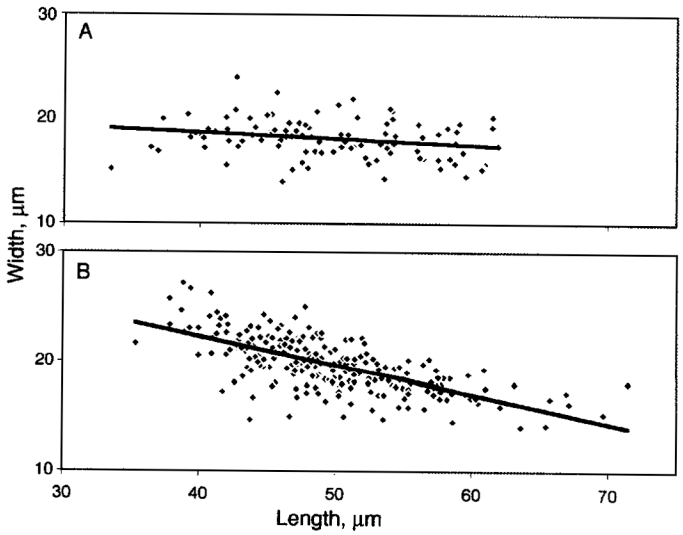

FIG. 5. Teliospore length plotted against width. A. *Puccinia striiformis* (regression slope = −0.06); B. *P. graminis* (regression slope = −0.26).

tion from drying out as do the other spore types, nor does it produce infection structures required for host cell wall penetration or entry through stomates, as in the case of aeciospores and urediniospores). Because of the similarity in relative spore size (except for pycniospores), differences between species tended to be similar whether based on teliospores, basidiospores, aeciospores or urediniospores. However differences in size between species were greater with basidiospores and aeciospores than with other spore types. Overall these relationships indicate that dimensions of basidiospores, aeciospores and urediniospores are potentially as useful as the more commonly used dimensions of teliospores in differentiating between species.

Digital image analysis aided measurement of spore length and width in that the spores could be measured more rapidly than if measured individually in the traditional way with an ocular micrometer on the microscope. The exception was measurement of teliospores, which individually required removal of the pedicel from spore images and, in many cases, separation of images when teliospores adhered to each other. For spores with relatively simple round or oval shapes, image analysis considerably speeds measurements of length and width, which is useful if large numbers of spores are to be measured. This, in turn, makes it easy to obtain enough spores for statistical analysis of results.

The principal advantage of image analysis, however, was the ability to measure projection area. Area was determined independently of length and width from the number of pixels within spore boundaries. This integrated the entire area of each spore regardless of shape. Projection area is potentially the most important single parameter for characterizing spore dimensions because it combines aspects of length, width and shape into a single value.

Furthermore, variation among spores tends to be less for square root values of projection area than for length or width, especially for teliospores. Although area measurements have not been used widely to characterize spores, image analysis makes area readily available as a potentially useful parameter, whether for taxonomic, genetic or physiological purposes.

Length and width of all spore types were determined as the major and minor axes of the best fitting ellipse for individual spores. The dimensions obtained in this way were similar to those obtained with an ocular micrometer (TABLE II). We did not evaluate alternative methods for determining length, such as use of the longest chord within the spore image or the "fiber" length as measured along the medial axis of the spore (Johnson et al 1999).

Orientation of the spore in the field of view influences measured spore length and probably projection area. This was not a problem with teliospores, which tended to lie with their longest dimension parallel to the microscope slide (FIGS. 1A, B, 3A, B). The same was true for the elongated urediniospores of *P. graminis* (not shown). Orientation also was not a problem with spores with only small differences between length and width, including urediniospores of species other than *P. graminis* and aeciospores of all species. On the other hand, some basidiospores and pycniospores were not parallel to the slide when measured for image analysis.

Basidiospores, allowed to fall onto slides from germinating teliospores (in preparation for measurement), sometimes landed on one end and became

affixed in a near vertical position, remaining in this position when mounted in lactophenol cotton blue. We could not dislodge such basidiospores without disrupting them. In a similar way some pycniospores were not parallel to the slide surface when mounted in lactophenol cotton blue. Nearly vertical pycniospores were eliminated because they were below the minimum area required to be included in the measured spore population. However, some spores at a low angle to the surface were included, which possibly reduced mean length values slightly. Because this error was probably similar for all species examined, we conclude that values obtained by image analysis for length or area of basidiospores and pycniospores (as in TABLE III) are valid for comparing species.

The length/width ratio (aspect ratio) can be used to differentiate between species. For example, the length/width ratios for *P. graminis* urediniospores was 1.62 compared to a mean of 1.14 for the other five species (values derived from data of TABLE III). The length/width ratio was one of 15 parameters Johnson et al (1999) found to be different between two races of *P. menthae.* Length/width ratios of urediniospores also have been used to distinguish subspecies. Urediniospores of *P. graminis* subsp. *graminis,* thought to have evolved on domesticated cereals, had length/width ratios of 1.8–1.9 whereas urediniospores of *P. graminis* subsp. *graminicola,* predominantly a parasite of wild grasses, had ratios of 1.4–1.6 (Savile and Urban 1982, Urban and Marková 1984). Clay models were used by Savile and Urban (1982) to estimate volume of urediniospore protoplasts. The resulting volumes of *P. graminis* subsp. *graminis* were 1.5–1.9 times greater than for *P. graminis* subsp. *graminicola.* Compared to such estimates of volume, area of urediniospores as determined by image analysis likely will be a more direct and useful way to compare subgroups with species. However length/width correlation coefficients also are useful for characterizing spores. Most spores in our investigation had coefficients that were positive or near zero (TABLE III). Exceptions were the long urediniospores of *P. graminis* (correlation coefficient −0.15) and teliospores of all six species examined (correlation coefficient −0.22 to −0.66).

For teliospores the reduced width associated with increasing length results in less variation in area (as expressed by variation in square root of area) than in length or width (TABLE III). As teliospores are formed in the crowded telium, length and width apparently are coordinated toward maintaining a constant projection area and, probably likewise, spore volume. The teliospore usually germinates in the telium, producing promycelia (basidia) and basidiospores there. It is not disseminated in air as are basidiospores, aeciospores and urediniospores. Having less need to maintain an aerodynamic shape, the teliospores can vary in length and width, while nevertheless maintaining uniform spore volume in support of ability to produce promycelia and basidiospores.

The basidiospores newly ejected from germinating teliospores frequently produced germ tubes before they were fixed for image analysis. The tubes fortuitously were eliminated in the threshold process used to generate binary images (FIG. 3D, E). This happened because the base of the germ tube near the spore surface was narrow and had low optical contrast. However germ tubes can be eliminated by a skeletonization (erosion) procedure in which pixels are removed from the surface until germ tubes are eliminated (Hilber and Schüepp 1992). Pixels then are restored over the surface of the image, which results in a full size image of the spore without the germ tube.

In addition to revealing patterns in spore dimensions among spore types and among species, the present investigation serves to emphasize projection area as an important parameter for characterizing rust spores. Useful for all spores in the rust life cycle, projection area is especially valuable for describing teliospores. Dimensions and morphology of teliospores are used widely as descriptors for rust species. Because of the negative length/width correlation as described earlier, variation in area is less than variation in length or width, potentially improving differentiation between species, especially when differences in teliospore dimensions are small.

ACKNOWLEDGMENTS

We thank Jaroslava Marková for discussion of this research. We also thank Jacolyn Morrison for preparation of figures and Ann Bushnell for word processing. Partial financing was provided by the Lieberman-Okinow Endowed Chair for Disease Resistance Breeding in Cereal Crops, University of Minnesota.

LITERATURE CITED

Adams HL, Thomas CR. 1988. The use of image analysis for morphological measurements. Biotechnol Bioeng 32:707–712.

Anikster Y, Bushnell WR, Eilam T, Manisterski J, Roelfs AP. 1997. *Puccinia recondita* causing leaf rust on cultivated wheats, wild wheats, and rye. Can J Bot 75:2082–2096.

———, Szabo LJ, Eilam J, Manisterski S, Koike ST, Bushnell WR. 2004. Morphology, life cycle biology, and DNA sequence analysis of rust fungi on garlic and chives from California. Phytopathology 94:569–577.

Ben Yehuda P, Eilam T, Manisterski J, Shimoni A, Anikster Y. 2004. Leaf rust on *Aegilops speltoides* caused by a new

forma specialis of *Puccinia triticina*. Phytopathology 94: 94–101.

Benyon FHL, Jones AS, Tovey ER. 1998. Spores of airborne, allergenic fungi are differentiated and identified by image analysis. J Aller Clinic Immunol 101:S132.

Cox PW, Thomas CR. 1992. Classification and measurement of fungal pellets by automated image analysis. Biotechnol Bioeng 39:945–952.

Eilam T, Bushnell WR, Anikster Y, McLaughlin DJ. 1992. Nuclear DNA content of basidiospores of selected rust fungi as estimated from fluorescence of propidium iodide-stained nuclei. Phytopathology 82:705–712.

Hernández JR, Palm ME, Castlebury LA. 2002. *Puccinia hemerocallidis*, cause of daylily rust, a newly introduced disease in the Americas. Pl Dis 86:1194–1198.

Hilber UW, Schüepp H. 1992. Accurate and rapid measurement of lengths of fungal germ tubes by image analysis. Can J Plant Pathol 14:185–186.

Johnson DA, Ball TA, Hess WM. 1999. Image analysis of urediniospores that infect *Mentha*. Mycologia 91:1016–1020.

Jones CL, Lonergan GT, Mainwaring DE. 1992. The use of image analysis for spore counts of white-rot fungi. Biotechnol Tech 6:417–422.

Makarov AA, Dorofeev AG, Panikov NS. 1998. Cell shape and size of starving microorganisms as determined by computer image analysis. Microbiol 67:264–270.

McCarthy AA, O'Shea DG, Murray NT, Walsh PK, Foley G. 1998. Effect of cell morphology on dead-end filtration of the dimorphic yeast *Kluyveromyces marxianus* var. *marxianus* NRRLy2415. Biotechnol Prog 14:279–285.

Mitchell AD, Walter M, Gaunt RE. 1997. Image analysis of *Agaricus* basidiospores for use in systematics. Biotechnol Tech 11:801–804.

Oh K, Chen Y, Matsuoka H, Yamamoto A, Kurata H. 1996. Morphological recognition of fungal spore germination by a computer-aided image analysis and its application to antifungal activity evaluation. J Biotechnol 45: 71–79.

O'Shea DG, Walsh PK. 1996. Morphological characterization of the dimorphic yeast *Kluyveromyces marxianus* var. *marxianus* NRRLy2415 by semi-automated image analysis. Biotechnol Bioeng 51:679–690.

Paul GC, Kent CA, Thomas CR. 1993. Viability testing and characterization of germination of fungal spores by automatic image analysis. Biotechnol Bioeng 42:11–23.

Savile DBO, Urban Z. 1982. Evolution and ecology of *Puccinia graminis*. Preslia, Praha 54:97–104.

———. 1984. Taxonomy of the cereal rust fungi. In: Bushnell WR, Roelfs AP, eds. The cereal rusts. Vol. I. Orlando, Florida: Academic Press. p 79–112.

Tucker KG, Kelly T, Delgrazia P, Thomas CR. 1992. Fully-automatic measurement of mycelial morphology by image analysis. Biotechnol Prog 8:353–359.

Urban Z, Marková J. 1984. Ecology and evolution of *Puccinia graminis* Pers. Rept Tottori Mycol Inst (Japan) 22: 91–96.